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PRIMER

LIN28: roles and regulation in development and beyond

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ABSTRACT

LIN28 is an RNA-binding protein that is best known for its roles in promoting pluripotency via regulation of the microRNA let-7. However, recent studies have uncovered new roles for LIN28 and have revealed how it functions, suggesting that it is more than just a regulator of miRNA biogenesis. Together, these findings imply a new paradigm for LIN28 – as a gatekeeper molecule that regulates the transition between pluripotency and committed cell lineages, in both let-7-dependent and let-7-independent manners. Here, we provide an overview of LIN28 function in development and disease.

KEY WORDS: LIN-28, RNA-binding protein, Pluripotency

Introduction

LIN-28 is one of the founding members of the *C. elegans* heterochronic pathway (Ambros and Horvitz, 1984), which comprises a network of genes that regulate the timing of developmental events. When these genes are mutated, whole larval stages are skipped or reiterated. In this pathway, LIN-28 promotes cell fates specific to early larval development while also regulating cell fates specific to later development, including the terminal differentiation of some cell types (Ambros, 1989; Ambros and Horvitz, 1984; Moss et al., 1997; Vadla et al., 2012).

In the 30 years since it was initially discovered in *C. elegans*, LIN28 has been shown to play expanding roles in development and it has generally been defined as a promoter of pluripotency. In mouse, human, *C. elegans*, *Xenopus*, zebrafish and *Drosophila*, LIN28 is expressed early during development and in undifferentiated tissues, and it is downregulated as development and cellular differentiation proceed (Faas et al., 2013; Moss et al., 1997; Moss and Tang, 2003; Ouchi et al., 2014; Yang and Moss, 2003). It is also highly expressed in embryonic stem cells (ESCs) and is downregulated in response to differentiation (Moss and Tang, 2003; Richards et al., 2004). Accordingly, LIN28 has been identified as one of several factors that can participate in the reprogramming of mammalian somatic cells to pluripotent cells (Hanna et al., 2009; Yu et al., 2007). Taken together, it appears that LIN28 has an evolutionarily conserved role promoting early, undifferentiated cell fates. In line with this, LIN28 expression in cancers is associated with less differentiated, more aggressive tumors (Hamano et al., 2012).

Despite its strong association with pluripotent cells, LIN28 has also been shown, in at least two cases, to be required for proper differentiation (Faas et al., 2013; Polesskaya et al., 2007). It was in light of these findings that LIN28 was first suggested to act as a molecular 'gatekeeper', regulating the transition from pluripotency to a committed cell lineage (Faas et al., 2013). Here, we review the roles of LIN28 in a variety of organisms and systems, in both normal development and diseased states.

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LIN28 gene and protein structure

A single *lin-28* gene is found in *C. elegans* and *Drosophila melanogaster* (Moss and Tang, 2003), whereas there are two *Lin28* paralogs in all vertebrates: *Lin28a* and *Lin28b* (Fig. 1). All LIN28 proteins can be identified by their unique pairing of a cold shock domain (CSD) and a cysteine cysteine histidine cysteine (CCHC) zinc knuckle domain (Fig. 1), both of which can bind RNA; LIN28 is the only animal protein to contain this combination of motifs. The CSD is a beta barrel and is similar to that of Y-box proteins of frog and human (Moss and Tang, 2003). The CCHC zinc knuckle domain is a motif found in retroviruses (Moss and Tang, 2003). LIN28A and LIN28B have high sequence identity in these regions, but differ in a few respects. For example, LIN28B contains an extended tail region at the C-terminus of the protein (Lee et al., 2014) and it also contains a nuclear localization signal (Fig. 1A) (Piskounova et al., 2008). LIN28A is predominantly located in the cytoplasm of cells, although it can shuttle to and from the nucleus (Balzer and Moss, 2007). LIN28B, by contrast, is found in the nucleus, specifically in the nucleolus (Piskounova et al., 2011). Another difference between LIN28A and LIN28B is in how they inhibit the microRNA (miRNA) let-7, as discussed below. This review will focus on LIN28A, which is canonically known as LIN28 and is referred to as such hereafter.

LIN-28 in the *C. elegans* heterochronic pathway

LIN-28 plays two roles in *C. elegans* larval development

The *C. elegans* heterochronic pathway controls the specification of cell fates in diverse cell types at each larval stage. The four larval stages (L1-L4) of *C. elegans* are characterized by stage-specific patterns of cell division and differentiation, punctuated with the synthesis of a new cuticle and the subsequent molting of the existing cuticle. When heterochronic genes are mutated, developmental events for a specific stage are skipped or reiterated (Ambros and Horvitz, 1984). Null mutations in *lin-28* cause precocious development, whereby L2 stage-specific events are skipped (Fig. 2) and later events occur one stage earlier relative to wild-type animals (Ambros and Horvitz, 1984). This is clearly seen in the lateral hypodermal seam cells, in which the proliferative L2 division is skipped and all later events in this lineage, including the terminal differentiation and synthesis of adult cuticle, occur precociously (Ambros and Horvitz, 1984). Furthermore, *lin-28* mutants cease molting after the L3, going through only three stages instead of the normal four. Additional tissues are affected, such as the hypodermal vulval precursor cells, which divide precociously and form a deformed, nonfunctional egg-laying system (Euling and Ambros, 1996). LIN-28 is also sufficient to cause reiteration of L2 cell fates (Moss et al., 1997); a constitutively expressed *lin-28* transgene causes reiteration of the L2 cell division, resulting in extensive hypodermal proliferation (Fig. 2). Additionally, vulval divisions are delayed or completely blocked in this mutant. Thus, LIN-28 is sufficient to drive reiteration of L2 cell fates, thereby blocking the execution of later fates.

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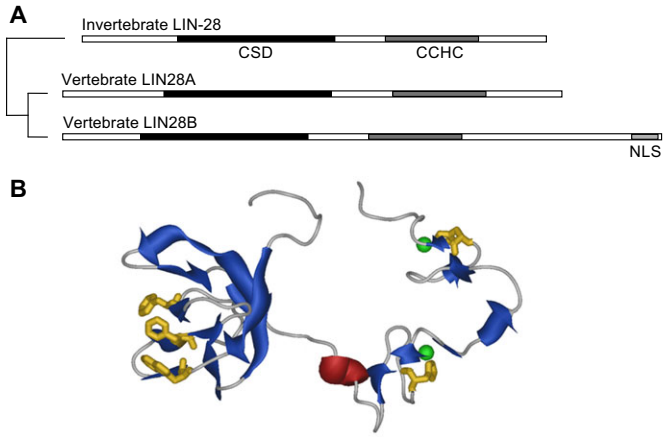

Fig. 1. LIN28 protein structure. (A) Conserved domains in invertebrate LIN-28 and the vertebrate paralogs LIN28A and LIN28B. LIN28 isoforms range in length from 195 to 260 amino acids, with vertebrate versions being among the longest, and LIN28B being slightly longer than LIN28A. CSD, cold shock domain; CCHC, cysteine cysteine histidine cysteine domain; NLS, nuclear localization signal. (B) Homology model of LIN28 based on the CSD of human YBX1 and NCp7 of HIV-1 (Balzer and Moss, 2007). Blue, beta sheets; red, alpha helix; yellow, aromatic residues important for LIN28 function; green, zinc atoms.

For many years it was believed that LIN-28 had only a single role promoting L2 cell fates, and that the precocious execution of later fates was simply a domino effect. This remained unquestioned until it was discovered that the mammalian LIN28 protein can bind to the primary and precursor forms of the let-7 miRNA and inhibit its processing, a function that was subsequently confirmed for the *C. elegans* ortholog (Lehrbach et al., 2009; Stefani et al., 2015; Van Wynsberghe et al., 2011; Viswanathan et al., 2008). *let-7* encodes a miRNA in the heterochronic pathway that promotes differentiation events (for a review see Mondol and Pasquinelli, 2012); this finding highlighted a novel form of gene regulation and offered the first molecular mechanism for how LIN-28 controls cell fate succession in *C. elegans*. It also suggested the possibility that LIN-28 acts at more than one stage of development.

The link between LIN-28 and let-7 was surprising because LIN-28 acts during the L2 stage, whereas let-7 was thought to act at the L4-adult transition, long after LIN-28 is downregulated (Moss et al., 1997; Reinhart et al., 2000; Slack et al., 2000). While attempting to understand this discrepancy, Vadla et al. (2012) found that LIN-28 acts in two mechanistically independent steps: first, a let-7-independent step to promote L2 cell fates; and second, a separate let-7-dependent step that controls L3 cell fates and subsequent differentiation (Fig. 3). Although the mechanisms and molecular targets by which LIN-28 regulates the L2 stage are still being elucidated, Vadla and colleagues found that let-7 and its family members are not required for the function of LIN-28 during the L2 stage, making it let-7 independent, and, mechanistically, they found no evidence that LIN-28 regulates any other miRNA in the way that it regulates let-7 (Vadla et al., 2012). However, by repressing let-7, LIN-28 promotes the expression of *lin-41*, a gene that promotes L3 cell fates (Fig. 3). This is seen most clearly in *lin-28(0)* mutants, in which a subset of seam cells display two-stage precociousness, skipping both L2 and L3 cell fates and secreting patches of alae at the end of L2 (Vadla et al., 2012). Vadla and colleagues demonstrated that the two-stage precociousness was dependent on let-7, as *lin-28(0); let-7(0)* double mutants do not display this phenotype. Taken together, *C. elegans* LIN-28 promotes early proliferative cell fates, while also promoting later events in the seam cell lineage, and it does so using two distinct mechanisms.

lin-28 is part of a complex genetic pathway

Although many questions remain, much is now known about the relationship between LIN-28 and other members of the heterochronic pathway. In 1989, the first hierarchy in the heterochronic pathway was established, when it was found that *lin-28* acted downstream of *lin-4* (Fig. 3) (Ambros, 1989). It was not until 1997, after the discovery that *lin-4* encoded the first known miRNA (Lee et al., 1993), that it was revealed that *lin-28* is a molecular target of lin-4 (Moss et al., 1997). In particular, it was shown that lin-4 targets *lin-28* via the lin-4-complementary element in its 3′UTR, making *lin-28* the second known miRNA target (Moss et al., 1997).

Another lin-4 target in the pathway is *lin-14*, which encodes a transcription factor that promotes both L1 and L2 cell fates during larval development (Fig. 2) (Ambros and Horvitz, 1984, 1987; Hristova et al., 2005). It was known that LIN-28 and LIN-14 acted at the same step of development (Ambros and Horvitz, 1984), but two main experiments elucidated their genetic relationship. First, it was shown that, in a *lin-4* null background, both LIN-14 and LIN-28 are expressed abnormally late into development, causing retarded development (Arasu et al., 1991; Moss et al., 1997). However, the late expression of LIN-14, whether in a *lin-4* null or a *lin-14(gf)* background, requires functional LIN-28 (Arasu et al., 1991). Second, it was shown that the late expression of LIN-28 in a *lin-4* null requires functional LIN-14 (Moss et al., 1997). Together, these findings indicated that *lin-14* and *lin-28* are in a positive-feedback loop, each promoting the expression of the other (Fig. 3). These findings also suggested that there is lin-4-independent repression of each gene in the other's absence (Arasu et al., 1991; Seggerson et al., 2002). To investigate this, Seggerson and colleagues used a strain containing a unique allele of *lin-14* [*lin-14(n355sd)*] in which the lin-4-responsive elements of the *lin-14* 3′UTR are missing and,

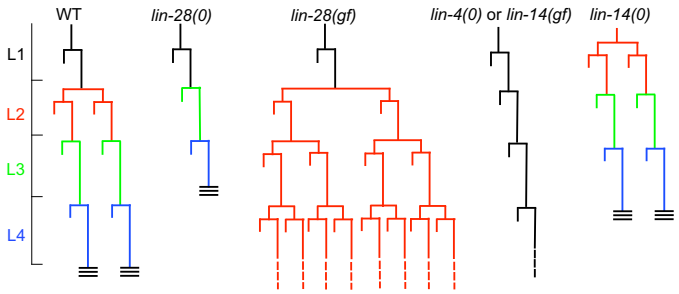

Fig. 2. Cell division patterns in the *C. elegans* lateral hypodermis. At the beginning of each *C. elegans* larval stage (L1-L4), seam cells undergo an asymmetric division (indicated by a horizontal line), whereby the posterior daughter remains an undifferentiated blast cell (represented by long vertical lines that continue to the next stage), and the anterior daughter differentiates and joins the neighboring hypodermal syncytium (represented by short vertical lines). However, in the L2 there is first a symmetrical, proliferative division, whereby both cells remain seam cells before undergoing a round of asymmetric division. When LIN-28 is either underexpressed [*lin-28(0)*] or overexpressed [*lin-28(gf)*], this stage is skipped or reiterated, respectively. This causes a change in the total number of seam cells, which presents an easily quantifiable phenotype. Unlike *lin-28*, mutations in *lin-4* and *lin-14* cause L1 cell fates to be skipped or reiterated. At the L4-to-adult transition, seam cells differentiate and secrete a special structure known as alae into the cuticle. Alae formation can occur precociously or not at all in heterochronic mutants. Three black horizontal lines indicate the formation of alae. Dashed lines indicate that this lineage is not followed past this stage. WT, wild type.

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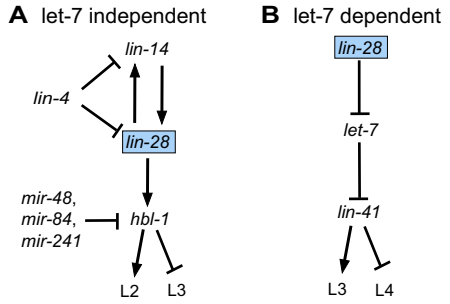

Fig. 3. *lin-28* is part of a complex genetic pathway. (A) In the let-7-independent pathway, *lin-28* positively regulates *hbl-1* to promote L2 cell fates, in opposition to the three let-7 family miRNAs: mir-48, mir-84 and mir-241. *lin-28* is also regulated at this stage by *lin-4* and *lin-14*. (B) In the let-7-dependent pathway, *lin-28* promotes later cell fates by blocking maturation of the let-7 miRNA and therefore relieves repression of its target *lin-41*, thereby promoting the execution of L3 cell fates.

consequently, *lin-14* expression is not downregulated (Arasu et al., 1991; Ruvkun et al., 1989; Wightman et al., 1991). They found that, in this strain, LIN-28 remained abundant late into development, at a level comparable to that observed in a *lin-4* null (Seggerson et al., 2002). Therefore, *lin-28* expression is not fully repressed when *lin-14* activity is elevated, despite the presence of lin-4. This discovery was surprising because it meant that lin-4 is not sufficient to fully repress *lin-28*, and that the lin-4-independent repression is likely to contribute substantially to *lin-28* regulation in normal development. They further discovered that the lin-4-independent repression of *lin-28* occurs post-transcriptionally and requires the 3′UTR of *lin-28* (Seggerson et al., 2002), although the molecular components of this mechanism remain unknown.

Perhaps the most informative experiments addressing the let-7-independent functions of LIN-28 are those regarding its relationship to *hbl-1*, which encodes an Ikaros family zinc-finger transcription factor and is an ortholog of *Drosophila hunchback* (Fay et al., 1999). In the heterochronic pathway, HBL-1 promotes L2 cell fates, similarly to LIN-28, and is thought to be the most proximal regulator of L2 (Fig. 3) (Abrahante et al., 2003; Fay et al., 1999; Lin et al., 2003). *hbl-1* is negatively regulated by the redundant activity of three let-7 family miRNAs: mir-48, mir-84 and mir-241 (Abbott et al., 2005). When these miRNAs are mutated, HBL-1 is expressed abnormally late in development (Abbott et al., 2005). However, this late expression requires a functional LIN-28, indicating that *lin-28* supports *hbl-1* expression (Fig. 3) (Vadla et al., 2012). Interestingly, the effect of *lin-28* on *hbl-1* is via the 3′UTR of *hbl-1* (Vadla et al., 2012). Although the mechanism is still unclear, the possibility that LIN-28 regulates mRNAs such as *hbl-1* to promote L2 cell fates is an exciting one. There is growing evidence that LIN-28 may use such a mechanism in mammalian systems, indicating an evolutionarily conserved role for LIN-28.

Lin28 in *Drosophila*

As previously mentioned, *Drosophila*, like *C. elegans*, contains one copy of *lin-28*. Like the *C. elegans* version, it has two isoforms that are the product of alternative splicing of the first exon (Moss and Tang, 2003). It also contains the unique pairing of the CSD and CCHC zinc knuckle motifs, as seen in many other species. Little is known about Lin28 in *Drosophila*. It is expressed early in embryogenesis, and this expression persists all the way through to the first instar larval stage (Moss and Tang, 2003). Lin28 is also detected during the pupal stage (Moss and Tang, 2003).

Recently, Lin28 was found to play an important role in oogenesis and fertility. Loss of Lin28 resulted in reduced fertility, disruption of normal germline stem cell differentiation, early fusion of the egg chamber, an abnormal quantity of egg chambers, and an irregular number of nurse cells within each egg chamber (Stratoulias et al., 2014). As in many other contexts, these effects of Lin28 are mediated via let-7 (Stratoulias et al., 2014).

LIN28 in vertebrates

The expression of LIN28 has been studied extensively in the whole mouse as well as in different pluripotent cell lines. This has resulted in a better understanding of the temporal and spatial expression patterns of LIN28 over the course of normal development and differentiation and of its function in vertebrates.

Developmental expression patterns

LIN28 is expressed early in development, with the protein showing accumulation in mouse nucleolar precursor bodies during the two-cell stage of embryogenesis (Vogt et al., 2012). Expression is seen throughout the developing embryo by E6.5, and extends to the extra-embryonic tissue as well (Yang and Moss, 2003). As development progresses, the previously broad expression of LIN28 becomes restricted to just some tissues: a subset of epithelial cells, including those of the branchial arches, the lungs and the kidney, cardiac muscle cells of the myocardium, and neuroepithelium; LIN28 is not expressed in all epithelial cells nor in the other two tissues of the developing heart (the endocardium and pericardium) (Yang and Moss, 2003). Expression in the lung epithelium decreases as differentiation occurs and is absent entirely by E17.5. Once development is complete, LIN28 remains expressed in the adult in the epithelial cells of the loop of Henle and collecting duct of the kidney, in cardiac and skeletal muscle (Yang and Moss, 2003) and in erythrocytes (de Vasconcellos et al., 2014).

In most instances, LIN28 is expressed in undifferentiated, pluripotent cell types. One notable exception is the cells of the small intestine. Here, LIN28 expression is restricted to a specific group of cells: the transit-amplifying population. These are cells that are committed to villous cell fates but that have not yet fully differentiated, establishing a virtual boundary zone in the tissue between more and less differentiated cell types (Yang and Moss, 2003). LIN28 expression is not observed in either the basal stem cells or fully differentiated villous cells (Yang and Moss, 2003). Therefore, in the small intestine, and perhaps elsewhere, LIN28 is expressed in a transit-amplifying population of cells within a tissue, rather than in its stem cells.

LIN28 in pluripotent stem cells

LIN28 is also highly expressed in ESCs, as well as in the NT-2 human teratocarcinoma cell line and in the mouse P19 embryonal carcinoma cell line, both of which exhibit pluripotent properties. When these cells differentiate, LIN28 expression is downregulated (Balzer et al., 2010; Yang and Moss, 2003). In ESCs, LIN28 regulates overall cell number and proliferation (Xu et al., 2009). Further investigation by Peng and colleagues identified a number of mRNAs regulated by LIN28 that are important for both growth and translation, supporting its role in the regulation of proliferation (Peng et al., 2011). When LIN28 is expressed, it regulates self-renewal in mouse ESCs as a function of AIRE, the autoimmune regulator (Bin et al., 2012). As in ESCs, proliferation in neural precursor cells is promoted by SOX2 through LIN28 (Cimadamore et al., 2013). LIN28 also regulates neural precursor cell proliferation promoted by MASH1 (ASCL1), via its inhibition of let-7

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(Cimadamore et al., 2013). Accordingly, the loss of LIN28 in neural progenitor cells results in fewer cells due to a reduction in proliferation (Yang et al., 2015).

Developmental and physiological roles for LIN28

At the organismal level, LIN28 has consequences for diverse biological processes. LIN28 overexpression causes an increase in total body size along with a proportional increase in organ size, which is likely to be due to an increase in cell number and proliferation. It also delays the onset of puberty, including postponement of the vaginal opening and first estrus (Zhu et al., 2010). LIN28 loss, by contrast, results in embryonic lethality, reduced growth and fat accumulation, and reduced brain size (Yang et al., 2015; Zhu et al., 2010). Additionally, LIN28 regulates glucose metabolism; its overexpression increases the ability of muscle cells to take up glucose (Zhu et al., 2010), while its loss results in insulin resistance (Shinoda et al., 2013; Zhu et al., 2011). Furthermore, sensitivity to insulin can be restored in obese adipose stem cells with the introduction of LIN28 (Perez et al., 2013). Finally, LIN28 overexpression increases regeneration during digit repair, epidermal hair regrowth and pinnal tissue regrowth in the mouse (Shyh-Chang et al., 2013). In many of these cases, the cellular basis and the mechanism of action of LIN28 are not fully understood.

LIN28 and the control of cell differentiation

In most mammalian cell culture models, cell differentiation is associated with downregulation of LIN28; in fact, LIN28 has been cited as a marker of 'stemness' (Richards et al., 2004). However, there are cases in which LIN28 appears to be required for efficient differentiation, notably in specifying mesodermal cell fates, as seen in both *Xenopus tropicalis* and mouse models (Faas et al., 2013; Polesskaya et al., 2007). Interestingly, in both these species, LIN28 does not require let-7 for this role.

For example, using *X. tropicalis* embryos Faas et al. (2013) found that knocking down LIN28 causes abnormalities in the differentiation and patterning of mesodermal structures at early larval stages. Several genes expressed in the very early mesoderm, including *Xbra*, *chordin* and *myoD*, were reduced both in their expression domains and expression levels. Furthermore, LIN28 knockdown inhibits the ability of pluripotent cells from the *Xenopus* embryo to differentiate into mesoderm in response to the growth factors FGF and activin (Faas et al., 2013). All of these effects occur in early development. Although *Xenopus* LIN28 can bind let-7, it was shown that, at this stage, there were no significant changes in let-7 expression following LIN28 knockdown (Faas et al., 2013). Therefore, the effects of LIN28 on mesoderm specification are unlikely to be mediated through a LIN28/let-7 pathway.

Another notable exception in which differentiation is associated with LIN28 induction is skeletal muscle (Polesskaya et al., 2007). Using *in vitro* gain- and loss-of-function assays, Polesskaya et al. uncovered the importance of LIN28 during myogenic differentiation. They found that overexpression of LIN28 in a myoblast cell line had a stimulating effect on terminal differentiation and, conversely, that repression of LIN28 dramatically decreased the efficiency of muscle differentiation (Polesskaya et al., 2007). Additionally, they found that LIN28 is upregulated during the differentiation and maturation of newly formed skeletal muscle fibers *in vivo*. Finally, they found that LIN28 is likely to act by promoting the translation of *Igf2* in this system, an mRNA that does not contain any predicted let-7 binding sites (Balzer et al., 2010). Taken together, these data suggest that LIN28 has an essential role in differentiating myoblasts and one that is not dependent on the regulation of let-7.

Molecular mechanisms of LIN28 action: two separate mechanistic pathways

The let-7-dependent pathway

Currently, the regulation of let-7 miRNAs is by far the best characterized mechanism of LIN28. Accordingly, a number of genetic pathways have been discovered in which LIN28 influences development via its regulation of let-7. The molecular aspects of the LIN28/let-7 axis have been studied in exquisite detail (Loughlin et al., 2012; Mayr and Heinemann, 2013; Nam et al., 2011). In brief, studies have shown that LIN28 can bind to both pri- and pre-*let-7* *in vivo* and block their processing (Fig. 4) (Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). LIN28 binds an evolutionarily conserved motif within let-7: GGAG. In mammals, this is present within the let-7 stem loop (Heo et al., 2009; Loughlin et al., 2012; Nam et al., 2011) and in nematodes it is found in the primary transcript, downstream of pre-*let-7* (Stefani et al., 2015).

In the nucleus, LIN28 co-transcriptionally binds pri-*let-7* and blocks its processing by the miRNA-processing enzyme Drosha (Van Wynsberghe et al., 2011). In human ESCs and neuronal stem/progenitor cells, it has been shown that the RNA-binding protein musashi 1 (MSI1) enhances the localization of LIN28 to the nucleus

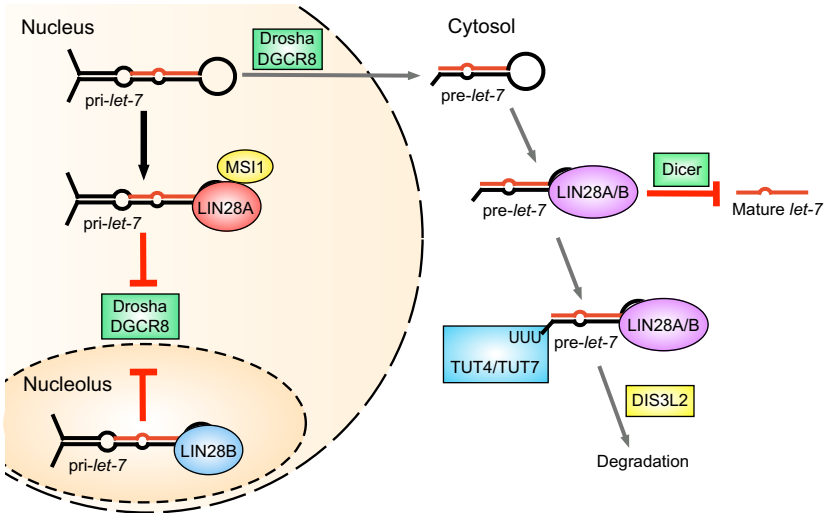

Fig. 4. LIN28 inhibits let-7 processing. LIN28 binds to both pri- and pre-*let-7* *in vivo* and blocks their processing (Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). In the nucleus, LIN28 co-transcriptionally binds pri-*let-7* and blocks its processing by Drosha in complex with DGCR8 (Van Wynsberghe et al., 2011). In human ESCs and neuronal stem/progenitor cells, the RNA-binding protein MSI1 enhances the localization of LIN28A to the nucleus and, synergistically with LIN28A, blocks the cropping of pri-*let-7* (Kawahara et al., 2011). By contrast, LIN28B predominantly localizes to the nucleolus, where it sequesters pri-*let-7* to block further processing (Piskounova et al., 2011). In the cytoplasm, LIN28 binds pre-*let-7* to block its processing by Dicer and instead induce its oligo-uridylation (Heo et al., 2008). In mammals, TUT4 and, to a lesser extent, TUT7 catalyze the oligo-uridylation (Hagan et al., 2009; Heo et al., 2009; Thornton et al., 2012). Once oligo-uridylated, pre-*let-7* is more rapidly degraded than unmodified pre-*let-7* (Heo et al., 2008); in mouse ESCs, the 3′-5′ exonuclease DIS3L12 catalyzes this decay (Chang et al., 2013).

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and, acting synergistically with LIN28, blocks the cropping of pri-*let-7*, which occurs in the nucleus (Kawahara et al., 2011). By contrast, LIN28B predominantly localizes to the nucleolus owing to its nuclear and nucleolar localization signals, where it sequesters pri-*let-7* and further blocks its processing (Piskounova et al., 2011).

In the cytoplasm, LIN28 binds pre-*let-7* to block its processing by Dicer and induces the oligo-uridylation of the 3′ overhang of pre-*let-7*′ (Heo et al., 2008). In mammals, terminal uridyl transferase 4 (TUT4; also known as ZCCHC11) and, to a lesser extent, TUT7 (ZCCHC6) catalyze this oligo-uridylation (Hagan et al., 2009; Heo et al., 2009; Thornton et al., 2012), whereas in *C. elegans* this is carried out by the poly(U) polymerase PUP-2 (Lehrbach et al., 2009). Once oligo-uridylated, pre-*let-7* is more rapidly degraded than unmodified pre-*let-7* (Heo et al., 2008), and it has been shown that, in mouse ESCs, the 3′-5′ exonuclease DIS3L12 catalyzes the decay of oligo-uridylated pre-*let-7* (Chang et al., 2013). For a recent, in-depth review of LIN28-mediated control of let-7, the reader is referred to Mayr and Heinemann (2013).

The let-7-independent pathway

Although the let-7-dependent mechanism is important for LIN28 function in development, it is only half the story of how LIN28 works. A number of papers have demonstrated that, just like in *C. elegans*, mammalian LIN28 exhibits let-7-independent roles. For example, Zhu and colleagues found that transgenic mice with a skeletal muscle-specific knockout of *Lin28* showed impaired glucose tolerance and insulin resistance, despite no significant change in let-7 levels (Zhu et al., 2011). In another study, transgenic mice that overexpressed LIN28 showed increased organ size compared with their wild-type littermates, even in tissues in which let-7 expression was unaffected (Zhu et al., 2010). LIN28 has also been found to alter gene expression during neurogliogenesis, prior to let-7 upregulation (Balzer et al., 2010). Finally, two studies found that LIN28 promotes ESC proliferation in part by binding to, and increasing the translation of, cell cycle-related mRNAs (Peng et al., 2011; Xu et al., 2009). Taken together, it is clear that let-7-independent roles of LIN28 are important in a number of developmental contexts.

In the search to understand these let-7-independent functions, small-scale studies have identified a handful of potential LIN28 targets and have begun to elucidate how some of these might function (Balzer et al., 2010; Polesskaya et al., 2007; Qiu et al., 2010; Xu and Huang, 2009; Xu et al., 2009; Yang et al., 2015). Additionally, several comprehensive and unbiased genome-wide studies have identified thousands more potential targets (Table 1) (Cho et al., 2012; Hafner et al., 2013; Peng et al., 2011; Stefani et al., 2015; Wilbert et al., 2012). These studies found that LIN28 primarily targets mature mRNAs in the gene ontology (GO) categories of cell cycle regulation, nuclear RNA-binding proteins and genes involved in translation.

In spite of this wealth of data, the exact targets that are relevant for the biological function of LIN28, and how LIN28 confers specificity when targeting these RNAs, is still unclear. For example, three genome-wide studies discovered distinct consensus sequences among their identified LIN28 targets: GGAGA (Wilbert et al., 2012), AYYHY (Y=U,C and H=A,C,U) (Hafner et al., 2013) and AAGNNG (Cho et al., 2012). It was surprising, given such large data sets, that these groups identified very divergent consensus sequences. However, it should be noted that Wilbert and colleagues found their consensus sequence enriched in the single-stranded RNA (ssRNA) within hairpin and other loop structures of target RNAs, Hafner and colleagues found that LIN28 preferentially bound ssRNA within the context of a stem-loop, and Cho and colleagues found their consensus sequence in the terminal loop of small hairpins. This binding pattern is reminiscent of the interaction of LIN28 with let-7 miRNA precursors, and might indicate the importance of secondary structure for LIN28 binding. One study that explored the secondary structure of several LIN28 target mRNAs found that the structural motif of an ‘A bulge’ flanked by two G:C base pairs within a larger stem loop motif mediated the LIN-28-dependent stimulation of translation (Lei et al., 2012). Despite these investigations, it is still unclear how LIN28 recognizes its targets.

Several studies have demonstrated that LIN28 targets are enriched in LIN28-containing polysome fractions, suggesting a role for LIN28 in modulating the translation of its target mRNAs (Fig. 5) (Balzer and Moss, 2007; Peng et al., 2011; Polesskaya et al., 2007; Qiu et al., 2010). However, although most studies found that association with LIN28 enhances translation (Peng et al., 2011; Polesskaya et al., 2007; Qiu et al., 2010; Xu and Huang, 2009; Xu et al., 2009), one demonstrated both enhancing and repressive effects (Wilbert et al., 2012), one primarily repressive effects (Cho et al., 2012), while another showed no statistically significant changes (Hafner et al., 2013). These discrepancies underscore our limited understanding of the molecular details of let-7-independent LIN28 functions.

LIN28 in disease and therapy

Understanding the role of LIN28 in disease is a very exciting area of research. Initial investigations have revealed roles for LIN28 in glucose uptake and tolerance, diabetes, sickle cell anemia and cancer (de Vasconcellos et al., 2014; Perez et al., 2013; Shinoda et al., 2013; Shyh-Chang et al., 2013; Zhu et al., 2011), suggesting that the modulation of LIN28 activity might be an attractive therapeutic approach. For example, the expression of LIN28 in cultured, sickle-shaped erythrocytes resulted in a significant decrease in their sickle morphology compared with control erythrocytes (de Vasconcellos et al., 2014). LIN28 has also proven to be useful in the fields of cellular reprogramming and regeneration. In each of these scenarios, however, the research is still in its infancy and many questions remain to be answered.

LIN28 and cancer

LIN28 has a powerful effect on the ability of the cell to maintain a pluripotent state. Aberrant LIN28 expression can therefore impact normal development, mainly the switch from the maintenance of a

Table 1. Classes of mRNAs targeted by LIN28

Cell cycle regulation	RNA-binding proteins	Histone components	Glucose metabolism	Early embryonic genes
Cyclin A, cyclin B, CDK4, CDK1/2, CCND1/2, CCNB1	FUS/TLS, hnRNP F, TDP-43, TIA-1	Histone H2A, histone H4H, linker histone H1FX	Insulin receptors, IGF receptors, IRS2/4, AKT1-3, IGF2BP1 to IGF2BP3, HMGA2	*Peg3*, *Sall4*, *Oct4*, *Lin28b*

Genome-wide as well as smaller scale studies (Balzer et al., 2010; Hafner et al., 2013; Peng et al., 2011; Polesskaya et al., 2007; Qiu et al., 2010; Wilbert et al., 2012; Xu and Huang, 2009; Xu et al., 2009; Yang et al., 2015) have identified thousands of potential LIN28 targets, a subset of which are shown here.

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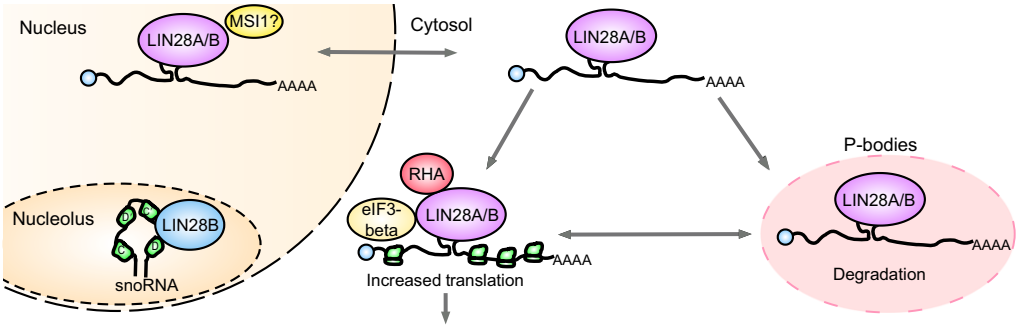

Fig. 5. LIN28 promotes the translation of mRNA targets. Researchers have identified thousands of potential LIN28 targets; however, it is unclear where LIN28 binds these targets and how they mediate LIN28 function. The effect of LIN28 on translation might be related to its cellular localization, as it has been found to localize to cellular processing bodies (P-bodies) and stress granules (Balzer and Moss, 2007). It may also be mediated through the action of co-factors. For example, LIN28 can bind to RNA helicase A (RHA) (Qiu et al., 2010), a protein that is known to facilitate efficient translation of target genes, possibly by relieving structural blockades in target mRNAs (Hartman et al., 2006). The recruitment of LIN28 RHA to polysomes might be a key feature of its mechanism, as depletion of RHA impairs LIN28-dependent stimulation of reporter genes (Jin et al., 2011; Qiu et al., 2010). Additionally, LIN28 can bind the translation initiation factor eIF3β, although how this interaction impacts LIN28 function is not known (Polesskaya et al., 2007; Qiu et al., 2010). In addition, both forms of LIN28 target a subset of box C/D small nucleolar RNAs (snoRNAs), consistent with reports that LIN28B and, to a much lesser extent, LIN28A localize to the nucleolus (Hafner et al., 2013; Piskounova et al., 2011).

pluripotent state to a cancerous state. Both LIN28 and LIN28B have been identified in a number of tumor and cancer cell types (for reviews see Thornton and Gregory, 2012; Zhou et al., 2013). The inappropriate expression of each LIN28 paralog in these different tumor types results in various combinations of cancer characteristics including invasiveness, larger tumor size, metastasis, poorer prognosis, and increased cell number (Feng et al., 2012; Hamano et al., 2012; King et al., 2011a,b; Liu et al., 2013; Qin et al., 2014). LIN28-mediated control of proliferation and cell division seems to be a key feature in the aggressive nature of these cancers (Feng et al., 2012; Nguyen et al., 2014; Urbach et al., 2014; Wang et al., 2015). For example, in breast cancer, high LIN28 expression increases the size of the tumor and increases cell growth, and this is likely to be a result of increased cell division and proliferation (Feng et al., 2012). LIN28 overexpression in the primitive cap mesenchyme cells of the kidney results in prolonged cell proliferation into adulthood and the prevention of normal postnatal differentiation (Urbach et al., 2014). Not only does the continued expression of LIN28 result in Wilms tumor (pediatric kidney cancer), but its continued expression is required for maintaining the proliferative nature of the cap mesenchyme cells within the tumor (Urbach et al., 2014).

LIN28 in reprogramming and regeneration

Currently, LIN28 is well known for its role in induced pluripotent stem cells (iPSCs). In combination with NANOG, OCT4 (POU5F1) and SOX2, it can reprogram somatic cells to pluripotent stem cells (Yu et al., 2007). OCT4 and SOX2 were shown to be essential components for this induction process, whereas LIN28 and NANOG are non-essential factors. LIN28 and NANOG each increase the reprogramming efficiency when inducing dedifferentiation – NANOG to a greater extent than LIN28 (Yu et al., 2007). In support of this proposed role, it has been shown that LIN28 promotes increased reprogramming by inducing a greater cell division rate and an increased number of cell divisions (Hanna et al., 2009). In the study of tissue regeneration, the overexpression of LIN28 results in increased digit repair, epidermal hair regrowth and pinnal tissue regrowth in the mouse (Shyh-Chang et al., 2013). The success of tissue regeneration is likely to be due to LIN28-mediated control of proliferation of the stem cell and transit-amplifying populations. LIN28 promotes expansion of these cell types leading to an increase in tissue size and regrowth of the damaged region.

Perspectives

In *C. elegans*, LIN-28 acts in a two-step mechanism: first, regulating early, proliferative cell fates; and second, promoting later differentiating cell fates via regulation of let-7 (Vadla et al., 2012). There is growing evidence that vertebrate LIN28 acts in a similar two-step fashion: first, promoting pluripotency by increasing proliferation; and second, directing cells down specific differentiation pathways.

Several studies suggest that LIN28 promotes pluripotency by promoting cellular proliferation. Human and mouse ESCs rapidly proliferate in a unique cell cycle that is characterized by a shortened G1 phase and a high proportion of cells in S phase. This unique cell cycle and rapid proliferation are thought to be biologically coupled to pluripotency (White and Dalton, 2005). Although it has not been shown that LIN28 promotes this specific cell cycle step, it does promote proliferation in several contexts. First, two separate studies have found that LIN28 expression promotes ESC proliferation (Peng et al., 2011; Xu et al., 2009). Second, LIN28 expression promotes reprogramming (to iPSCs) by accelerating the reprogramming process through more rapid cell divisions (Hanna et al., 2009; Yu et al., 2007). Third, adult mice overexpressing LIN28 are significantly larger (in height and weight) than their wild-type littermates, a phenotype that results from increased cell number (Zhu et al., 2010). Finally, *Lin28* knockout mice weigh 20% less at birth than wild-type pups (Zhu et al., 2010), and they also have reduced brain size due to decreased proliferation and enhanced cell cycle exit of neural progenitors (Yang et al., 2015).

The role of LIN28 in development is not limited to promoting pluripotency; it also plays a key role in directing cells to specific committed lineages. During skeletal muscle differentiation, LIN28 induction is essential for terminal differentiation (Polesskaya et al., 2007). Additionally, in *Xenopus* embryos, cells require LIN28 in order to respond to mesoderm-inducing growth factors and to differentiate properly (Faas et al., 2013). Finally, there is evidence of at least one case in which LIN28 appears to act somewhere between pluripotency and differentiation; in murine adult intestinal crypts, the transition from stem cell to differentiated villus can be spatially visualized and, here, LIN28 is highly expressed in a population of cells transitioning from pluripotency to a differentiated state, possibly the transit-amplifying cells (Yang and Moss, 2003).

Based on this evidence, we believe that the two-step mechanism first elucidated in *C. elegans* is an evolutionarily conserved feature

of LIN28, placing it as a gatekeeper between pluripotency and differentiation (Faas et al., 2013; Vadla et al., 2012). In *C. elegans*, the distinct let-7-dependent and let-7-independent molecular mechanisms of LIN-28 strongly correlate with its two separate roles in development, but in vertebrates this distinction is less clear. Elucidating how the let-7-independent and let-7-dependent functions of LIN28 work separately and together to execute its two roles in development will provide the key to understanding this unusual regulator of gene expression and cell behavior.

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Competing interests

The authors declare no competing or financial interests.

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