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An elegant miRror: microRNAs in stem cells, developmental timing and cancer

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Abstract

MicroRNAs (miRNAs) were first discovered in genetic screens for regulators of developmental timing in the stem-cell-like seam cell lineage in *Caenorhabditis elegans*. As members of the heterochronic pathway, the *lin-4* and *let-7* miRNAs are required in the seam cells for the correct progression of stage-specific events and to ensure that cell cycle exit and terminal differentiation occur at the correct time. Other heterochronic genes such as *lin-28* and *lin-41* are direct targets of the *lin-4* and *let-7* miRNAs. Recent findings on the functions of the *let-7* and *lin-4/mir-125* miRNA families and *lin-28* and *lin-41* orthologs from a variety of organisms suggest that core elements of the heterochronic pathway are retained in mammalian stem cells and development. In particular, these genes appear to form bistable switches via double-negative feedback loops in both nematode and mammalian stem cell development, the functional relevance of which is finally becoming clear. *let-7* inhibits stem cell self-renewal in both normal and cancer stem cells of the breast and acts as a tumor suppressor in lung and breast cancer. *let-7* also promotes terminal differentiation at the larval to adult transition in both nematode stem cells and fly wing imaginal discs and inhibits proliferation of human lung and liver cancer cells. Conversely, LIN-28 is a highly specific embryonic stem cell marker and is one of four “stemness” factors used to reprogram adult fibroblasts into induced pluripotent stem cells; furthermore, *lin-28* is oncogenic in hepatocellular carcinomas. Therefore, a core module of heterochronic genes—*lin-28*, *lin-41*, *let-7*, and *lin-4/mir-125*—acts as an ancient regulatory switch for differentiation in stem cells (and in some cancers), illustrating that nematode seam cells mirror miRNA regulatory networks in mammalian stem cells during both normal development and cancer.

Stem cells

Stem cells have risen to prominence in all areas of biology since the discovery that embryonic development and adult tissue growth and maintenance result from a cellular hierarchy in which multiple cell types are derived from single, self-renewing stem cells. The key to understanding stem cells is likely to lie in discovering how they are able to divide indefinitely without differentiating (self-renew) while retaining the ability to produce progeny that are able to differentiate into multiple cell types. While the undifferentiated stem cell fate is maintained during self-renewal, differentiating cells derived from these stem

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cells must remain committed to an increasingly restricted range of fates through multiple mitotic divisions. The stable maintenance of these dichotomous states implies that epigenetic mechanisms are involved. Further, the differentiation process clearly has an important temporal element; changes in gene expression over time are required for the correct sequence of events resulting in progressive restriction of cell fate. Temporal patterning and fate determination have of course been well studied in developmental model organisms, and indeed, several recent studies have shown intriguing parallels between the regulation of vertebrate stem and progenitor cells and developmental timing by MicroRNAs (miRNAs) in a stem-cell-like lineage of *Caenorhabditis elegans*. As such, the powerful genetic tools available to nematode researchers provide a useful means to identify novel components of the pathways regulating stem cell function and development.

C. elegans seam cells as a model stem cell population

The heterochronic genes (Moss 2007) determine the relative timing of developmental decisions in *C. elegans*. These genes were initially identified by the isolation and cloning of mutants with alterations in the relative order of a set of developmental events during postembryonic development of the hypodermis (skin) of the nematode (Ambros and Horvitz 1984). The seam cells are hypodermal stem cells that divide in a stereotyped, invariant manner to produce different types of hypodermal and neural cells (Sulston and Horvitz 1977; Fig. 1). Generally, seam cells divide asymmetrically once at the beginning of each larval stage to produce an anterior cell that differentiates and fuses with the hypodermal syncytium and a posterior daughter that retains the seam stem cell fate, dividing again at the next larval stage. Seam cells also undergo a symmetrical proliferative division at the beginning of the second larval stage as well as at the third larval stage in the male posterior seam (Sulston et al. 1980; Sulston and Horvitz 1977). Both types of division result in self-renewal of the seam cells, but it is the asymmetric divisions that allow production of differentiated neural and epidermal cells (Fig. 1). Thus, seam cells provide a simple model for the development of stem cells and their progeny.

Each larval stage—developmental periods terminated by molting—has a distinct pattern of cell division and cell fates. The order and progression of these events is regulated by a network of genes that includes two miRNA families— the *lin-4* and *let-7* families and the protein coding genes *lin-14, lin-28, lin-41, hbl-1, daf-12*, and *lin-29*, amongst others (Fig. 2; Moss 2007; Rougvie 2005; Slack and Ruvkun 1997). Interestingly, most of the heterochronic genes are targets of either *lin-4* or *let-7* miRNAs; thus, miRNA switches regulate developmental timing in this pathway (Figs. 2 and 3). The L1-to-L2 switch is mediated by *lin-4* repression of *lin-14*, the L2-to-L3 switch by *lin-4* and *let-7* family (*mir-48*, *-84*, and *-241*) repression of *lin-28* and *hbl-1*, and, finally, the L4-to-adult switch by *let-7* repression of *lin-41* and *hbl-1* (Fig. 2). *daf-12* appears to integrate hormonal signals into this timing pathway and determine the timing of *lin-28* down-regulation; it in turn is repressed by *let-7* (Grosshans et al. 2005; Morita and Han 2006). The most downstream target of the heterochronic pathway identified genetically is *lin-29*, which is a Krüppel-like transcription factor required for terminal differentiation of the seam stem cells. Other heterochronic genes have been identified, but it is not clear how they fit into the pathway. For example, *lin-46* encodes a putative scaffolding protein involved in the repression of L2

fates, which was identified as a mutant that suppressed *lin-28* mutant defects (Pepper et al. 2004), and *lin-42* is a homologue of the circadian gene *Period* that prevents precocious terminal differentiation of the seam stem cells (Jeon et al. 1999), but the molecular roles of these genes in the heterochronic pathway are not well understood.

lin-4 and *let-7* were the first miRNAs to be identified; at the time of the discovery of *lin-4* in the early 1990s (Lee et al. 1993), the significance of this small regulatory RNA was overlooked by all but a few dedicated nematode geneticists (Ruvkun et al. 2004). The subsequent identification of the gene responsible for the retarded *let-7* heterochronic mutant as another miRNA (Reinhart et al. 2000) led to the discovery that almost all eukaryotes produce these short ~22 nucleotide RNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Pasquinelli et al. 2000) as well as other more recently described classes of small non-coding RNAs such as piRNAs and endo-siRNAs (Ambros et al. 2003; Brennecke et al. 2007; Czech et al. 2008; Ghildiyal et al. 2008; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Okamura et al. 2008; Ruby et al. 2006; Tam et al. 2008). In addition, the discovery of RNAi and the finding that miRNAs are processed and act via a related pathway has resulted in a small RNA revolution (Ambros 2001; Ruvkun 2008; Sharp 2001). The importance of miRNAs in regulating gene expression is only just beginning to be elucidated, but with the massive expansion of this new field and the advent of improved technologies, our understanding of this new class of regulators is rapidly growing.

miRNAs are processed in two steps from RNA polymerase II transcribed primary transcripts called “pri-miRNAs” (primary miRNAs) via short, approximately 70 nucleotide stem-loop hairpin precursors [precursor miRNAs (pre-miR-NAs)] into the mature 21–23 nucleotide mature miRNA (Kim et al. 2009). The initial processing is performed in the nucleus by the Drosha/DGCR8 (Pasha) microprocessor complex, which cleaves the pri-miRNA around the hairpin at the base of the stem. The resulting pre-miRNA hairpin is transported out of the nucleus by Exportin 5/RanGTP and, once in the cytoplasm, is cleaved by the RNase Dicer in the RISC loading complex (RLC), which is also composed of TRBP and Argonaute proteins, to produce a double-stranded RNA duplex of the 22 nucleotide mature miRNA and the partially complementary passenger (miRNA*) strand (Kim et al. 2009). The duplex is unwound and the mature miRNA is loaded into the miRNA-induced silencing complex (miRISC, the Argonaute-containing effector complex), while the passenger strand is displaced and degraded. The single-stranded miRNA acts as a sequence-specific guide for the silencing complex to target particular messenger RNAs (mRNAs) and repress gene expression either by translational inhibition and exonuclease digestion of the mRNA or, in the case of highly complementary interactions, mRNA cleavage (Kim et al. 2009). At least some miRISC complexes are associated with subcellular compartments involved in mRNA degradation and storage known as processing bodies (P bodies) and stress granules (Leung and Sharp 2006).

The seed sequences (core, specificity-defining regions located two to eight nucleotides from the 5′ end of the 22nt mature miRNA sequences) of *let-7* and *lin-4* are conserved between worms, flies, and mammals. The *lin-4* family members are known as *mir-125* miRNAs in other organisms, while the *let-7* miRNA family is so highly conserved (the nematode *let-7* sequence is absolutely identical to human *let-7a*) that this family is the only one not to

follow the *mir-X* nomenclature and are known as *let-7* family miRNAs in vertebrates (Pasquinelli et al. 2000). There are multiple members of the *lin-4/mir-125* and *let-7* families in nematodes and mammals, but only one of each in flies (Pasquinelli et al. 2000; Roush and Slack 2008; miRNA families are defined by an identical seed sequence, whereas the remainder of the mature miRNA sequence varies between members). The function of *let-7* and *lin-4* miRNAs and their targets that were identified in *C. elegans* have, in some cases, been proven to be conserved; significantly, two of the protein-coding genes in the nematode heterochronic pathway, *lin-28* and *lin-41*, have now been identified as having major roles in regulating miRNA expression and processing in stem cells in other organisms (discussed below). As such, it may be that further research into the nematode heterochronic pathway will reveal further insights into conserved mechanisms of control—both miRNA and otherwise—of stem cell traits.

Cancer as a retarded heterochronic defect

miRNAs are increasingly associated with cancer, either as tumor suppressors or oncogenes; such miRNAs have been dubbed “oncomiRs”. The *let-7* miRNAs are prime examples of oncomiRs since they act as tumor suppressors in numerous cell types. The important role that *let-7* has in both developmental timing and cancer leads us to speculate that other regulators of developmental timing also are involved in oncogenesis. It is easy to envisage how the disruption of the temporal regulation of proliferation and differentiation could lead to the aberrant expansion of immature cells, especially in light of the recent validation of the cancer stem cell hypothesis (Visvader and Lindeman 2008). The hierarchy of development from stem cells through to terminally differentiated, tissue-specific cell types is critically dependent on the temporal coordination of proliferation, commitment, differentiation, and maturation. Cancer can be viewed as both the failure of cells to properly transition from one developmental phase to another and the decoupling of normally coordinated behaviors. In heterochronic terminology, cancer is a retarded heterochronic defect in which the transition to the mature differentiated stage is delayed or blocked and earlier developmental programs are reiterated, leading to the proliferation and self-renewal of progenitors at later stages than normal. In this review, we discuss the conserved roles of the *C. elegans* heterochronic genes, both miRNA- and protein-encoding, in regulating developmental timing in stem cells and cancer. Detailed discussions of the heterochronic genes in *C. elegans* and the role of other miRNAs in cancer are reviewed elsewhere (Esquela-Kerscher and Slack 2006; Moss 2007; Rougvie 2005; Slack and Ruvkun 1997; Stefani and Slack 2008).

Conservation of relative temporal expression of the heterochronic genes, *let-7* and *lin-4/mir-125*, and their targets, *lin-41* and *lin-28*

Many miRNAs show spatiotemporally regulated expression where the mature species is present only at certain times in development or in particular cell types. Indeed, profiling studies have provided evidence that the lineage-specific expression of some mammalian miRNAs may control downstream effectors of differentiation (Sempere et al. 2004; Smirnova et al. 2005; Wulczyn et al. 2007). In addition, the temporally regulated expression and function of miRNAs in mammalian stem cells and during development of insects and vertebrates is particularly interesting, as it suggests that their role as switch genes for

differentiation along the temporal axis in *C. elegans* seam cells has been conserved in other organisms.

Drosophila let-7 and *mir-125* (the fly *lin-4* ortholog) are expressed in a developmentally regulated manner at the juvenile-to-adult transition mirroring the expression of *let-7* family miRNAs in mid to late larval stage nematodes to direct the larval-to-adult transition (Caygill and Johnston 2008; Sempere et al. 2002, 2003; Sokol et al. 2008). The expression of the vertebrate *let-7* and *mir-125* family members is restricted to late stages of vertebrate embryonic development; mature *let-7* and *mir-125* miRNAs are largely absent in early embryos, embryonic stem (ES), and embryonal carcinoma (EC) cells, but are produced once cells are induced to differentiate (Lee et al. 2005; Rybak et al. 2008; Schulman et al. 2005; Thomson et al. 2004; Wienholds et al. 2005; Wulczyn et al. 2007; Fig. 3). Intriguingly, in an EC neural differentiation model and in whole mouse embryos, the *lin-4* family miRNA, *mir-125* starts to be expressed before the *let-7* family miRNAs, as it does in nematodes (Lee et al. 2005; Schulman et al. 2005).

Orthologs of *lin-41* and *lin-28* also exhibit temporally regulated expression during embryonic development and in vitro ES or EC cell differentiation and importantly are expressed reciprocally to the *let-7* and *mir-125* family miRNAs (Lee et al. 2005; Richards et al. 2004; Schulman et al. 2005; Wu and Belasco 2005; Yang and Moss 2003; Yokoyama et al. 2008; Fig. 3). Moreover, the miRNA regulation of these genes is conserved: *lin-41* genes are conserved as *let-7* family targets in human, mouse, chick, zebrafish, *Drosophila*, and *C. elegans* (Kanamoto et al. 2006; Kloosterman et al. 2004; Lin et al. 2007; O'Farrell et al. 2008; Slack et al. 2000; Vella et al. 2004a, b), and the *lin-4*/*mir-125* and *let-7* miRNA families down-regulate *lin-28* genes via complementary sites in their 3′ UTRs in both mammals and *C. elegans* (Morita and Han 2006; Rybak et al. 2008; Wu and Belasco 2005). The core heterochronic pathway genes, *let-7* and *lin-4/mir-125* miRNAs, and the *lin-41* and *lin-28* gene families are functionally interlinked in a variety of processes, and this network, or elements of it, appears to have been utilized to control developmental timing and transitions between developmental states in diverse organisms and cell types throughout evolution.

let-7 family miRNAs in developmental timing, stem cells, and cancer

let-7 is a conserved regulator of cell cycle exit and differentiation (Reinhart et al. 2000). In *C. elegans*, *let-7* mutants have a retarded heterochronic phenotype such that the seam cells fail to exit the cell cycle at the correct time and instead undergo an extra round of division and terminally differentiate (by fusing and producing alae cuticle) one stage later than normal (Reinhart et al. 2000; Fig. 2). This phenotype combined with high conservation of this miRNA family through evolution implies that *let-7* might act as a master regulator of proliferation and differentiation, and subsequent work has indeed confirmed this hypothesis. In human cancer cell lines, *let-7* inhibits cell proliferation, and microarray analysis showed that this is due to repression of cell cycle genes such as *cyclin D2*, *CDK6*, and *CDC25*, all of which contain predicted *let-7* complementary sites in their 3′ UTRs, implying that this regulation is direct (Johnson et al. 2007). *let-7* acts as a tumor suppressor in various cancers, notably lung and breast cancer (Esquela-Kerscher et al. 2008; Johnson et al. 2005, 2007;

Kumar et al. 2008; Lee and Dutta 2007; Takamizawa et al. 2004; Yu et al. 2007a). *let-7* was found to directly down-regulate *RAS* in nematodes and humans (Johnson et al. 2005; Takamizawa et al. 2004), and *HMGA2* has been found to be another major oncogenic target of *let-7*. Exogenous expression of *let-7* inhibits tumor growth in lung and breast cancer models in part by down-regulating these oncogenic targets (Esquela-Kerscher et al. 2008; Kumar et al. 2008; Yu et al. 2007a). Expression profiling studies of a variety of human tumor samples and cancer cell lines have found *let-7* to be significantly down-regulated in a variety of human cancer cells compared to normal tissue. Indeed, miRNAs in general have been found to be down-regulated in cancer cells (Lu et al. 2005; Takamizawa et al. 2004; Thomson et al. 2006) and miRNAs are frequently mutated in cancer (Calin et al. 2004). SNP analysis of 3′ UTRs has identified a cancer-associated mutation in a *let-7* complementary site in the *K-RAS* 3′ UTR in lung cancer patients (Chin et al. 2008), and many tumors with high HMGA2 levels have translocations that remove the 3′ UTR of *HMGA2* containing multiple *let-7* complementary sites and thus abrogate down-regulation by *let-7* (Lee and Dutta 2007; Mayr et al. 2007). Consistent with these observations, many other miRNAs have been found to act as tumor suppressors, although some have also been identified as oncogenes (Esquela-Kerscher and Slack 2006). Furthermore, miRNA expression patterns of cancer cells are more distinctive of different cancer types than the overall mRNA profile (Lu et al. 2005). Thus, the promise of miRNAs for cancer research is twofold: both diagnostic aids and potential targets and tools for therapy (Slack and Weidhaas 2006).

let-7 family miRNAs seem to be associated with the transition from juvenile or embryonic stages to adulthood in worms, flies, and vertebrates. In *Drosophila, let-7* was recently shown to regulate various processes associated with ecdysone-induced metamorphosis at the larval to adult transition (Caygill and Johnston 2008; Sokol et al. 2008). In particular, *let-7* showed a remarkable conservation of function in regulating the timing of cell cycle exit. The wing imaginal discs normally undergo terminal cell cycle exit 24 h after puparium formation, but in *let-7, mir-125* mutant clones, the cells continued to divide and had increased apoptosis and smaller size. This defect was attributed specifically to loss of *let-7*, as misexpressing *let-7* in larval stage wing discs caused precocious cell cycle arrest (Caygill and Johnston 2008). Therefore, *let-7* appears to have a conserved role in programming cell cycle exit and terminal differentiation of larval cells in *Drosophila* wing imaginal discs and *C. elegans* seam cells (Reinhart et al. 2000). In addition, the timing of remodeling of the neuromuscular junction (NMJ) is delayed in the *let-7, mir-125* mutant flies due to failure to repress the BTB-POZ zinc finger protein Abrupt at the pupal stage (Caygill and Johnston 2008; Sokol et al. 2008). Indeed, the cell cycle exit and NMJ remodeling delays of the *let-7, mir-125* mutant flies are highly analogous to retarded phenotypes of the *let-7* and *lin-4* mutant nematodes, suggesting that the heterochronic pathway miRNAs have a conserved function in developmental timing, promoting the larval to adult switch in both nematodes and flies.

In another parallel with *C. elegans*, where *let-7* is expressed late in development of the seam cells to promote differentiation and inhibit proliferation and self-renewal (Esquela-Kerscher et al. 2005; Johnson et al. 2003), mammalian *let-7* appears to be selectively depleted in stem cell populations of both normal and malignant tissue. In breast cancer, *let-7* was down-regulated in the self-renewing, multipotent breast-tumor-initiating cells (BT-ICs) compared

to the non-self-renewing population of cancer cells and was up-regulated as the cells differentiated, while RAS and HMGA2 had the opposite expression profile (Yu et al. 2007a). Enforced expression of *let-7* in these cells inhibited self-renewal and promoted multilineage differentiation via repression of both RAS and HMGA2 expression. The idea that these BT-ICs may be derived from, or similar to, normal mammary stem cells is corroborated by a separate study examining *let-7* in the mouse mammary epithelial progenitor cell line, Comma-Dβ (Ibarra et al. 2007). Exogenous expression of *let-7* depleted the proportion of self-renewing mammary stem/progenitor cells, and this was consistent with lower levels of expression of *let-7* in this compartment (Ibarra et al. 2007). Strikingly, a *let-7* sensor construct was more highly expressed in the stem/progenitor cells and could be used to prospectively enrich for self-renewing multipotent cells. Similarly, in ES cells, mature *let-7* is not expressed until the cells are induced to differentiate, and in vivo, *let-7* is not expressed until late in embryonic development (Lee et al. 2005; Newman et al. 2008; Rybak et al. 2008; Schulman et al. 2005; Thomson et al. 2004; Viswanathan et al. 2008). Recently, *let-7a* was shown to be required for the differentiation of neural progenitors in mice (Schwamborn et al. 2009). It will be interesting to discover whether low levels of *let-7* are also associated with other stem cell populations, that is, is *let-7* a general inhibitor of self-renewal and promoter of differentiation in stem cells and, if so, which of its targets determine this role?

LIN-28 RNA binding proteins promote stemness and inhibit *let-7* processing

LIN-28 is an RNA binding protein with a cold shock domain and a CCHC zinc finger motif that was first identified as a regulator of developmental timing in *C. elegans* (Ambros and Horvitz 1984; Moss et al. 1997). *lin-28* mutants skip the L2 fates, thus undergoing L3 fates precociously and causing the larval to adult transition to occur one or two stages earlier than normal. In the hypodermis, this results in reduced seam stem cell self-renewal and proliferation and early terminal differentiation (Fig. 2). Conversely, mutation of the *lin-4* complementary site in the 3′ UTR or complete removal of the endogenous 3′ UTR prevents down-regulation of *lin-28* and results in reiteration of the symmetrical self-renewing division of the L2 stage seam cells at later stages and failure to differentiate at the larval to adult transition (Moss et al. 1997; Seggerson et al. 2002). *lin-28* is targeted by both the *lin-4* and *let-7* miRNAs in *C. elegans* (Morita and Han 2006), a pattern of regulation that is remarkably conserved as mammalian *lin-28* is subject to direct repression by both *let-7* and *mir-125* family miRNAs. This repression results in mutually exclusive temporal expression of *lin-28* versus *mir-125* and *let-7* in embryogenesis and ES/EC cell differentiation (Rybak et al. 2008; Wu and Belasco 2005).

The increase in mature *let-7* in differentiating ES/EC cells and embryos is not mirrored by the primary transcript, which shows little change during differentiation, implying that a processing block is present in early embryonic cells to prevent production of the mature *let-7* miRNA. Many other miRNAs also display this processing block in early embryonic cells and in cancer cells (Thomson et al. 2004). The fact that the inhibition is specific to cancer cells and undifferentiated cells implies that temporal regulation of miRNA processing

is important for the regulated differentiation of cells during development. Four groups have recently reported that that the identity of the inhibitor of *let-7* processing is LIN-28 (Heo et al. 2008; Newman et al. 2008; Rybak et al. 2008; Viswanathan et al. 2008). The inhibitory activity of LIN-28 appears to be relatively specific to *let-7* family miRNAs, but there is some disagreement as to the point at which LIN-28 functions in the *let-7* processing pathway. The Daley and Hammond labs reported inhibition at the Drosha step (Newman et al. 2008; Viswanathan et al. 2008), while the Wulzcyn and Kim groups found that Dicer endonuclease activity was affected (Heo et al. 2008; Rybak et al. 2008). It may be that both steps are affected and the magnitude of the effect at each stage is dependent on the cell type and context. LIN-28 binds to the *let-7* hairpin loop present in both the pri- and pre-miRNA (Newman et al. 2008; Piskounova et al. 2008), and so it may act to prevent access of both processing enzymes by steric hindrance. However, Heo et al. (2008) found that the *let-7* pre-miRNA is uridylated at the 3′ terminus in a LIN-28-dependent manner, preventing Dicer processing and increasing degradation. Alternatively, the association of LIN-28 with P-bodies (Balzer and Moss 2007) suggests that it may shuttle the primary transcript and the pre-miRNA to P-bodies for storage or degradation.

The functional relevance of this inhibition in terms of effects on embryonic development or ES cell self-renewal and differentiation is not yet clear. However, the recent discovery that LIN-28 is one of a combination of four factors that can promote reprogramming of differentiated human somatic cells into induced pluripotent stem (iPS) cells suggests that LIN-28 has an important role in promoting “stemness” (Yu et al. 2007b). The other factors are well-known regulators of ES cell pluripotency, OCT4, SOX2, and NANOG. Therefore, the discovery that *let-7* processing is inhibited by *lin-28* in ES cells, and that *let-7* inhibits self-renewal and promotes differentiation of normal and malignant mammary stem/progenitor cells, implies that LIN-28 may promote self-renewal and pluripotency in ES and iPS cells by down-regulation of *let-7*. Recently, LIN-28 was found to promote proliferation of mouse ES cells and directly associate with the 3′ UTRs of cyclin A and B and *Cdk4* mRNAs to enhance their translation (Xu et al. 2009). LIN-28 has also been shown to up-regulate IGF2 translation in differentiating muscle cells (Polesskaya et al. 2007). Therefore, LIN-28 appears to have dual roles in promoting proliferation of stem cells by inhibiting *let-7* processing and directly up-regulating translation of cell-cycle-related genes.

Since *lin-28* is expressed specifically in stem cells, promotes stemness in iPS cells, and inhibits the tumor-suppressive *let-7* miRNA, it seems likely that it will have important roles in cancer. Consistent with this hypothesis, LIN-28B, a paralog of LIN-28, was recently discovered to be over-expressed in human hepatocellular carcinoma cells and to promote growth of cancer cells (Guo et al. 2006). It seems likely that this is caused, at least partially, by down-regulation of *let-7*, as LIN-28B inhibits *let-7* processing in hepatocellular carcinoma cell lines such as Huh7 (Heo et al. 2008). Recently, LIN-28B was found to be induced by Myc in both a human B cell lymphoma model and a mouse colon cancer model and was responsible for the down-regulation of *let-7* family members and the increased proliferation of these cells (Chang et al. 2009). It will be important to further investigate the role of *lin-28* family proteins in carcinogenesis, especially since down-regulation of *let-7* has been associated with a wide range of cancers in many recent studies (discussed above). Is

LIN-28 upregulation responsible for this? Furthermore, cancer stem cells have similarities to normal stem cells, and since LIN-28 is an important stem-determining factor, it may be aberrantly activated in tumor-initiating cells.

LIN-28 is mainly localized to the cytoplasm in a variety of cell types, but it has been shown to shuttle to the nucleus in a cell-cycle-dependant fashion (Guo et al. 2006). During S and G2 phases, LIN-28 was found localized to the nucleus in cells from the Huh7 hepatocellular carcinoma cell line, and other groups noticed some nuclear localization in other cell types (Balzer and Moss 2007; Guo et al. 2006). This raises the intriguing possibility that LIN-28 may act differently in actively cycling cells. The cell cycle dependence of this change in localization and the role of LIN-28 in promoting proliferation of both cancer cells and nematode seam cells may indicate that this change in intracellular localization helps promote cell proliferation and self-renewal. Indeed, *lin-28* is normally expressed exclusively in undifferentiated rapidly cycling, early embryonic cells, or cancer cells. As LIN-28 inhibits the processing of the anti-proliferative miRNA, *let-7*, it may be that LIN-28 shuttling to the nucleus promotes cell cycle progression. Perhaps, nuclear LIN-28*i*sequesters pre-*let-7* from the cytoplasmic miRISC or increases inhibition of the Drosha step of pri-*let-7* processing in proliferating cells. It will be interesting to discover the significance of this nuclear-cytoplasmic shuttling of LIN-28 during the cell cycle and if this affects the processing or activity of *let-7* according to the cell cycle status of the cell. The cell cycle has been found to alter *let-7* miRNA activity—synthetic *let-7* miRNA up-regulates translation in cells in G0/G1, but reverts to the canonical role in inhibiting translation in actively proliferating cells, indicating that it is subject to cell-cycle-dependent regulation (Vasudevan and Steitz 2007; Vasudevan et al. 2007, 2008). However, this experiment used a synthetic mature miRNA without the hairpin loop, and so this effect may be independent of LIN-28. Cell-cycle-dependent changes in 3′ UTR length and the number of miRNA complementary sites have also been reported; shorter transcripts with fewer sites are preferentially expressed in proliferating cells, though it is not clear if this is caused by higher miRNA activity in cycling cells or if it is a strategy to avoid miRNA inhibition (Sandberg et al. 2008).

LIN-28 has been known to regulate the timing of *let-7* activity in *C. elegans* for many years: *let-7* miRNAs are expressed earlier in *lin-28* mutants and later in *lin-4* mutants in which *lin-28* expression is not down-regulated (Moss et al. 1997; Seggerson et al. 2002). These recent findings in mammalian cells suggest that LIN-28 may be acting directly on the *let-7* hairpin to inhibit production of the mature miRNA, but a direct role for LIN-28 in inhibition of *C. elegans let-7* has not yet been shown. In fact, *let-7* appears to be mainly regulated at the transcriptional level in nematodes (Johnson et al. 2003). However, forced transcription of *pri-let-7* in early L2 stage animals results in the accumulation of the pre-miRNA relative to the mature form at this stage compared to L4 stage animals (Hayes and Ruvkun 2006). Significantly, this correlates with the timing of LIN-28 down-regulation that occurs between L2 and L4 stage animals (Moss et al. 1997). It would be interesting to test if this effect is dependent on LIN-28, as both transcriptional and posttranscriptional mechanisms may collaborate to ensure robust switching of *let-7* activity in order to precisely time developmental transitions in *C. elegans*.

The similarity between the early larval expression of LIN-28 in *C. elegans* seam cells and its expression in early mammalian embryos and embryonic stem cells, as well as the conserved down-regulation of LIN-28 by *let-7* and *lin-4*/*mir-125* miRNAs during development and differentiation in nematodes and mammals, suggests that this protein has a highly conserved role in regulating developmental timing across evolution (Fig. 3). The inhibitory effect of LIN-28 on *let-7* processing and the inhibition of *lin-28* expression by *let-7* family miRNAs forms a double-negative feedback loop that creates a bistable switch in LIN-28 versus *let-7* activity (Fig. 4). In mammals, LIN-28 promotes stemness and cancer cell proliferation, and *let-7* is a tumor suppressor with growth-suppressive effects. In nematodes too, these genes act antagonistically in a temporally regulated manner to regulate stem cell proliferation and self-renewal, suggesting that this regulatory motif may act as a conserved switch for differentiation in stem cells.

LIN-41 TRIM-NHL family proteins as conserved elements of miRNA regulatory networks in stem cells

LIN-41 is a founding member of the TRIM-NHL family and was first identified as a suppressor of the *let-7* mutant (Fig. 2) and subsequently identified as a co-purifying factor with DCR-1, the *C. elegans* DICER protein (Duchaine et al. 2006). The regulation of *lin-41* by *let-7* has been strikingly well conserved within this subclade of the TRIM superfamily. *lin-41* family members from *C. elegans, Drosophila*, zebrafish, mouse, and human contain binding sites for *let-7* miRNAs that have been all been experimentally validated (Kanamoto et al. 2006; Kloosterman et al. 2004; Lin et al. 2007; O'Farrell et al. 2008; Slack et al. 2000). But despite the similarity of LIN-41 to both E3 ubiquitin ligases and the tumor repressor Brat, it is still unclear how LIN-41 functions in developmental timing in *C. elegans* seam cells to prevent terminal differentiation. Now, however, recent findings from *Drosophila* may provide a tantalizing clue. Brat and two other TRIM-NHL factors, Mei-P26 and Dappled, the closest fly LIN-41 homologue, have been found to physically interact with Ago1, the Argonaute member of the miRNA RISC complex. This interaction appears to mediate an inhibitory effect on miRNA expression, as miRNAs were globally repressed by Mei-P26 over-expression and upregulated in Mei-P26 mutant ovaries (Neumuller et al. 2008). The mechanistic basis for this effect is not yet clear, but the strikingly conserved domain architecture of this protein may provide important insights. The RING finger present in LIN-41 and Mei-P26 is often found in E3 ubiquitin ligases and has also been implicated in sumoylation, and the coiled coil domain of the tripartite motif (TRIM) is associated with homo-interaction and formation of high-molecular-weight complexes, suggesting a role for LIN-41 family members in posttranslational modification of proteins (Meroni and Diez-Roux 2005). The NHL domain is required for the interaction of Mei-P26 with Ago1 and for its ability to inhibit self-renewal of ovarian stem cells. Interestingly, this domain is most often mutated in *lin-41* loss of function mutants of *C. elegans* and is the most highly conserved region between *C. elegans* LIN-41 and its human ortholog, HLIN41/TRIM71 (Schulman et al. 2005; Slack et al. 2000). Therefore, LIN-41 may inhibit miRNA processing by ubiquitinating or sumoylating Dicer/Ago1/pre-miRNA complexes, resulting in degradation, inhibition, or disruption of the complex.

The *C. elegans* NHL-2 TRIM-NHL protein is required for the activity of a subset of miRNAs including *let-7* family miRNAs and *lsy-6* (Hammell et al. 2009). In contrast to Mei-P26, the processing of these miRNAs is apparently unaffected by NHL-2, suggesting that TRIM-NHL proteins can modulate miRNA function in different ways, although both functions are dependent on the presence of the NHL Argonaute interaction domain. Indeed, the mouse TRIM-NHL protein TRIM32 also interacts with Argonaute-1 and increases the activity of specific miRNAs, including *let-7a*, to promote neural stem cell differentiation (Schwamborn et al. 2009). TRIM-NHL proteins also function in other ways, independent of miRNA regulation; for example, TRIM32 binds and ubiquitinates c-Myc through its RING finger domain during the neuronal differentiation of NSCs, resulting in c-Myc degradation. c-Myc promotes self-renewal and proliferation of NSCs and the combined activities of TRIM32: Protein degradation of c-Myc and enhancement of translational inhibition by *let-7* represent an important strategy for the switch from self-renewal to differentiation. It is not yet known if LIN-41 shares any of these roles with other TRIM-NHL proteins, although the suppression of *let-7* mutant phenotypes by *lin-41* loss of function in *C. elegans*, the conserved targeting of LIN-41 by *let-7* miRNAs, and the presence of the Argonaute-interacting NHL domain strongly suggest that LIN-41 also participates in miRNA regulatory networks, most likely including *let-7* family miRNAs. Indeed, LIN-41 co-immunoprecipitates with DCR-1 in *C. elegans* (Duchaine et al. 2006), so perhaps, LIN-41 acts to repress formation or function of mature *let-7* (and possibly other miRNAs), thus ensuring that it is active only at the larval to adult transition, and not earlier. This would explain why in *lin-41* mutants the seam cells undergo premature differentiation at the end of the L3 stage (Slack et al. 2000; Fig. 2). *lin-41* was the first validated direct *let-7* target; *let-7* is both necessary and sufficient to cause down-regulation of *lin-41* via two complementary sites in the 3′ UTR, and genetic experiments showed that *lin-41* is epistatic to *let-7*, suggesting that it acts downstream of *let-7* in the heterochronic pathway (Slack et al. 2000; Vella et al. 2004a, b). However, this suppression could be equally well explained by the up-regulation of other more recently identified members of the *let-7* family such as *mir-48*, *-84*, and *-241* (Abbott et al. 2005) in *lin-41* mutants, which may compensate for loss of *let-7* itself. It will therefore be important to revisit the role of LIN-41 in nematodes in light of the role of other TRIM-NHL family proteins in miRNA regulation.

Both Mei-P26 and Brat have been classified as tumor suppressors based on their mutant phenotypes in flies: They are required to restrict self-renewal and growth of ovarian and neural stem cells, respectively (Betschinger et al. 2006; Neumuller et al. 2008). In worms, however, LIN-41 prevents terminal differentiation and so appears to act in the opposite manner to its *Drosophila* homologues. If the major function of these NHL family proteins is to negatively regulate miRNAs, then this probably reflects differences in the roles of miRNAs between nematode seam cells and fly ovarian stem cells. Consistent with this idea, in *Drosophila dicer-1* and *ago-1* mutants, ovarian stem cells fail to self-renew, while in nematodes mutant for these RISC components, the seam cells fail to terminally differentiate at the correct time and undergo extra rounds of self-renewing division, similar to *let-7* and *lin-4* mutants (Grishok et al. 2001; Jin and Xie 2007). Therefore, TRIM-NHL proteins are important regulators of stem cell self-renewal and differentiation, in part through their ability to regulate miRNA activity or expression. Some TRIM-NHL proteins, such as Mei-

P26 in fly ovarian stem cells and LIN-41 in *C. elegans* seam cells, have roles that are antagonistic to miRNA function in regulating stem cell self-renewal and differentiation, whereas other members of the TRIM-NHL family, such as NHL-2 in *C. elegans* seam cells and TRIM32 in mammalian neural progenitors, enhance miRNA activity in order to promote differentiation of stem cells.

The role of mammalian LIN-41 orthologs is not known, but mouse and chick *lin-41* genes show intriguing similarities in expression to *lin-28*. Like *lin-28, lin-41* is expressed at a high level in ES cells and vertebrate early embryos and becomes down-regulated as the cells differentiate, with reciprocal timing of expression to *let-7* and *mir-125* (Lee et al. 2005; Schulman et al. 2005; Fig. 3). As *lin-41* is a conserved target of *let-7*, it seems likely that this mutually exclusive expression may result partly from *let-7* inhibition of *lin-41* expression (Kanamoto et al. 2006; Kloosterman et al. 2004; Lin et al. 2007). It will be interesting to discover the role of LIN-41 in mammalian stem cells in light of the roles of the TRIM/RBCC/NHL family proteins in stem cells in mice, flies, and nematodes and the recent indication that these proteins might have the ability to regulate miRNA activity. Functional evidence that LIN-41 plays a role in vertebrate development comes from knockdown experiments in fish and mouse. *Mlin41*-defective mice die during mid-embryogenesis and display exencephaly, demonstrating that *Mlin41* is an essential mammalian gene (Maller Schulman et al. 2008). Knockdown of zebrafish LIN-41 in one-cell stage embryos causes defective embryogenesis and death (Lin et al. 2007). Interestingly, the phenotype of the embryos— reduced tail and yolk sac deformities—is almost identical to those into which a synthetic *let-7*a miRNA had been injected (Kloosterman et al. 2004). The expression of *lin-41* in the early vertebrate embryo is therefore essential, and it seems likely that its reciprocal expression to *let-7* results from LIN-41 and *let-7* acting antagonistically upon each other during embryogenesis.

Dynamic expression patterns of heterochronic genes in vertebrate limb and brain development

There are compelling expression pattern data to suggest that the heterochronic genes *lin-28, lin-41, let-7*, and *lin-4*/*mir-125* act together as a module in limb development. *lin-41* and *lin-28* are gradually down-regulated over time in a specific pattern in vertebrate limb buds; both are expressed ubiquitously initially in mesoderm and become restricted to distal sub-ridge mesoderm, with *lin-41* showing slower kinetics of down-regulation than *lin-28* (Lancman et al. 2005; Schulman et al. 2005; Yokoyama et al. 2008). Distal *lin-41* expression shows posterior bias which correlates with *let-7*c activity in the anterior of the limb bud, and the complete absence of both *lin-41* and *lin-28* from the apical ectodermal ridge (AER) correlates with high levels of activity of *let-7*e in this region (Mansfield et al. 2004). *lin-41* has been found to be downstream of fibroblast growth factor and sonic hedgehog (Shh) signaling in the limb—signals which emanate from the AER and zone of polarizing activity, respectively (Lancman et al. 2005). Interestingly, correct limb development depends on temporal patterning along both the proximal-distal (PD) and the anterior-posterior (AP) axes: Proximal cells differentiate before distal cells and cells take on posterior fate because they have expressed Shh the longest. Perhaps, *lin-41* and *lin-28* are

involved in developmental timing of the AP and PD axial fates by preventing differentiation occurring too early in the posterior and distal regions. The timing of down-regulation of *lin-41* and *lin-28* is regulated by miRNAs of the *let-7* and *mir-125* families, which are up-regulated in the embryo as these genes are repressed (Schulman et al. 2005). Therefore, these miRNAs may determine the timing of differentiation of the posterior and distal cells of the limb bud. However, it is not yet known how these genes impact proliferation, differentiation, and morphogenesis of the limb bud, and knockouts will need to be made in order to progress with functional analysis of these genes in limb development.

lin-41 and *lin-28* show overlapping dynamic expression in other tissues in embryogenesis, most notably in the developing brain where they are both down-regulated in late embryogenesis (Schulman et al. 2005; Yokoyama et al. 2008). *let-7* and *mir-125* family miRNAs are highly expressed in both developing brain and show high level expression in mature brain (Lagos-Quintana et al. 2002; Sempere et al. 2004). Moreover, both *let-7* and *mir-125* family miRNAs showed specific neuronal lineage expression (Smirnova et al. 2005; Wulczyn et al. 2007). The recent identification of LIN-28 as an important inhibitor of *let-7* processing in ES cells and the reciprocal expression of *mir-125* and *let-7* family miRNAs and their targets, *lin-28* and *lin-41*, during neural differentiation of ES and EC cells (Lee et al. 2005; Rybak et al. 2008; Wu and Belasco 2005), as discussed earlier, suggests that this quartet of factors may act as a functional regulatory circuit in the developing brain and in neurogenesis. In *C. elegans*, *lin-4* and *lin-28* are neuronally expressed, and a target of *lin-4*, *lin-14*, regulates the timing of ventral cord neuron synaptic remodeling (Hallam and Jin 1998). In addition, *let-7* is highly expressed in many types of neurons where it inhibits *hunchback-like-1* (*hbl-1*) expression (Abrahante et al. 2003; Lin et al. 2003) and *let-7* mutant nematodes have locomotive defects (Reinhart et al. 2000). In *Drosophila*, *let-7* is highly expressed in the adult central nervous system, motor neurons, and muscle and was found to be required for the correct timing of maturation of neuromuscular junctions (Caygill and Johnston 2008; Sokol et al. 2008). Furthermore, there are multiple behavioral and locomotory defects in *let-7* mutant flies implying that there are other as-yet uncharacterized defects in neural development or function in these animals. In addition, *dappled*, the closest *lin-41* homologue, is expressed ubiquitously in the early embryo but becomes restricted to the developing central nervous system and peripheral nervous system (PNS; O'Farrell et al. 2008), and misexpression results in defective PNS development (O'Farrell and Kylsten 2008). Therefore, the neuronal roles of this module of genes may well have been conserved through evolution, perhaps even in vertebrates.

Model: bistable switches composed of *let-7* and its conserved heterochronic gene targets regulate the timing of differentiation in stem cells and development

The conservation of *let-7* target sites in the 3′ UTRs of *lin-41* and *lin-28* orthologs, combined with the finding that these genes often have opposing roles and timing of expression in development, strongly suggests that these regulatory interactions have been retained throughout evolution. Why have these miRNA:target pairs been so well conserved when many other miRNA:target interactions are not? A clue may come from the fact that

both the *lin-28* and *lin-41* gene families have now been implicated in repression of miRNA production. *let-7* miRNAs may have evolved early on in metazoal lineage to negatively regulate *lin-41* and *lin-28* family genes in order to counteract the inhibitory effects of these factors on *let-7* activity and create a bistable switch composed of cross-antagonistic, double-negative feedback loops (Fig. 4). This regulatory module involving *let-7* and its targets *lin-41* and *lin-28* may have subsequently been co-opted to act as a switch for global changes in gene expression during the development of multiple cell types across phylogeny.

A model is emerging in which LIN-28 may specifically repress *let-7* in early development and in stem cells to promote proliferation and self-renewal; later, a signal tips the balance between *let-7* and *lin-28* in favor of *let-7* in order to promote differentiation (Fig. 4). A good candidate for the factor mediating this tipping point is *mir-125*, which is up-regulated before *let-7* and causes down-regulation of *lin-28*, a function that the *mir-125* ortholog, *lin-4*, also performs in *C. elegans* (Lee et al. 2005; Schulman et al. 2005). This would allow induction of *let-7*, which would then inhibit both *lin-41* and *lin-28*, as well as genes involved in cell proliferation such as *RAS*, *HMGA2*, *CDK6*, and *cyclin D2*. In addition, down-regulation of the negative regulators of miRNAs, *lin-28* and *lin-41*, by *mir-125* and *let-7* could promote global derepression of miRNAs, which in turn might facilitate differentiation. *Dicer1* and *Dgcr8* mutant ES cells fail to differentiate (Kanellopoulou et al. 2005; Wang et al. 2007), supporting the notion that global up-regulation of miRNAs is required for differentiation to occur; note also that miRNAs are generally present at much higher levels in differentiated cells than in ES cells and cancer cells (Kloosterman et al. 2006; Thomson et al. 2004, 2006; Wienholds et al. 2005). This model also nicely matches the roles of these four genes in the heterochronic pathway in *C. elegans*, and so we suggest that this core module of genes has been conserved across evolution to regulate the timing of differentiation during development.

Interestingly, hormonal signaling may also be a conserved mechanism for triggering the timing of induction of differentiation in nematodes, flies, and mammals. In *C. elegans*, the down-regulation of *lin-28* and induction of differentiation by *let-7* is regulated via signaling through the nuclear hormone receptor DAF-12 (Morita and Han 2006); in flies, ecdysone hormones regulate the timing of metamorphosis and induction of *let-7* (Sempere et al. 2002, 2003); and in mammals, the hormone retinoic acid induces differentiation of ES cells, down-regulation of *lin-28* and *lin-41*, and up-regulation of *let-7* and *mir-125* (Lee et al. 2005; Richards et al. 2004; Wu and Belasco 2005; Yang and Moss 2003). It therefore may also be useful to study model organisms such as nematodes and flies to understand how endocrine and intercellular signaling pathways are integrated with the intrinsic intracellular heterochronic pathway to regulate developmental timing.

Heterochronic genes, *lin-28*, *lin-4/mir-125*, *lin-41*, and *let-7*, as a conserved module for temporal regulation of development

The gradual restriction of pluripotency and determination of cell fate during development is highly dependent on the temporal restriction of cell fates and the switching of genes on and off in the correct order. Gene expression is highly dynamic, and it is as important to understand how genes are regulated in a temporal manner as it is to understand spatial

control of gene expression. In the last quarter of a century, we brought a great deal of knowledge about spatial patterning beginning with the discovery of Hox genes and other spatial patterning genes in *Drosophila*; similarly, the discovery of miRNAs as switches for developmental timing of cell fates in *C. elegans* at the end of the twentieth century now promises to help unearth the mechanisms of temporal patterning.

Recent findings, summarized above, suggest that the *lin-41* and *lin-28* family proteins and the *let-7* and *mir-125* families of miRNAs are functionally interlinked and dynamically expressed in a reciprocal manner in a variety of situations during juvenile development in nematodes and flies and in vertebrate embryogenesis. As such, this module of heterochronic genes appears to have been used throughout evolution to regulate developmental timing in numerous contexts. *let-7* generally seems to promote differentiation and inhibit proliferation, whereas *lin-28* has opposing roles in this regard. miRNAs of the *let-7* family in particular have recently been shown to have a vital role in differentiation and inhibiting proliferation in stem cells of the mammary gland and in breast cancer. Furthermore, the temporally regulated accumulation of *let-7* miRNAs during differentiation of ES and EC cells and embryogenesis suggests that it is important for the regulated differentiation of stem cells in the early embryo. Since *lin-28* is an oncogene and a stemness-promoting factor, and *let-7* is an important tumor suppressor that is down-regulated in stem cells, it will be important to discover the roles of other conserved heterochronic genes such as *lin-41* in stem cells and cancer. Indeed, *lin-41* homologues in *Drosophila—brat* and *mei-P26*—are tumor suppressors that act by inhibiting stem cell self-renewal in the neuroblast and ovarian stem cell lineages, respectively.

It has been observed that cancer cells are more embryonic in nature and that stem cell programs are aberrantly activated in cancer (Ben-Porath et al. 2008; Krivtsov et al. 2006; Park et al. 2007; Visvader and Lindeman 2008); therefore, the roles of the heterochronic genes in stem cells, development, and cancer may well be fundamentally interlinked. The interest in using stem cells for regenerative purposes and the need to more specifically target cancer-initiating cells in cancer therapy therefore propels this module of heterochronic genes, *lin-28, lin-41, lin-4/mir-125*, and *let-7*, to a position of high priority for further research. The roles of these core heterochronic genes in the stem-cell-like seam cells of *C. elegans* mirrors the roles of their homologues in stem cells of higher systems. Therefore, we believe that the powerful genetic tools available for use with *C. elegans*, combined with the ease of studying seam cells at single cell resolution, will make the nematode an important tool for the further understanding of miRNA regulatory networks in development, stem cells, and cancer, just as it was in their discovery.

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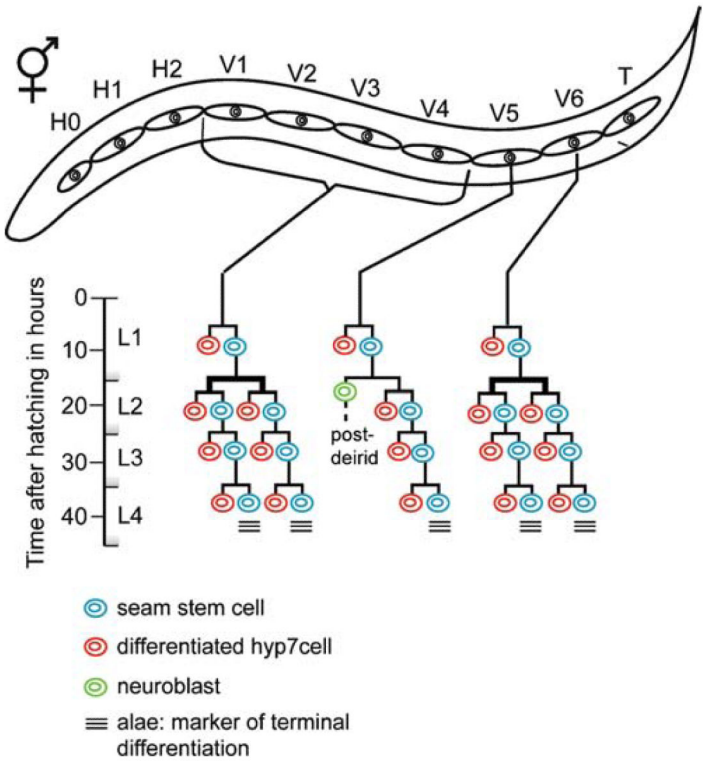

Fig. 1.

The *C. elegans* seam stem cells. The seam stem cells divide asymmetrically at each larval stage such that they self-renew and produce multiple differentiated ectodermal cell types—epidermal cells and various types of neuronal and glial cells (only the hermaphrodite V lineages are shown for simplicity). At the end of the L4 stage, the seam stem cells undergo the larval to adult (*L/A*) switch and terminally differentiate by exiting the cell cycle, fusing, and secreting alae (a cuticular structure). A symmetrical proliferative division (shown in *bold*) occurs early in the L2 stage to expand seam cell number

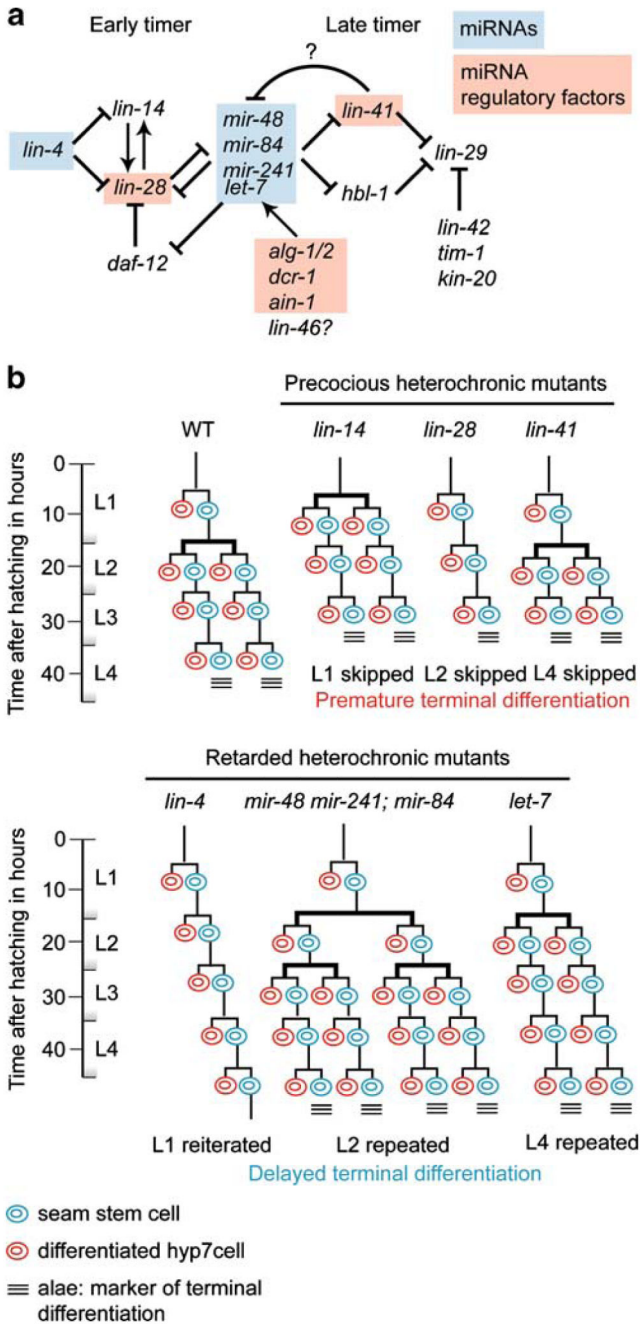

Fig. 2.

The heterochronic genes, including the *lin-4* and *let-7* family miRNAs, regulate developmental timing in seam stem cells. **a** The heterochronic pathway is an excellent model for miRNA regulatory networks acting as binary switches to pattern development along the temporal axis. In nematodes, heterochronic genes regulate the transitions between stage-specific patterns of division in the seam stem cells. The early timer consists of *lin-4*-mediated down-regulation of *lin-14* and *lin-28* to program the L1/L2 and L2/L3 transitions, respectively. The late timer involves the repression of *hbl-1* and *lin-41* by miRNAs of the

let-7 family in order to program both the L2/L3 transition and the L/A switch. **b** The seam cell lineages in the *lin-28* and *lin-41* mutants (both miRNA targets) are termed precocious due to the skipping of L2 and L4 stage events, respectively. This results in the L/A switch occurring in the seam, one stage earlier than normal. The *lin-4* and *let-7* miRNA mutants, in contrast, have retarded phenotypes in that the L1 and L4 stages, respectively, are reiterated, resulting in a delay of terminal differentiation

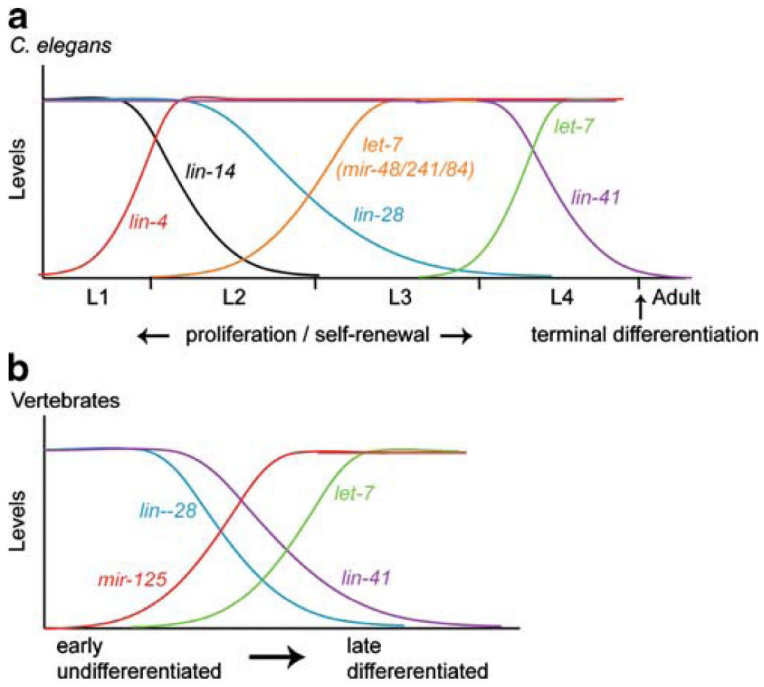

Fig. 3.

Conserved reciprocal temporal expression of the *lin-4/mir-125* and *let-7* family miRNAs and their targets, *lin-28* and *lin-41*. *lin-28* and *lin-41* are expressed specifically in undifferentiated mouse ES and EC cells and early embryos. These genes are down-regulated during differentiation by increasing levels of *let-7* and *mir-125* miRNAs (**b**), mirroring their regulation in *C. elegans* stem cells during development (**a**)

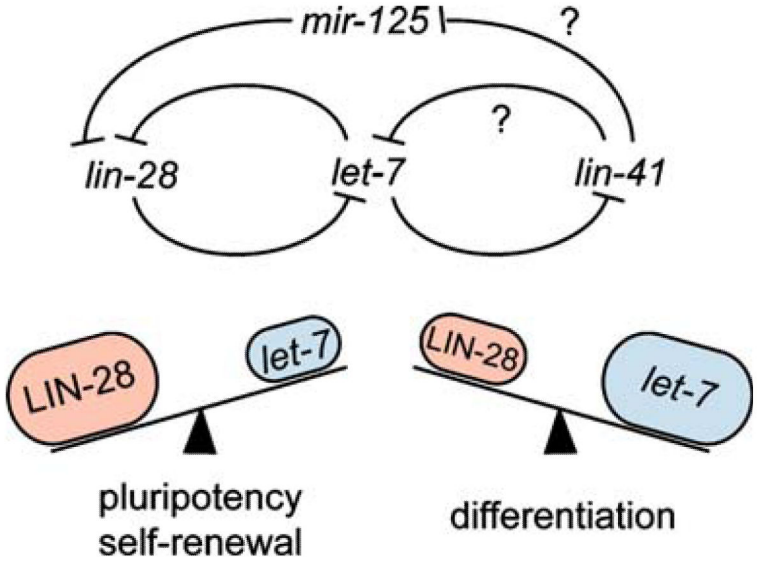

Fig. 4.

Model for conserved functions of the heterochronic genes in stem cells, development and cancer. Bistable switches may regulate stem cell differentiation via double-negative feedback loops between *let-7* and its targets, *lin-28* and *lin-41*. The roles of *lin-28* and *let-7* as an oncogene and tumor suppressor suggests that stem cells and cancer cells share a common strategy for regulating the balance between self-renewal and differentiation