Intrinsic and extrinsic regulators of developmental timing: from miRNAs to nutritional cues

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Summary

A fundamental challenge in biology is to understand the reproducibility of developmental programs between individuals of the same metazoan species. This developmental precision reflects the meticulous integration of temporal control mechanisms with those that specify other aspects of pattern formation, such as spatial and sexual information. The cues that guide these developmental events are largely intrinsic to the organism but can also include extrinsic inputs, such as nutrition or temperature. This review discusses the well-characterized developmental timing mechanism that patterns the *C. elegans* epidermis. Components of this pathway are conserved, and their links to developmental time control in other species are considered, including the temporal patterning of the fly nervous system. Particular attention is given to the roles of miRNAs in developmental timing and to the emerging mechanisms that link developmental programs to nutritional cues.

Introduction

How to synchronize developmental events among different tissues as an organism progresses from a fertilized egg to its adult form is a problem that all animals have solved. The molecular mechanism responsible for integration of such events is emerging from studies in *C. elegans*, where a forward genetics approach has identified components of a pathway that temporally specifies cell identities. The original four members of this 'heterochronic' gene pathway were identified from screens for animals with cell lineage or egg-laying defects (Ambros and Horvitz, 1984; Chalfie et al., 1981), while additional members were revealed, in part, through screens targeted to identify genes that regulate the timing of developmental events. These screens included searches for mutants with temporal alterations in reporter gene expression (Abrahante et al., 1998) or stage-specific locomotion behavior (Abrahante et al., 2003), as well as screens for suppressors of known heterochronic mutants (Pepper et al., 2004; Reinhart et al., 2000). In addition to advancing our understanding of developmental timing mechanisms in the worm, the subsequent analyses of the genes and proteins identified by these studies has had two especially significant outcomes. First, phylogenetic analyses have revealed that many of the key timing genes are conserved among diverse organisms, and some of these homologs are also known or suspected to be involved in the temporal patterning of development. Second, these studies have spawned a new area of biology that has seen exponential growth in the past few years: the biogenesis and function of microRNAs (for reviews, see Bartel and Chen, 2004; He and Hannon, 2004).

This review focuses on temporal control mechanisms employed in *C. elegans* and considers how components of this pathway intersect with biological processes in other organisms. Particular attention is devoted to the temporal specification of

cell identities in the fly central nervous system (CNS), a process that employs a worm heterochronic gene homolog, thereby raising the possibility of functional conservation. Finally, interplay between the nutritional status of an organism and the execution of developmental timing mechanisms is also considered.

The *C. elegans* heterochronic gene pathway: keeping time with microRNAs

Diverse developmental events are under heterochronic gene control in the worm, including the specification of cell lineage patterns in the epidermis (often called hypodermis) and vulva, neuronal rewiring and formation of the dauer larva (Box 1) (Ambros and Horvitz, 1984; Hallam and Jin, 1998; Liu and Ambros, 1989). Mutations in heterochronic genes alter the timing of such stage-specific events relative to other unaffected events (e.g. gonad development or the molting cycle). In general, these mutations do not act by appreciably accelerating or retarding the life cycle or life span of the animal. Nor do they alter cell fate per se. Rather, they change a the temporal identity of a cell (or perhaps 'temporal fate') to one normally expressed at a different time within the same lineage, but usually restricted to a distinct life stage. The observed temporal transformations in heterochronic mutants have been likened to the homeotic mutants of flies, in which cell identities are spatially, rather than temporally, transformed (Ambros and Horvitz, 1987; Slack and Ruvkun, 1997; Thummel, 2001).

The core members of the heterochronic gene pathway appear to act as developmental switches that program stage-specific cell identities. Thus, the activation or repression of a given heterochronic gene at a specific developmental time is often a crucial event, such that the corresponding change in activity level modulates the progression of a cell to its next temporal fate. Thus, mutations that increase or decrease heterochronic

Box 1. The worm heterochronic gene pathway times diverse developmental events
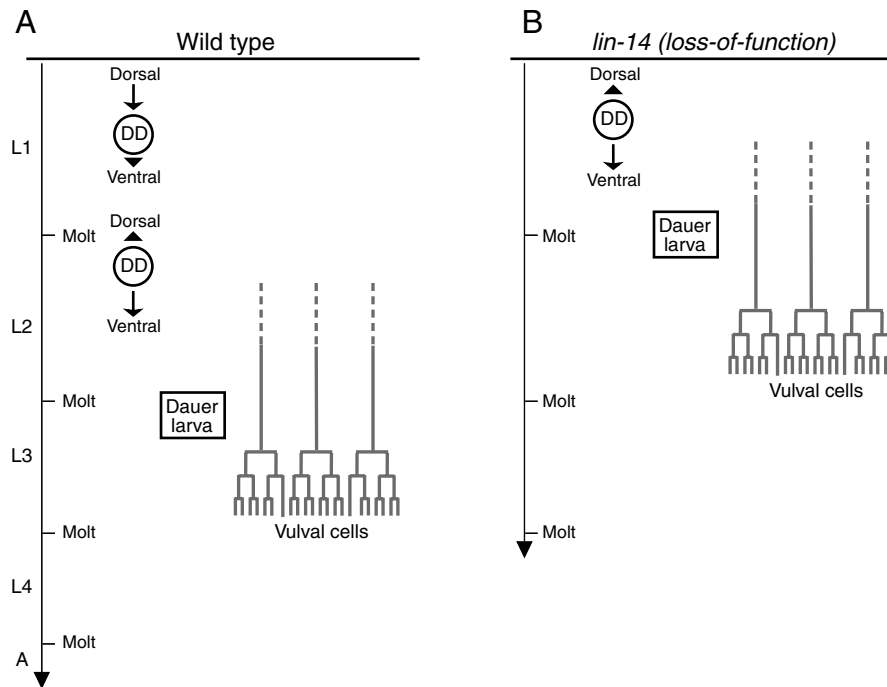
The heterochronic genes time diverse developmental events in addition to controlling temporal identity in the epidermis, such as controlling stage-specific cell division patterns in the vulva, intestine and sex myoblasts (Ambros and Horvitz, 1984; Chalfie et al., 1981). However, heterochronic gene function is not restricted to programming the stage-appropriate division patterns of mitotically competent cells. Other types of events timed by these genes include neuronal rewiring, dauer formation and the cessation of the molting cycles. Events that are controlled by *lin-14* are shown in the figure. In young L1 larvae, six GABAergic motoneurons known as the DDs, the cell bodies of which are positioned along the ventral cord, receive synaptic inputs (arrow) from the dorsal side and innervate (arrowhead) ventral body wall muscles. At the end of the L1 stage, the synaptic connectivity of these neurons is reversed, such that during the remainder of development they innervate dorsal body wall muscles while receiving synaptic inputs ventrally (Hallam and Jin, 1998). Loss of *lin-14* activity (right) causes this synaptic rewiring event to occur precociously. Its loss also causes worms to enter the dauer diapause stage at the L1, rather than at the L2 molt; vulval cell divisions to advance from the mid-L3 to the mid-L2 stage; and molting cycles to terminate after the third molt (Ambros and Horvitz, 1984; Liu and Ambros, 1989).

Although the genetic pathways that time these events have yet to be fully delineated, it is clear that there is some division of labor between the heterochronic genes with respect to the timing of these events. The genes genetically upstream in the heterochronic pathway tend to time events in a broader range of tissues than those further downstream (Chalfie et al., 1981; Ambros and Horvitz, 1984). Additional timing genes will undoubtedly be identified through directed screens, and the identification of shared and additional components will help to determine how developmental time is maintained and synchronized throughout the animal.

gene activity at inappropriate times often result in opposite temporal transformations. The ultimate readout of heterochronic gene activity in the worm epidermis in particular is the behavior of specialized 'seam' cells, which are situated on the lateral midlines of the worm. These cells terminally differentiate during the final molt, the transition from the fourth larval stage to the adult, and they contribute to the synthesis of a morphologically distinct adult cuticle (Fig. 1). As described below, specific heterochronic genes alter the time of adult cuticle synthesis indirectly, by deleting or reiterating earlier cell identities, whereas other genes act more directly.

LIN-14 and LIN-28 are core components of the mechanism that programs early epidermal cell fate transitions, which can be recognized by specific cell division patterns (Fig. 1A) as development proceeds through the first three larval stages (L1→L2→L3) (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987; Moss et al., 1997). LIN-14 and LIN-28 protein

levels are high at hatching and subsequently decay; LIN-14 disappears from the epidermis by the end of the L1 stage and LIN-28 by the end of the L2 stage (Fig. 1B) (Ruvkun and Giusto, 1989; Seggerson et al., 2002). LIN-14 is a nuclear protein, whereas LIN-28 is cytoplasmic and contains hallmark RNA-binding domains (Moss et al., 1997; Ruvkun and Giusto, 1989). Although the precise functions of these proteins and the identities of their potential targets remain unknown, molecular genetic studies have provided insights into their roles in developmental timing.

lin-14 is required for the execution of wild-type L1-stage cell division patterns; in its absence, L2 patterns occur instead, and subsequent patterns are each advanced by one stage, leading to a precocious phenotype and to the synthesis of an adult-type cuticle one stage too early (Fig. 1A,E) (Ambros and Horvitz, 1984). *lin-28* mutants skip the proliferative double division at the start of the L2 stage, and substitute the L3
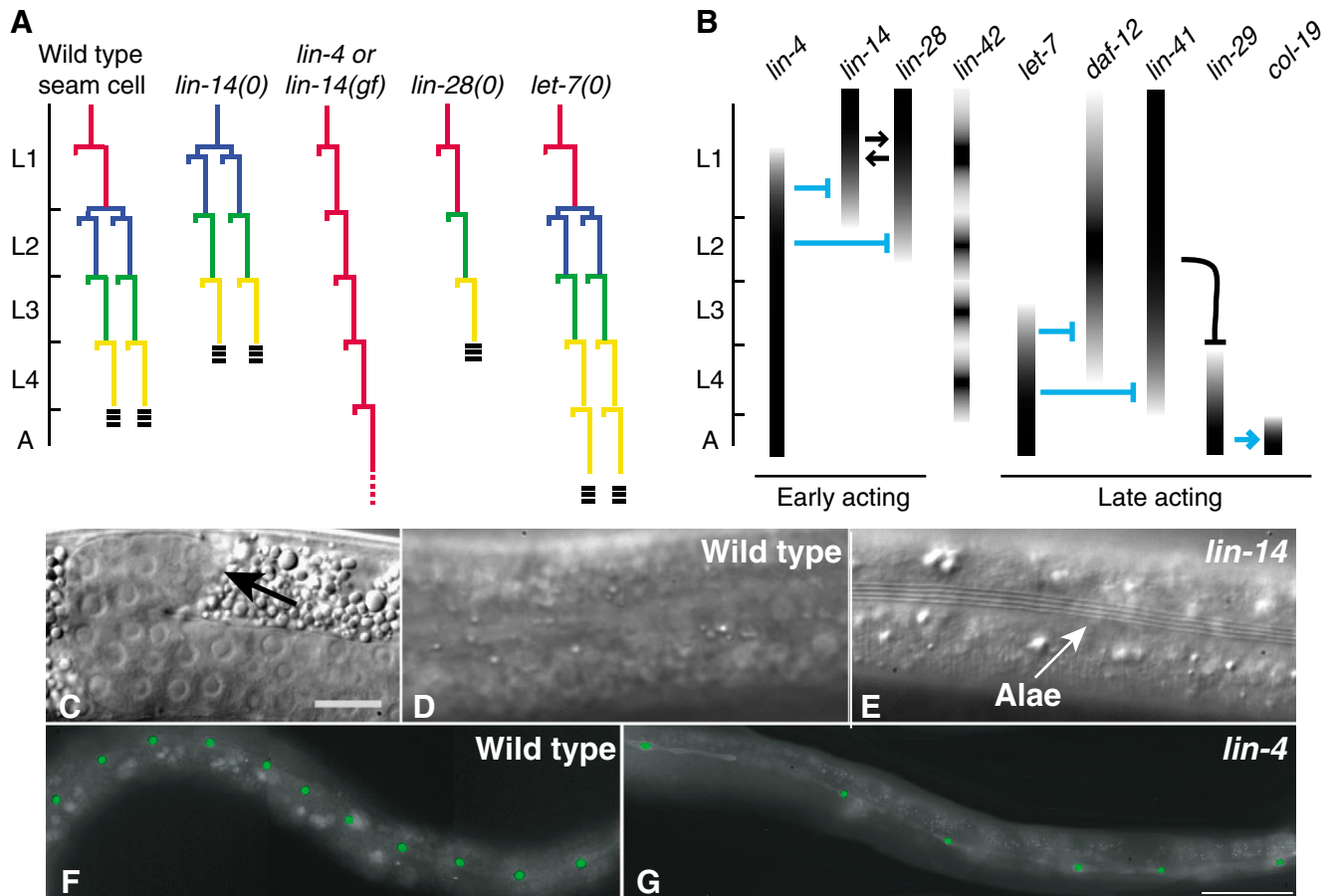
Fig. 1. Seam cell lineages and expression patterns of selected heterochronic genes. (A) The postembryonic cell division pattern of a generic seam cell (left), with the divisions specific to each larval stage color coded. Horizontal bars indicate the time of cell divisions. The triple horizontal bars at the bottom represent alae, hallmark cuticular ridges that are specific to the adult cuticle. Larval (L) stages are indicated on the left, with ticks denoting the molts. Seam cell lineage patterns for several null (0) mutations are shown on the right, together with that of *lin-14* gain-of-function (*gf*) alleles. The mutant lineages are color coded with respect to the wild-type lineage. (B) The expression patterns of selected heterochronic genes. Arrows indicate positive regulatory interactions and bars indicate negative regulatory interactions (direct interactions are shown in blue). (C-E) L3 molt animals identified by a characteristic just-reflexed gonad indicated by the black arrow in C. The cuticle of L3 molt stage wild-type animals is smooth (D), whereas in *lin-14(lf)* mutants it contains alae (E), cuticular ridges normally synthesized during the L4 molt. (F,G) L3 stage worms expressing *scm:gfp*, which marks seam cell nuclei. The nuclear *gfp* signal has been pseudo-colored green to distinguish it from background autofluorescence. The 11 seam blast cell nuclei in the left mid-body (V cells) are shown in wild type (F). In *lin-4* mutants at the same stage, there are only six V cells (G), owing to the omission of the L2 stage proliferative division (blue in A), which is normally executed by five V cells. Scale bars: in C, 5 μm for C-E; in G, 50 μm for F,G. *lin-4* mutants are longer than wild type.

pattern in its place, again leading to a precocious adult cuticle phenotype, albeit with a somewhat different underlying basis. The *lin-28* phenotype suggests that LIN-28 has a role in promoting the L2 stage proliferative division. However, this appears not to be the case; mutations in *lin-46*, a *lin-28* suppressor, allow the L2 stage fate to be expressed in the complete absence of *lin-28* activity (Pepper et al., 2004). Thus, in wild-type animals, the activity of *lin-28* postpones L3 fates, allowing for the expression of the L2 stage division pattern. The temporal decay of LIN-14 and LIN-28 levels is therefore a key factor for seam cell identity to progress through the early larval fates. Indeed, the continued expression of these proteins at inappropriately late times produces phenotypes that are essentially opposite to their loss-of-function (*lf*) phenotypes: development is retarded because of the reiteration of L1 (*lin-14*) or L2 (*lin-28*) stage patterns, and subsequent patterns are delayed (Ambros and Horvitz, 1984; Moss et al., 1997). A

crucial question to answer in terms of selecting temporal fates during early larval stages then logically moves upstream is how is the temporal decay of LIN-14 and LIN-28 managed? The answer is found in *lin-4*.

lin-4 is the founding member of the now extensive microRNA gene family (Lee et al., 1993) (see the miRNA registry at: http://microrna.sanger.ac.uk). In animals, these ~22 nucleotide non-coding RNAs generally act by binding sites of partial complementarity in the 3′UTR of target genes and inhibiting productive translation (Fig. 2A,B) (Olsen and Ambros, 1999; Seggerson et al., 2002). The *lin-4* miRNA begins to accumulate midway through the first larval stage and downregulates *lin-14* and *lin-28* by binding to their 3′UTRs, leading to a decrease in their protein levels as development progresses through the early larval stages (Fig. 1B) (Feinbaum and Ambros, 1999; Lee et al., 1993; Moss et al., 1997; Olsen and Ambros, 1999; Seggerson et al., 2002; Wightman et al.,

1993). Thus, in the absence of *lin-4* activity, LIN-14 and LIN-28 levels remain high, and seam cells reiterate the L1 division pattern during subsequent larval stages. There is no proliferative division at the start of the L2 stage and, consequently, these mutants have fewer seam cells than wild type (Fig. 1A,F,G; see also Box 1). The *lin-4* retarded phenotype is also characterized by extra 'larval' stages not observed in wild type. This mechanism of *lin-4* regulation correlates precisely with the gain-of-function (*gf*) experiments mentioned above. *lin-14(gf)* is caused by 3′UTR deletions that remove *lin-4* binding sites (Wightman et al., 1991), and *lin-28(gf)* arises in transgenic animals where a *lin-4* site has been deleted from a construct that is otherwise capable of rescuing *lin-28(lf)* mutations (Moss et al., 1997).

The regulation of the early larval stage timer is more complex than the simple downregulation of *lin-14* and *lin-28* by the *lin-4* miRNA. Additional levels of control are built into the temporal regulation of these early cell lineages. A complex feedback mechanism acts to further fine-tune *lin-14* and *lin-28* levels; each is required for optimal expression of the other (Arasu et al., 1991; Moss et al., 1997). Moreover, elegant genetics experiments have revealed that *lin-28* is also controlled by a *lin-4*-independent mechanism (Moss et al., 1997). Downregulation of LIN-28 occurs in the absence of *lin-4* when *lin-14* levels are reduced. Here too, the regulation occurs through the *lin-28* 3′UTR (Seggerson et al., 2002), raising the possibility that additional miRNAs act to modulate *lin-28* expression. Various experiments indicate that other heterochronic genes (including *lin-42*, *hbl-1* and *daf-12*, which are discussed in more detail later) also act in this early time window to ensure the proper temporal progression of development in the epidermis.

Given the hundreds of miRNAs that are now known, it is amazing that the second miRNA to be discovered, *let-7*, was also identified in worms as a key temporal regulator of seam cell identity (Reinhart et al., 2000). *let-7* miRNA is detected during the L3 and later stages, and is deployed to downregulate targets stage specifically in the epidermis, temporally guiding development to the adult stage. A major target of the *let-7* miRNA in the epidermis is *lin-41*, which encodes a cytoplasmic protein that has RNA-binding motifs (Slack et al., 2000). Loss of *lin-41* function causes a precocious phenotype in which seam cell lineage patterns appear to be wild type until the L3 molt, when terminal differentiation occurs one stage too early. The disappearance of LIN-41 from the hypodermis during the L4 stage depends upon the presence of its 3′UTR and the *let-7* miRNA (Slack et al., 2000). Moreover, the *lin-41* 3′UTR can direct temporal downregulation of a heterologous reporter gene, and this control is abrogated in a *let-7* mutant background (Reinhart et al., 2000). The temporal decay of LIN-41 activity presages the accumulation of LIN-29, the most downstream member of the pathway (Ambros, 1989; Slack et al., 2000). LIN-29 is a zinc-finger transcription factor that triggers the switch to the adult fate, in part through the stage specific control of collagen gene expression (Fig. 1B) (Rougvie and Ambros, 1995).

In summary, the *lin-4* and *let-7* miRNAs are key to guiding the gene expression transitions that temporally pattern the worm epidermis. Although many of the core components of the *lin-4*-directed early timer and the *let-7*-directed late timer have been identified and characterized, our knowledge of how these genes collectively convey temporal information during development is still incomplete. The identification of the precise functions, possible interaction partners and direct regulatory targets of LIN-14, LIN-28 and LIN-41 are future challenges. Direct regulators of *lin-29* activity also await identification, as does the mechanism that links the early and late timers. Whether molecularly related developmental timing mechanisms operate in other organisms also remains unknown. As *lin-4* and *let-7* miRNAs, and some of their targets, are conserved in other species (Lagos-Quintana et al., 2002; Pasquinelli et al., 2000), some temporal control mechanisms might share a common origin.

Keeping time with microRNAs in other organisms

We now know that miRNAs are neither restricted to *C. elegans* nor are they unique to developmental timing pathways. miRNAs function in diverse processes, including the specification of neuronal asymmetry (Chang et al., 2004; Johnston and Hobert, 2003), insulin secretion (Poy et al., 2004) and programmed cell death (Brennecke et al., 2003; Xu et al., 2003), and their mis-regulation has been linked to cancer (Calin et al., 2002; Calin et al., 2004; Takamizawa et al., 2004; Johnson et al., 2005). The study of these small non-coding RNAs has become one of the fastest-paced fields of research in both animal systems and plants (for reviews, see Ambros, 2004; Bartel and Chen, 2004; He and Hannon, 2004; Kidner and Martienssen, 2005).

let-7 led the march for the expansion of miRNA biology outside of *C. elegans*, when Ruvkun and colleagues discovered that it is conserved broadly in bilaterian animals, including humans (Pasquinelli et al., 2000). In fact, *let-7* is extremely highly conserved: the mature *let-7* miRNA sequence is identical between worms and humans (it is encoded by multiple genes in humans) (Fig. 2C). Moreover, *let-7* is a member of a larger gene family in both species; additional members contain imperfect sequence identity (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2002; Lau et al., 2001; Lim et al., 2003). miRNA cloning efforts have now also identified *lin-4*-related genes in worms, flies and vertebrates (Fig. 2D) (Ambros et al., 2003; Lagos-Quintana et al., 2002; Lim et al., 2003).

Whether *lin-4* and *let-7*-related miRNAs have conserved roles in developmental timing in other organisms has not been established at the level of mutational analysis; however, there is considerable evidence in favor of such a role. For example, in flies and zebrafish, *let-7* expression is activated late during development (Pasquinelli et al., 2000; Wienholds et al., 2005), indicating that its function in promoting late-stage cell fate decisions, such as the induction of terminal differentiation programs, may be a general feature of the gene. In flies, *let-7* miRNA is first detected as pupal formation begins and remains high in adults (Pasquinelli et al., 2000; Sempere et al., 2002). The timing of this activation suggests that it is regulated by ecdysone, a steroid hormone that triggers stage transitions during fly development, including pupal formation and metamorphosis (Riddiford, 1993). However, depletion of the ecdysone receptor by RNAi has little, if any, effect on *let-7* miRNA levels, yet it effectively eliminates expression of known ecdysone early response genes (Bashirullah et al., 2003). Moreover, these early response genes are rapidly induced in cultured cells in response to ecdysone treatment,
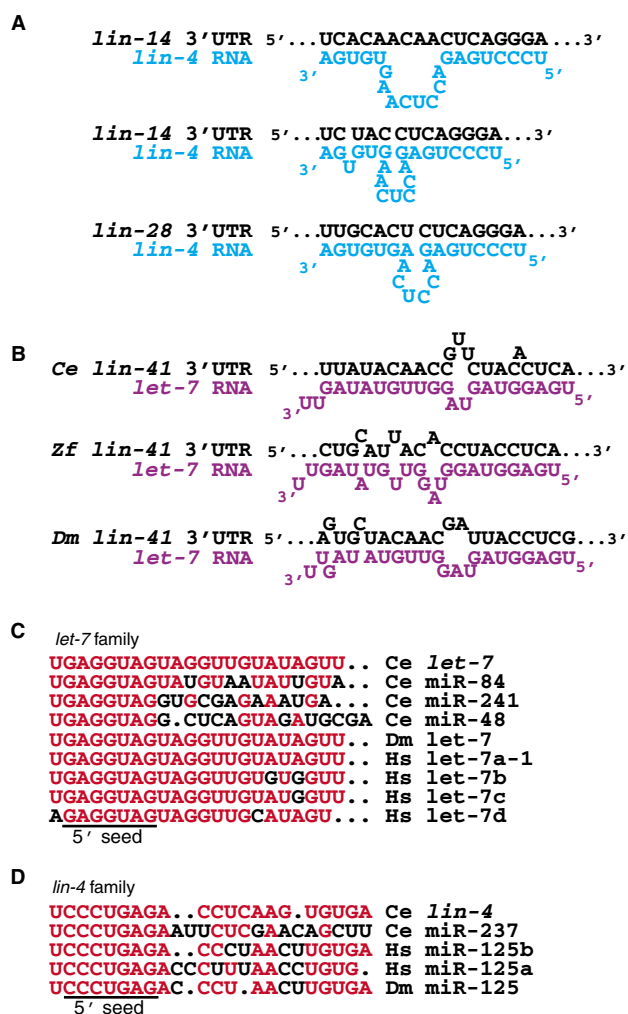
A
```
lin-14 3'UTR 5'...UCACAACAACUCAGGGA...3'
  lin-4 RNA      AGUGU      GAGUCCCU
              3'      G  A           5'
                       A  C
                      ACUC

lin-14 3'UTR 5'...UC UAC CUCAGGGA...3'
  lin-4 RNA      AG GUG GAGUCCCU
              3'    U  AA         5'
                       A  C
                       CUC

lin-28 3'UTR 5'...UUGCACU CUCAGGGA...3'
  lin-4 RNA      AGUGUGA GAGUCCCU
              3'          A A      5'
                         C  C
                          UC
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B
```
                           U   GU    A
Ce lin-41 3'UTR 5'...UUAUACAACC  CUACCUCA...3'
      let-7 RNA      GAUAUGUUGG  GAUGGAGU
                  3,UU          AU        5'

                        C  U  A
Zf lin-41 3'UTR 5'...CUG AU AC  CCUACCUCA...3'
      let-7 RNA      UGAU UG UG  GGAUGGAGU
                  3'     A  U GU           5'
                                A

                        G C      GA
Dm lin-41 3'UTR 5'...AUG UACAAC  UUACCUCG...3'
      let-7 RNA      UAU AUGUUG  GAUGGAGU
                  3,U G          GAU      5'
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C
let-7 family
```
UGAGGUAGUAGGUUGUAUAGUU.. Ce let-7
UGAGGUAGUAUGUAAUAUUGUA.. Ce miR-84
UGAGGUAGGUGCGAGAAAUGA... Ce miR-241
UGAGGUAGG.CUCAGAUGCGA Ce miR-48
UGAGGUAGUAGGUUGUAUAGUU.. Dm let-7
UGAGGUAGUAGGUUGUAUAGUU.. Hs let-7a-1
UGAGGUAGUAGGUUGUGUGGUU... Hs let-7b
UGAGGUAGUAGGUUGUAUGGUU.. Hs let-7c
AGAGGUAGUAGGUUGCAUAGU... Hs let-7d
 5' seed
```

D
lin-4 family
```
UCCCUGAGA..CCUCAAG.UGUGA Ce lin-4
UCCCUGAGAAUUCUCGAACAGCUU Ce miR-237
UCCCUGAGA..CCCUAACUUGUGA Hs miR-125b
UCCCUGAGACCCUUUAACCUGUG. Hs miR-125a
UCCCUGAGAC.CCU.AACUUGUGA Dm miR-125
 5' seed
```

Fig. 2. *C. elegans lin-4* and *let-7* miRNA family members and 3'UTR interactions. (A) Examples of heteroduplexes between *lin-4* and two out of seven binding sites in the *lin-14* 3'UTR and the single binding site in the *lin-28* 3'UTR (Moss et al., 1997; Wightman et al., 1993). The duplexes are imperfect and overall duplex structure varies because of binding site sequence variations; however, the 5' seed region is usually paired. (B) Representative heteroduplexes demonstrating conservation of *let-7*-binding sites in the 3'UTRs of *lin-41* homologs in worm (Ce), flies (Dm) and zebrafish (Zf) (Pasquinelli et al., 2000; Slack et al., 2000). (C,D) Alignments of *let-7* and *lin-4* family members. Residues identical to *C. elegans let-7* or *lin-4* are shown in red. The 5' seed region, which is important for binding-site selection, is underlined. (C) Alignment of the four worm (Ce) *let-7* miRNA family members with *let-7* genes from *Drosophila* (Dm) and human (Hs). Only a subset of known human *let-7* miRNAs is shown (Lim et al., 2003). (D) Alignment of *lin-4* miRNA family members (Ambros et al., 2003; Lim et al., 2003).

transcribed with *let-7* (Bashirullah et al., 2003). Unraveling whether these genes are required for specifying late-stage cell identities in the fly is thus likely to require targeted mutation of the *let-7/miR-125* locus coupled to transgenic expression of each single miRNA.

Not only are the *lin-4* and *let-7* miRNAs maintained and temporally regulated in other animals, but many of the genes they control in worms also have conserved homologs, including *lin-28* and *lin-41* (Moss and Tang, 2003; Pasquinelli et al., 2000). Moreover, putative binding sites for these miRNAs have been identified in the 3'UTRs of the *lin-28* and *lin-41* homologs (e.g. Fig. 2B), raising the possibility that *lin-4* and *let-7* miRNA-target pairs may be evolutionarily maintained across species (Moss and Tang, 2003; Pasquinelli et al., 2000). *lin-28* is found in flies and vertebrates, and tends to be expressed in early development and in undifferentiated cells, consistent with its expression profile in worms (Moss and Tang, 2003), hinting at a similar function. Remarkably, when the 3'UTRs of mouse and human *lin-28* are compared and examined for conserved miRNA-binding sites, *lin-4* and *let-7* homolog (miR-125b and let-7b, respectively) binding sites have the strongest miRNA basepairing predictions, supporting the idea of a conserved control mechanism. However, initial tests of this mechanism in mammalian tissue culture cells yield somewhat conflicting results. Luciferase reporter constructs bearing sequences from the mouse *lin-28* 3'UTR are expressed at lower levels than are constructs with control 3'UTRs, and this difference depends on the integrity of the *let-7*-binding site (Kiriakidou et al., 2004; Nelson et al., 2004). By contrast, decreased LIN-28 levels observed during the differentiation of specific cell lines appears to occur at the transcriptional level, and the depletion of *let-7* or *mir-125b* (*lin-4*) miRNA levels does not result in *lin-28* mis-expression (Lee et al., 2005; Sempere et al., 2004).

let-7 family member binding sites are also conserved in the *lin-41* 3'UTRs of flies and fish (Fig. 2B) (Pasquinelli et al., 2000). A reporter gene bearing a *lin-41* 3'UTR can be post-transcriptionally downregulated if artificially co-expressed with *let-7* in early fish embryos (Kloosterman et al., 2004), but additional experiments are required to test whether the *lin-41::let-7* partnership has been maintained in vivo and whether control of temporal identity is an aspect of its function. These vertebrate studies are confounded by the presence of multiple *lin-4* and *let-7* family members that are identical in their 5' 'seed' sequence (nucleotides 2-8; Fig. 2C,D) (Doench and Sharp, 2004). Basepairing between this region and a 3'UTR-binding site is a key factor in target recognition (see Fig. 2A,B), and, consequently, these miRNA family members have the ability to regulate common targets if co-expressed. This property complicates the functional analysis of these miRNAs through gene knockout experiments and makes it difficult to assess the miRNA dependence of target gene expression patterns.

Additional players in the *C. elegans* heterochronic gene pathway

Two central players in the *C. elegans* pathway do not fit neatly into either the early *lin-4*-directed timer or the late *let-7*-directed timer: *lin-42* and *hbl-1* (also known as *lin-57).* Mutations in these genes cause strong precocious phenotypes, with seam cell terminal differentiation occurring during the L3

whereas *let-7* miRNA is not. These experiments suggest that *let-7* induction occurs by a mechanism distinct from that of ecdysone early responders and is independent of the ecdysone receptor. Nevertheless, the temporal *let-7* miRNA accumulation profile, together with its widespread expression pattern (Sempere et al., 2002), suggests that its expression is under systemic hormonal control possibly via a distinct receptor. Curiously, the best match to the *lin-4* miRNA in flies, miR-125 (Fig. 2D) (Lagos-Quintana et al., 2002), is co-

molt (Abrahante et al., 2003; Jeon et al., 1999; Lin et al., 2003), but additional analyses suggest that they act at multiple points in the pathway.

LIN-42 is a worm homolog of the Period (Per) family of proteins, originally identified in insects and mammals (Jeon et al., 1999). The Per proteins function in a second biological timing mechanism, the control of circadian rhythms, raising the possibility that this protein family has a conserved role in timing events. Per proteins contain a hallmark protein interaction domain, the PAS domain, and the fly and mammalian homologs have been shown to function, at least in part, by interfering with transcriptional activator proteins (for a review, see Glossop and Hardin, 2002). Another feature of Per genes is their extremely dynamic expression pattern: their message and protein levels oscillate with a 24-hour period. A truly striking aspect of *lin-42* conservation is that, similar to *per*, its expression levels cycle, although with a shorter period that is coupled to molting cycles rather than to day length. This reiterative expression pattern (Fig. 1B), with high mRNA levels during each intermolt [a pattern that is reflected at the protein level (J. Tennessen and A.R., unpublished)] sets *lin-42* apart from the other members of the pathway and is suggestive of multiple or repeated roles for *lin-42* during postembryonic development. Genetic interactions with a weak *lin-14* allele suggest that *lin-42* has an early role in controlling the proliferative L2 division (Z. Liu, PhD Thesis, Harvard University, 1990). In this sensitized background, the proliferative division is omitted, indicating an early, albeit redundant, role for *lin-42*. By contrast, inactivation of *lin-42* alone does not appear to alter the early lineages, suggesting that it may have a later role in the pathway, a position supported by genetic studies that place *lin-42* in parallel to, or downstream from, *lin-46* and *let-7* (Pepper et al., 2004; Reinhart et al., 2000).

Although there have been reports of circadian behaviors in worms (Kippert et al., 2002; Saigusa et al., 2002), they have yet to be associated with *lin-42* function. However, worm genes with sequence relatedness to other circadian rhythm proteins have also been identified in the genome (Banerjee et al., 2005; Clayton et al., 2001; Jeon et al., 1999). Postembryonic RNAi-based depletion experiments for two of these, *kin-20* [a fly *doubletime* (*dbt*; *dco* – FlyBase) homolog] and, to a lesser extent, *tim-1* [a *timeless* (*tim*)/*timeout* homolog], reveal phenotypes and genetic interactions that suggest that these genes act in the timing pathway; their loss of function causes some aspects of the terminal differentiation program (e.g. cell fusion) to be activated precociously (Banerjee et al., 2005). However, the observed phenotypes are weaker and less penetrant than that produced by of *lin-42* inactivation, suggesting that these genes may play less central roles in the timing mechanism. In flies, Tim binds Per, contributing to its ability to interfere with transcriptional activators and the generation of its oscillatory expression pattern (Darlington et al., 1998; Lee et al., 1999). Dbt is a casein kinase that phosphorylates Per, thereby possibly potentiating its repressor activity (Nawathean and Rosbash, 2004). Future studies will reveal whether the worm homologs of these proteins act to modulate LIN-42 activity in ways similar to their control of Per. *C. elegans tim-1* is also a component of the cohesin complex (Chan et al., 2003), but how and whether this function relates to the reported postembryonic RNAi phenotype has not been addressed.

Similar to *lin-42*, *hbl-1*, which encodes the worm homolog of the *Drosophila Hunchback* (*Hb*) transcription factor, may influence seam cell temporal identity at multiple points. Omission of the L2 proliferative division occurs when *hbl-1* activity is depleted by RNAi (Abrahante et al., 2003), suggesting it has an early timing role. *hbl-1* might also have a later function, as indicated by the presence of many putative *let-7*-binding sites in its 3′UTR, and by the observation that, genetically, *hbl-1* appears to be partially redundant with the *let-7* target *lin-41* (Abrahante et al., 2003; Lin et al., 2003). Curiously, *hbl-1* mis-expression has not been detected in the epidermis of *let-7* mutants, a finding that may be explained by the existence of the three *let-7*-related miRNA genes: *mir-48*, *mir-84* and *mir-241* (Fig. 2C) (Lim et al., 2003). The presence of multiple *let-7* family members in worms, as in vertebrates, raises the possibility of functional redundancy and implicates these additional family members in developmental time control. Because these four miRNAs share perfect identity in the 5′ seed, they are likely to act through similar or overlapping sets of binding sites. All four family members show temporally restricted expression patterns (Lau et al., 2001; Lim et al., 2003), and if also expressed in the epidermis, these other family members might regulate *hbl-1* and perhaps other members of the pathway. Indeed, overexpression of *mir-84* results in precocious seam cell phenotypes, suggesting that it may time the terminal differentiation of this tissue in wild-type animals (Johnson et al., 2005). The ultimate test of whether *hbl-1* is regulated by *let-7* family member(s) in the epidermis awaits the generation of *C. elegans* strains that are null for all these miRNA genes, a project that is now under way (V. Ambros, personal communication).

HBL-1 and fly Hb share strongest sequence identity in their central four Cys_2-His_2 zinc fingers, which bind DNA in the fly, indicating that this function might be conserved. More impressive than this simple sequence conservation are recent experiments that suggest that these proteins share a common biological function – control of developmental time.

Hunchback homologs in worms and flies time developmental events

The conservation of Hb sequence between flies and worms provides a molecular link between developmental timing mechanisms in these organisms. Although perhaps best known for its role in spatial patterning (Lehmann and Nusslein-Volhard, 1987; Tautz et al., 1987), *hb* is a key regulator of temporal identity in the *Drosophila* CNS (Isshiki et al., 2001). The fly CNS also provides an exquisite system for examining temporal control mechanisms in animals. Similar to the blast cells of the *C. elegans* epidermis, fly neuroblasts (NBs) divide in invariant stem cell-like lineage patterns (Fig. 3), and cell identities can be determined by their position and the expression of molecular markers (for a review, see Skeath and Thor, 2003). The NBs divide asymmetrically, giving rise to a smaller ganglion mother cell (GMC), which divides to produce post-mitotic neurons, and a NB, which retains stem cell character and repeats the cycle. The sequential expression of several transcription factors [Hb→Krüppel (Kr)→Pdm1→Castor (Cas)] in the NB, and maintained in the GMC progeny, specifies temporal identity. Hb determines the identity of the first born progeny, Kr the second, and so on. In a manner remarkably reminiscent of heterochronic genes in
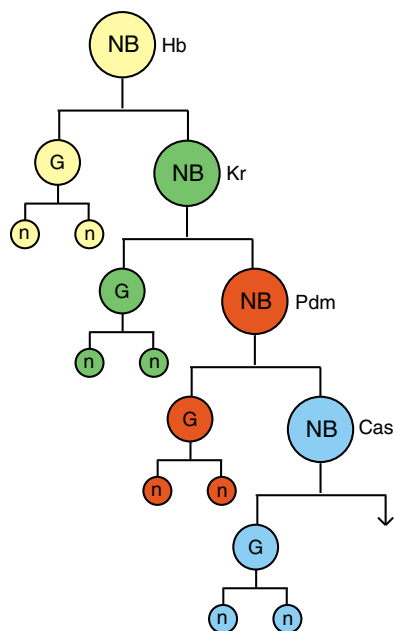
Fig. 3. Generic neuroblasts from the *Drosophila* CNS. The neuroblasts (NBs) divide in a stem-cell like fashion, giving rise to a ganglion mother cell (GMC, G) at each division. The GMCs divide to give rise to neurons (n). Although drawn symmetrically for simplicity, the GMC divisions are often asymmetric, giving rise to neurons of different types. The temporal progression of transcription factor expression in the NB specifies temporal identity in the successive GMCs and is indicated by color coding. Cas, Castor; Hb, Hunchback; Kr, Krüppel.

worms, Hb loss- and gain-of-function situations cause opposite temporal transformations in cell fate: Hb loss of function causes the first born fate to be omitted and subsequent identities to be expressed too early, whereas Hb gain of function (i.e. continued Hb expression in NBs at inappropriately late developmental times) results in the reiteration of the first-born fate (Isshiki et al., 2001).

Analysis of wild-type CNS development led to the proposition that intrinsic cues are largely responsible for controlling the NB timing mechanism because the division times of neighboring NBs can vary, generating a field of cells in which temporally distinct fates are juxtaposed (Isshiki et al., 2001). This hypothesis was confirmed by recent studies from the Doe laboratory, which elaborated upon the crucial role of Hb in the neuronal temporal control mechanism (Grosskortenhaus et al., 2005). The timing of the transcription factor cascade is maintained when isolated NBs divide in culture, and therefore it must be programmed largely by cell intrinsic cues. However, if NBs are cell cycle arrested, neuronal identity does not progress beyond the first-born fate; the cells remain Hb+, rather than switching to the subsequent Hb– Kr+ state. This transcription factor switching also fails to occur when the nuclear cell cycle continues in the absence of cell division [i.e. in *pebble* mutants (Hime and Saint, 1992; Lehner, 1992)]. Thus, a nuclear division-based counter is not employed to mark time, but cytokinesis is required for the cell to progress beyond the Hb-specified fate. These studies suggested that the generation of the GMC daughter may be required to signal to

the NB to change expression profiles, or that molecules required for the switch may be asymmetrically partitioned during the required cell division (Grosskortenhaus et al., 2005).

Intriguingly, the temporal transitions downstream of the Hb→Kr switch do not require cell division (Grosskortenhaus et al., 2005); when the cell cycle is blocked in the absence of Hb, the Kr→Pdm→Cas transcription factor progression is correctly specified. The temporal transitions in this late timer are thus triggered in a mechanistically distinct manner from the Hb→Kr transition. Moreover, Hb activity must play a key role in the inhibition of the cell cycle-independent late timer. But how? Grosskortenhaus et al. distinguish between two models. In one, Hb would block the initiation of the late timer. Think of a power-cut at 3 AM – the clock stalls until power returns at, say, 7 AM, at which time it resumes and marks the intervening hours 4, 5, etc. In the other model, Hb would simply inhibit the expression of the transcription factors, while the timer itself continues to progress, as in a power-cut to a computer – the timer progresses and when power returns its clock resumes from 7 AM, with the omission of the intervening times (or transcription factor profiles). Hb function fits the former model. Continued Hb expression at inappropriately late times causes the reiteration of first-born fates, and when it is subsequently removed, the temporal program resumes sequential transcription factor expression (Kr→Pdm→Cas) without the deletion of intermediate expression patterns. Hb is required to specify the first-born fate and to postpone the subsequent fates until the appropriate time. Thus, Hb negatively regulates the late timer, and its level must decline to allow the temporal progression of neuronal differentiation in the wild-type CNS. One can consider the downregulation of Hb expression as playing an important role in the diversification of neurons in the fly CNS; Hb decay allows the temporal progression of NB identity and the subsequent expansion of neuronal cell types.

Hb activity is thus a key component of the temporal identity timer that specifies neuronal cell fates. However, important questions remain. How does Hb activity specify the first-born fate? How does it inhibit the late timer? How is Hb expression spatially and temporally restricted within the NB lineage and downregulated to allow the transition to later fates? Answers to the first two questions await the identification of the direct Hb targets in the CNS and determination of its mode of action. Answers to the last question are beginning to emerge from studies of Hb expression. The regulation of Hb in the CNS appears to be largely transcriptional (Grosskortenhaus et al., 2005), although additional post-transcriptional modulation has not been ruled out. Such a post-transcriptional system could reinforce a primarily transcriptional mechanism, contributing to the observed rapid decay in Hb levels. Translational control of *Hb* plays a major role in the spatial patterning of the early fly embryo. Interestingly, this mechanism acts through the 3′UTR and employs Brat, a protein with sequence relatedness to LIN-41 (Sonoda and Wharton, 2001), which, similar to HBL-1, also acts downstream of *let-7* in the worm heterochronic gene pathway. Although *let-7*-binding sites are not present in the fly *hb* 3′UTR, other miRNAs could potentially play a secondary role in fine-tuning Hb levels (Abrahante et al., 2003; Lin et al., 2003).

Hb is expressed in the NB and first GMC daughter, and this expression is maintained in the GMC and its post-mitotic neuronal descendents. Although activators of Hb in the NB

lineage have not been identified, some models of Hb regulation have been ruled out. In contrast to the situation in the fly epidermis, where Hb maintains its own expression, neuronal expression of Hb in the GMC and its progeny is not maintained by an auto-feedback loop and must rely on heterologous activators. The loss of Hb from the NB, a key to late fate transitions, is not simply due to its asymmetric localization to the GMC when the NB divides (Grosskortenhaus et al., 2005). Rather, recent studies indicate that Hb expression is transcriptionally downregulated in the NB (Kanai et al., 2005).

One key to the puzzle of Hb regulation is found in the orphan nuclear receptor Seven-up (Svp) (Kanai et al., 2005). Alterations in *svp* expression cause temporal cell fate transformations that are essentially opposite to those caused by varying *hb* expression patterns. *svp(lf)* leads to reiteration of the early-born fate, similar to that observed with prolonged *hb* expression, whereas forced precocious expression of *svp* causes loss of the early-born fate, similar to *hb(lf)*. These observations suggest that *svp* might negatively regulate *hb* expression in early stage NBs. Indeed, *svp* is activated in the NB after the first GMC daughter is born, at the time of the transition from Hb→Kr expression. Moreover, prolonged expression of *hb* is observed in *svp(lf)* mutants and proper downregulation of a *hb* promoter:*lacZ* fusion is dependent on *svp* activity, raising the possibility that this regulation may be direct. These studies demonstrate nicely that the Hb→Kr transition is mediated by Svp, thereby allowing the transition to the late timer. In addition to identifying a new component of the NB identity timer, these studies also highlight the issue of how developmental time is controlled in the NB lineage. How does *svp* expression become activated in the early NB in order to orchestrate the subsequent temporal transitions? Is cytokinesis required? If so, why and how? Is there a signal from the newly born GMC? Only time will tell.

Nutritional inputs into developmental timing

The temporal control of neuronal differentiation has also been revealed through genetic studies of fly eye development, but with a unique twist: these studies have unexpectedly linked temporal control mechanisms to nutritional inputs and growth control. Target of rapamycin (Tor) and insulin receptor (InR) signaling pathways monitor nutrient status and mediate cell growth in animals (Fig. 4A) (for reviews, see Long et al., 2004; Neufeld, 2004). An intriguing new study reveals that increased signaling through the Tor/InR pathways causes precocious neuronal differentiation, whereas reduced activity delays differentiation (Bateman and McNeill, 2004).

The fly eye consists of hundreds of ommatidia, clusters of eight photoreceptors (R1-R8) that are arrayed in a stereotypic pattern. The differentiation of these receptors occurs in the larval eye imaginal disc, which is initially an undifferentiated epithelium. As the visually distinct morphogenetic furrow passes through the epithelium from posterior to anterior, the differentiation of photoreceptors is induced by an epidermal growth factor (Egf)/Ras/Mapk pathway (Wolff, 2003).

Bateman and McNeill (Bateman and McNeill, 2004) monitored the temporal profile of photoreceptor differentiation by assaying neuronal markers in clones of mutant cells that spanned the morphogenetic furrow. Clones of cells bearing mutations that increased Tor/InR signaling differentiated
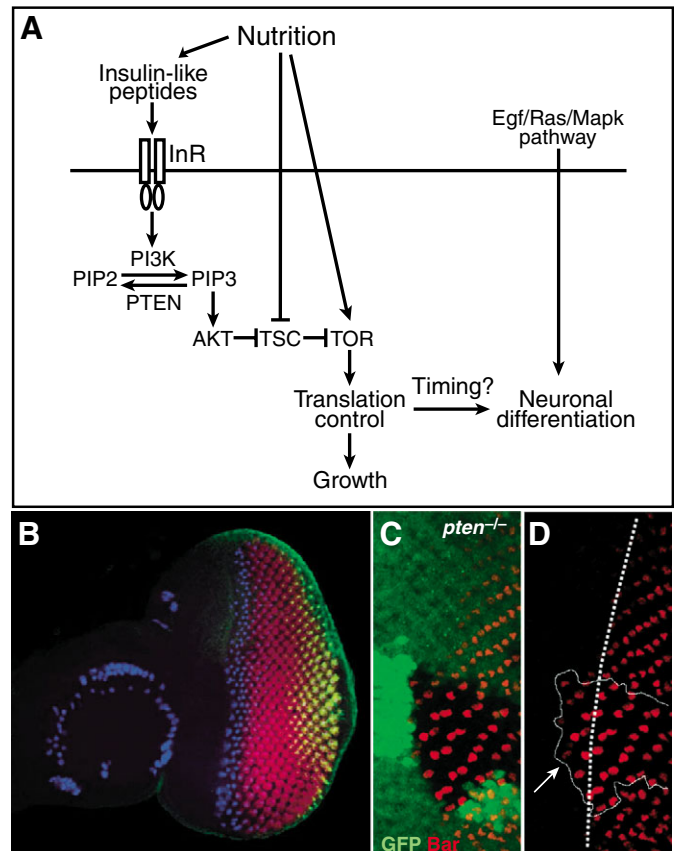

Fig. 4. Temporal control of neuronal differentiation in the fly eye. (A) Simplified diagram of the InR/Tor pathways. (B) Differentiation in the fly eye disc occurs in a temporal gradient from anterior to posterior, as demonstrated by the expression of differentiation markers (anterior is towards the left). Blue marks early fates, red marks intermediate and yellow indicates expression of late identities. Disruptions in Tor/InR signaling alter this temporal progression. (C,D) Loss-of-function mutations in *pten* cause the precocious expression of neuronal differentiation markers, including Bar. A *pten*^{-/-} cell clone, marked by loss of GFP expression (C) and outlined in D, expresses the neuronal transcription factor Bar (red) ahead of the normal differentiation front (D, broken line). Similar precocious expression is caused by overexpression of a PI3K subunit. By contrast, loss of InR, PI3K or Tor activity delays differentiation. See Bateman and McNeill (Bateman and McNeill, 2004) for further details. PI3K, phosphoinositide 3 kinase; PIP2 and PIP3, phosphatidylinositol (4,5)-diphosphate and (3,4,5)-triphosphate, respectively; PTEN, phosphatase and tensin homology; TSC, tuberous sclerosis complex. Reproduced, with permission, from Bateman and McNeill (Bateman and McNeill, 2004).

before their wild-type neighbors and expressed neuronal markers too early (Fig. 4B-D). By contrast, decreased Tor/InR signaling delayed differentiation. Moreover, the expression of components of the Egf/Ras/Mapk pathway appeared normal in mutant clones, suggesting that the Tor/InR pathways exert temporal control on the differentiation program at a downstream step in this signal cascade or through a parallel pathway. Importantly, the observed disruption of temporal control was not simply a result of altered cell size caused by the disruption of Tor/InR signaling. Increased cell mass caused

by other means (such as increased activity of cyclin D) failed to induce precocious differentiation.

These temporal fate alterations have morphological consequences in the adult fly. Although the composition and arrangement of the eight photoreceptors in each ommatidia appears normal, the organization of eye tissue in adults is disrupted (Bateman and McNeill, 2004), illustrating that precise temporal control is important for correctly patterning the tissue to ensure normal vision. These studies indicate that temporal control mechanisms can be linked to environmental cues, in this case nutritional status. Conceivably, such a link could allow the organism to coordinate developmental events during conditions of nutritional deprivation that could otherwise cause developmental defects. Such synchrony is particularly important for organs that are composed of cells from distinct lineages or cell types, or, in the case of neurons, where axonal projections may need to find target tissues at great distance. Interestingly, although the disruption of the Tor/InR pathway also temporally altered neuronal differentiation in leg imaginal discs, it failed to shift the timing of non-neuronal cell differentiation in wing discs, suggesting that Tor/InR signaling is not an essential global temporal regulator of differentiation programs (Bateman and McNeill, 2004). Rather, these pathways may provide a more specific link between neuronal programs and nutrition.

Although this example of temporal transformation is not on the same scale as the life-stage temporal transformations seen in *C. elegans*, it nevertheless significantly expands our repertoire for studies of developmental time control in animals. Once more, a common theme arises – timing molecules need to be precisely controlled to establish developmental synchrony between tissues; too much or too little signaling activity causes opposite temporal transformations. In addition, the possibility that timing molecule activity is modulated by miRNAs again looms on the horizon because a miRNA has been implicated in the control of insulin secretion in mammals (Poy et al., 2004). Future work will need to determine whether there is an intersection between this nutrient-sensitive mechanism in flies and the heterochronic gene pathway, and to test, for example, whether Tor or InR signaling pathways, parts of which are conserved in worms (Hara et al., 2002; Jia et al., 2004; Long et al., 2002), influence developmental time control in the nematode relative to nutritional status.

It has been established that nutritional status influences multiple steps of the heterochronic gene pathway. If worms hatch in the absence of food, the L1 hatchlings arrest development and can survive several weeks. In this state of 'L1 diapause', postembryonic cell divisions do not occur and the heterochronic gene pathway is not initiated. LIN-14 levels remain elevated (Arasu et al., 1991) and *lin-4* is not activated. The heterochronic gene timer is thus ultimately dependent upon external food signal(s), although how directly this timing mechanism is linked to nutritional status is unknown. One approach to investigating this problem would be to work backwards from *lin-4* activation – at present *lin-4* activation, which occurs ~12 hours after L1 larvae are place on food (Feinbaum and Ambros, 1999), is the most upstream step in the heterochronic gene pathway. The identification of the transcriptional regulator(s) of *lin-4* and the testing of whether their activity is altered by nutritional status, will be important next steps.

Nutritional cues also affect the heterochronic gene pathway with respect to the developmental choice between proceeding directly through the L3 stage or instead forming a dauer larva, an alternative third larval stage specialized for stress-resistance and dispersal (Cassada and Russell, 1975). Adverse conditions such as food shortage and high population density trigger dauer larva formation. A key player in this decision is *daf-12*, which encodes a nuclear hormone receptor that also acts in the heterochronic pathway (Antebi et al., 1998; Antebi et al., 2000). The *daf-12* locus is complex. Null mutations are dauer defective, whereas specific alleles have highly penetrant heterochronic phenotypes, including reiteration of the proliferative L2 division during subsequent stages, a retarded heterochronic phenotype. In an interesting twist to the heterochronic gene pathway, *daf-12* has recently been identified as a *let-7* target in the hypodermis, and its mis-regulation at late larval stages is likely to contribute to the *let-7* mutant phenotype (Großhans et al., 2005).

Interestingly, the *daf-12* alleles that produce strongly retarded phenotypes contain mutations in the ligand-binding domain of the protein (Antebi et al., 2000), suggesting that hormonal inputs are key to wild-type DAF-12 function and prevention of these phenotypes. The hormonal control of *daf-12* through its ligand binding domain is also supported by the finding that *daf-9*, which acts just upstream of *daf-12*, encodes a cytochrome P450, a class of enzyme required for steroid hormone biosynthesis (Gerisch et al., 2001; Jia et al., 2002). Binding of DAF-12 by hormone has been proposed to promote reproductive development at the L2 molt, while the unbound form triggers dauer formation (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). Wild-type levels of *daf-9* expression, and possibly downstream hormone levels, depend on a variety of factors, including activity of the worm insulin receptor (DAF-2) and feedback regulation by *daf-12*. Such nutritional and hormonal inputs to the heterochronic gene pathway, mediated through DAF-12, could provide a means for integrating nutritional signals and coordinating the progression of temporal cell fates throughout the animal.

Conclusions and future directions

Temporal control is an important facet of the developmental mechanisms employed to produce the complex body plans of multicellular organisms. Considerable progress has been made in elucidating the molecular components deployed to time developmental events, particularly within the *C. elegans* epidermis. However, several key issues remain to be resolved. Aside from a few miRNA:target interactions, direct connections between heterochronic gene pathway members are essentially unknown. This problem is exemplified by the dynamic expression pattern of *lin-42*, which must reflect short, and possibly regulated, half-lives of both its protein and mRNA. How this pattern is established and what its functional significance is are unknown, as is the precise mechanism by which LIN-42 acts and the identity of its interaction partners. This deficit of functional information is true of most pathway members, including the miRNA components to some extent, and its remedy is key to deciphering the timing pathway in the epidermis. And yet, elucidation of the molecular mechanism that times stage identity in the epidermis is one piece of a much larger puzzle: how are developmental events throughout the animal synchronized? Answering this question will require

delineating the timing pathway components used in other tissues and the molecules that coordinate these pathways. This coordination is likely to employ cues that act systemically, and highlights the importance of searching for hormonal inputs, such as those predicted to modulate of DAF-12 activity (Antebi et al., 2000).

Recent advances in understanding temporal control have also been made in *Drosophila*, including the important finding that alterations in signaling through the insulin/Tor pathway can alter the time of cell differentiation (Bateman and McNeill, 2004). This finding brings environmental cues and the issue of the nutritional status of an organism into the equation of developmental time control, thereby suggesting additional avenues to explore in worms for the signals that mediate food inputs to *lin-4* activation. Understanding the mechanism that links the Tor/InR pathway to fly neuronal differentiation will require the identification of its downstream components, perhaps involving translational control mechanisms, as used by these pathways in growth control.

Still unanswered is the extent to which conserved components of the worm heterochronic gene pathway time developmental events in other organisms. At present, the only clear example is the timing of NB identity by *Drosophila hb*, but it is still not clear whether there are other molecules shared by these two timing pathways. Tests of other conserved genes in flies and vertebrates (including *lin-28*, *lin-41*, and the *lin-4* and *let-7* miRNAs) will require inactivation studies, preferably by mutational analysis, to determine whether they act in temporal control mechanisms. However, the possibility remains that timing mechanisms in other organisms might be largely distinct from that of nematodes. Thus, further insights into developmental time control in other species, vertebrates in particular, might also require forward genetics, e.g. the design of zebrafish screens around reporter gene temporal misexpression strategies.

Importantly, in each of the systems discussed in this review, the mutations analyzed alter temporal cell identities independently of cell fate. Thus, such studies are greatly expanding our understanding of developmental time control, thereby closing the knowledge gap between our understanding of spatial and temporal control mechanisms. An additional challenge for the future will be to decipher the mechanisms that integrate spatial and temporal information, together with cues that specify sexual cell identity, as an organism develops.

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