REVIEW

LIN-41/TRIM71: emancipation of a miRNA target

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lin-41 (lineage variant 41)/*TRIM71* (tripartite motif 71) is well known for being a conserved target of the *let-7* (lethal 7) microRNA (miRNA), a regulatory relationship found in animals evolutionarily as distant as *Caenorhabditis elegans* and humans. It has thus been studied extensively as a model for miRNA-mediated gene silencing. In contrast, the developmental and molecular functions of LIN41 have historically received less attention. However, LIN41 proteins are now emerging as important regulators of cell proliferation and differentiation in stem and progenitor cells. Moreover, LIN41's functions appear to involve two distinct molecular activities; namely, protein ubiquitylation and post-transcriptional silencing of mRNAs. Thus, LIN41 is ready for a scientific life of its own.

Caenorhabditis elegans lin-41 (lineage variant 41) was originally discovered more than a decade ago as a target of the highly conserved *let-7* (lethal 7) microRNA (miRNA) (Reinhart et al. 2000; Slack et al. 2000). This was also the time when miRNAs were beginning to emerge as a large and important class of regulators of gene expression in plants and animals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart et al. 2002). However, very few miRNA targets had been validated, and among the known targets, *lin-41* stood out in that orthologous proteins could be identified in other organisms, including mice and humans (Slack et al. 2000). Yet more strikingly, even the regulation by *let-7* was conserved for these orthologs (Kloosterman et al. 2004; Schulman et al. 2005; Lin et al. 2007). It is thus hardly surprising that *lin-41* quickly became an intensely studied model miRNA target. Somewhat paradoxically, then, a focus on the mechanism of *lin-41* regulation by *let-7* also meant that our understanding of the molecular and developmental functions of LIN41 itself has lagged behind. Recent studies have begun to change this and identified LIN41 and the orthologous tripartite motif 71 (TRIM71) proteins as regulators of stem and progenitor

cell proliferation and differentiation that can silence mRNA and drive protein ubiquitylation (Rybak et al. 2009; Chang et al. 2012; J Chen et al. 2012; Loedige et al. 2012). Here we discuss these exciting novel insights into the molecular and developmental biology of *lin-41*. For simplicity, we avoid, in the following, species-specific nomenclatures and refer to the gene and mRNA as *LIN41* and the protein product as LIN41, respectively.

Prelude: *LIN41* as a target of the *let-7* miRNA

LIN41 is a member of the TRIM-NHL family of proteins (for review, see Wulczyn et al. 2011). The family name derives from the tripartite motif of RING (really interesting new gene) finger, B-box(es), and coiled-coil domain (accordingly also named RBCC) and, typically, six NHL repeats at the C terminus (Fig. 1). (Here, NHL stands for NCL-1, HT2A2, and LIN-41, after the proteins in which this motif was first discovered [Slack and Ruvkun 1998].) Initially, *LIN41* was identified as a suppressor of phenotypes caused by a *let-7* loss-of-function (*lf*) mutation as well as a regulator of temporal cell fates in *C. elegans* larvae (Slack et al. 2000), as discussed in more detail in a later section. The genetic data supported *let-7* functioning as a negative regulator of *LIN41*, and parallel work identified *let-7* as a short noncoding RNA (Reinhart et al. 2000), now known as a miRNA, which had potential to bind to partially complementary sites in the *LIN41* 3' untranslated region (UTR). Subsequent studies could confirm direct regulation of *LIN41* by *let-7* and identified sequence elements that generate functional *let-7* target sites (Vella et al. 2004a, b; Long et al. 2007) and revealed *LIN41* mRNA degradation (Bagga et al. 2005) and translational repression (Ding and Großhans 2009) as modes of *let-7* activity. Collectively, these findings were highly influential in building a general framework for our understanding of miRNA function, helped in part by the fact that *let-7* sequence and function appeared highly conserved in animals. Specifically, *let-7* orthologs, known as *let-7a* in some organisms, are 100% conserved in sequence and present in most animals (Pasquinelli et al. 2000; Lagos-Quintana et al. 2002). Moreover, *let-7* was shown to repress fly (O'Farrell et al. 2008), zebrafish (Kloosterman et al. 2004), mouse (Kanamoto et al. 2006; Rybak et al. 2009), chicken (Kanamoto et al. 2006), and

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Figure 1. Domain architecture of LIN41 proteins from different species. The indicated domains were identified using SMART (<http://smart.embl-heidelberg.de>) and CDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) algorithms. Domain sizes are approximately to scale. Note that two isoforms have been reported for *C. elegans*, LIN41a and LIN41B, which only differ by three amino acids (Slack et al. 2000).

human (Lin et al. 2007) *LIN41* orthologs in 3' UTR reporter assays. Finally, ectopic expression of *let-7* decreased endogenous LIN41 protein levels in a mouse embryonic carcinoma cell line (Rybak et al. 2009).

Surprisingly then, whereas extensive research on *let-7* over the past decade has established it as a regulator of a wide range of processes in development (Mondol and Pasquinelli 2012), metabolism (Zhu et al. 2011), and human disease (Büssing et al. 2008), a contribution of *LIN41* repression to these *let-7* functions has not been investigated. This is despite the fact that the evolutionary conservation of *LIN41* regulation by *let-7* makes a strong case for *LIN41* being a key *let-7* effector, even when taking into account that most experiments involved the use of reporters and/or *let-7* overexpression rather than regulation of endogenous *LIN41* under physiological conditions. By extension, the evident importance of *LIN41* regulation suggests that *LIN41*'s yet elusive molecular functions might have significant impact on cellular homeostasis, and this idea is strongly supported by recently published data discussed in the following sections.

An emerging theme: LIN41 controls cell proliferation and differentiation in vertebrate and invertebrate development

C. elegans seam cells divide in an asymmetric, stem cell-like fashion in a characteristic pattern during each larval stage (Sulston and Horvitz 1977). At the larval-to-adult (L/A) transition, the seam cells exit the cell cycle, fuse, and differentiate to secrete specialized collagenous structures called alae. The timing of these events is governed by the heterochronic pathway (Ambros and Horvitz 1984). Reduced levels of LIN41 lead to precocious execution of the L/A transition, as evidenced, for instance, by alae secretion and cell cycle exit in larvae. In contrast, *LIN41* overexpression—or *let-7lf*—results in a retarded L/A transition, which promotes continued division of even adult seam cells, while preventing them from fusing and secreting alae (Reinhart et al. 2000; Slack et al. 2000). Epistasis analysis placed *LIN41* downstream from the *let-7* miRNA and upstream of the *lin-29* zinc finger transcription factor (Slack et al. 2000). Indeed, it has been suggested that *let-7* acts primarily through *LIN41*

because RNAi-mediated depletion of *LIN41*, but not another putative target, *hbl-1*, almost completely suppressed aberrant seam cell divisions in *let-7(0)* mutant adult animals (Vadla et al. 2012). However, we note that *lin-41lf; let-7(0)* double-mutant animals generate alae, like wild-type animals, only at the L4-to-adult molt (Slack et al. 2000). In contrast, precocious alae formation at the L3 molt, a hallmark of *lin-41lf* single-mutant animals, is rare (Slack et al. 2000). These observations are inconsistent with the notion of a simple, linear pathway. It is therefore possible that distinct *let-7* targets are key to regulating proliferation and differentiation, respectively, although this remains to be determined.

Although the hypodermis is the tissue where *LIN41* function has been studied in most detail, *LIN41* appears to play broader roles in *C. elegans* development. In addition to the hypodermis, a GFP-LIN41 fusion protein was observed in neurons, muscle cells, and the somatic gonad, in all of which it might accumulate in a *let-7*-insensitive manner (Slack et al. 2000). As *LIN41* mutant worms display gut defects (Del Rio-Albrechtsen et al. 2006) and are sterile due to a failure to produce oocytes (Slack et al. 2000), widespread expression appears functional, although the basis of the respective phenotypes remain to be determined. In *C. elegans* males, *LIN41* further functions in morphogenesis of the tail tip. This process is temporally regulated (Nguyen et al. 1999) and highly sensitive to *LIN41* dosage, as demonstrated by graded RNAi and allelic complementation studies (Del Rio-Albrechtsen et al. 2006). Similar to the hypodermis, reduced *LIN-41* activity results in precocious, increased activity in retarded phenotypes (Del Rio-Albrechtsen et al. 2006). Mutations altering the LIN41 N terminus outside annotated domains cause the male tail tip but no hypodermal phenotypes, suggesting either a different threshold or, alternatively, different functions or interaction partners of LIN41 in these two tissues. Indeed, LIN-29, which we discuss in more detail in a later section, is a major effector of LIN41 functions in the hypodermis but dispensable in the tail (Del Rio-Albrechtsen et al. 2006).

The conservation of *LIN41* sequence and regulation by *let-7* in various animals as well as the fact that *LIN41* expression is temporally and spatially regulated during development in animals where this has been investi-

gated, strongly imply that LIN41 proteins have important functions in a wide range of animals and developmental contexts. This notion is supported by studies of specific loss-of-function phenotypes in zebrafish (Lin et al. 2007) and mice (Maller Schulman et al. 2008; J Chen et al. 2012). In zebrafish, knockdown of *LIN41* leads to partially penetrant embryonic lethality, with surviving embryos showing developmental abnormalities such as a short trunk, abnormal yolk shape, and an S-shaped tail (Lin et al. 2007). Mice carrying a homozygous *LIN41* “gene trap” mutation, which depletes *LIN41* by providing a strong splice acceptor site that will cause endogenous *LIN41* transcripts to missplice and terminate prematurely, start to die at around embryonic day 8.5 (E8.5), and all embryos are dead by E13.5–E16.5 (Maller Schulman et al. 2008; J Chen et al. 2012). The most striking phenotype of these *LIN41*^{fl} mice is a neural tube closure defect (Maller Schulman et al. 2008; J Chen et al. 2012), although this is apparently not the cause of lethality (Maller Schulman et al. 2008). J Chen et al. (2012) could subsequently pinpoint decreased proliferation rate and increased differentiation of neural progenitor cells, caused at least in part through defects in fibroblast growth factor (FGF) signaling, as the basis of the neural tube closure defect.

It is also in mice where *LIN41* expression has been studied in the most detail, including by analysis of endogenous promoter activity (Maller Schulman et al. 2008; Yu et al. 2010; J Chen et al. 2012), mRNA in situ hybridization (Lancman et al. 2005; Schulman et al. 2005), immunohistochemistry (Rybak et al. 2009; Yu et al. 2010), and Western blot analysis (Rybak et al. 2009; Yu et al. 2010; J Chen et al. 2012). Generally, *LIN41* expression decreases over development (Schulman et al. 2005; Yu et al. 2010; J Chen et al. 2012). In particular, following ubiquitous expression early, *LIN41* transcription becomes gradually restricted to some neural tissues and the limb buds during embryogenesis (Maller Schulman et al. 2008). In neural tissue, LIN41 protein levels, measured by Western blotting, are high in neuroepithelial cells of early embryos (until E11.5) but undetectable as neurogenesis—and thus neural differentiation—proceeds (J Chen et al. 2012).

LIN41 expression not only correlates temporally with proliferative processes, but is also generally high in the proliferative, progenitor compartments of tissues where this has been characterized in more detail. Whole-mount staining of early embryos at E7 showed that LIN41 distribution is similar to that of the pluripotency factor Oct4, with staining in the embryonic ectoderm and, less strongly, the ectoplacental cone (Rybak et al. 2009). LIN41 was also detected in the interfollicular stem cells of both embryonic and adult epidermis as well as in postnatal testis, where it was found in gonocytes, early spermatogonia, and spermatocytes (Rybak et al. 2009). Interestingly, LIN41 mRNA or protein and *let-7* display largely inverse expression patterns in skin and testis, with *let-7* staining, for instance, being visible in the subbasal cell layer rather than the basal stem cell layer, where LIN41 accumulates (Rybak et al. 2009). Taken together with the demonstration that LIN41 levels in

mouse embryonic stem (mES) cells decrease during differentiation in parallel with up-regulation of *let-7* (Chang et al. 2012), this suggests that *let-7* plays a major role in shaping *LIN41* expression during mouse—and, possibly more global, animal—development.

At the same time, it is worth keeping in mind that even for a notorious miRNA target such as *LIN41*, regulation by a miRNA is just one among many regulatory inputs. For instance, whole-mount in situ hybridization against endogenous *LIN41* mRNA in FGF signaling-deficient mice suggests that expression of *LIN41* in limb buds is dependent on FGF signaling, similar to the situation in chickens in which Shh and Fgf signaling induces *LIN41* expression (Lancman et al. 2005). Interestingly, *LIN41* itself appears to promote FGF signaling during mouse embryonic brain development (J Chen et al. 2012), which implies that LIN41 might stimulate its own expression. Furthermore, the *LIN41* promoter and first intron contain three putative E-boxes that can be bound by the MYC transcription factor in cell lines (YL Chen et al. 2012). In luciferase reporter experiments, these elements can mediate transactivation of the *LIN41* promoter by ectopic MYC expression (YL Chen et al. 2012). Although the physiological significance of this finding is not clear, MYC is, similar to *LIN41*, highly expressed and has an important function in ES cells (Kim et al. 2010). Moreover, in addition to transcriptionally activating *LIN41*, MYC relieves it from post-transcriptional repression by repressing *let-7* directly (Chang et al. 2008) and through increased transcription of the *let-7* inhibitor *LIN28* (Chang et al. 2009; Dangi-Garimella et al. 2009). This exquisite regulation by at least two different mechanisms might point to a need to ensure sufficiently high *LIN41* expression in ES cells in a robust and faithful manner.

Taken together, *LIN41* expression in mice and worms appears to be a marker of a uni- or multipotent self-renewing state. Given this expression pattern as well as the functions of *LIN41* in mouse neural progenitor (J Chen et al. 2012) and *C. elegans* seam cells (Slack et al. 2000), an emerging principle of *LIN41* activity in developmental processes and tissues seems to be promotion of proliferation and inhibition of differentiation. Consistent with this notion, *LIN41* has been suggested to have a tumor-promoting role in human hepatocellular carcinoma (HCC): It is overexpressed in many tumor samples, and high levels correlate with poor patient survival (YL Chen et al. 2012). Moreover, *LIN41* overexpression in HCC cell lines resulted in increased tumor growth in a subcutaneous xenograft model, which can probably be explained by an increased proliferation rate (YL Chen et al. 2012). In this regard, LIN41 may generally, in diverse organisms and developmental events, function as an important effector of the LIN28–*let-7* “switch” between a self-renewing, proliferative stem cell or progenitor state, and differentiation (Melton et al. 2010). This idea is consistent with available data indicating that LIN28, a small RNA-binding protein, promotes proliferation and represses differentiation at least in part through repressing *let-7* biogenesis (Rybak et al. 2008; Viswanathan et al.

2008; Vadla et al. 2012) and that *let-7* conversely promotes differentiation and represses proliferation while repressing *LIN41*. How *LIN41* in turn controls the two processes of differentiation and proliferation is less clear, and we discuss in the next section potential molecular targets of, and mechanisms of regulation by, *LIN41* (Fig. 2).

A multitude of targets: cell cycle mRNAs and a signaling protein as *LIN41* effectors

Genetic analysis in *C. elegans* identified *lin-29* as a factor functioning downstream from *lin-41* in the control of differentiation and proliferation of seam cells (Slack et al. 2000). *LIN-29* is a transcription factor and appears to provide a direct link to the cell cycle machinery, as it can stimulate transcription of the cell cycle inhibitor *cki-1* (Hong et al. 1998). Moreover, *LIN-29* promotes cell differentiation, although, with the exception of collagen genes (Rougvie and Ambros 1995), the relevant targets in

this process largely remain to be established. The fact that *lin-29* mRNA accumulates two larval stages before *LIN-29* protein becomes detectable (Rougvie and Ambros 1995; Bettinger et al. 1996), that *LIN41lf* mutations cause precocious *LIN-29* accumulation in seam cells (Slack et al. 2000), and that a GFP-*LIN41* fusion protein localizes to the cytoplasm (Slack et al. 2000) was taken to speculate that *LIN41* could be a translational repressor of *lin-29* (Slack et al. 2000).

Although experimental data for such an activity of *LIN41* on *lin-29*—and, in fact, for *lin-29* as a direct target of *LIN41*—are still lacking, evidence is now accumulating that *LIN41* proteins are indeed post-transcriptional, potentially translational, silencers of mRNAs. Consistent with this notion, modulation of *LIN41* levels changes the accumulation of numerous mRNAs and proteins in mouse and human cells, and in several instances, the 3' UTRs of potential target genes are sufficient to recapitulate these effects for target reporters (Chang et al. 2012; Loedige et al. 2012). Moreover, *LIN41* can directly repress translation of a luciferase reporter mRNA when artificially tethered to its 3' UTR of a luciferase reporter (Chang et al. 2012; Loedige et al. 2012). Silencing of either type of reporter is typically accompanied by some decrease in mRNA levels (Loedige et al. 2012). Finally, for a subset of these targets, it was shown that ectopically expressed *LIN41* could coimmunoprecipitate the relevant endogenous mRNAs (Loedige et al. 2012). Thus, at least when expressed in HEK293 or mES cells, *LIN41* displays several hallmarks of a translational repressor, although the mechanisms of repression remain to be elucidated.

How is *LIN41* recruited to mRNAs? The *LIN41* paralog and translational repressor BRAT might provide a model, as it is thought not to bind mRNAs directly, but rather in a complex with the pumilio protein PUF and the unspecific RNA-binding protein Nos (Sonoda and Wharton 2001). However, although both the *hb* mRNA and the *cyclin B* 3' UTR contain Nos response elements (NREs), which recruit a NOS/PUF complex, BRAT only binds to the complex on the *hb* NRE (Sonoda and Wharton 2001). Thus, mRNA features such as specific sequences or secondary structures seem to contribute to BRAT recruitment. It has not been possible so far to establish the requirements of target repression by *LIN41*. Although it interacts with several RNA-binding proteins, these interactions are mostly RNA-dependent (Loedige et al. 2012) and thus presumably not responsible for recruitment of *LIN41* to RNA. Moreover, human pumilio proteins are not required for *LIN41* activity in HEK293 cells, and on the mRNA side, there is no apparent consensus sequence or binding motif that would explain the interaction with *LIN41* (Loedige et al. 2012).

Insights into *LIN41*'s mode of action come from structure–function analyses. The coiled-coil and the filamin domain mediate mRNA repression in tethering assays but alone are not sufficient to recapitulate *lin-41* activity in the context of a free mRNA (Loedige et al. 2012). Moreover, whereas a construct lacking the TRIM domain (i.e., RING, B-box, and coiled-coil domains) can coimmunoprecipitate *LIN41* target mRNAs, a construct lacking

Figure 2. Proposed models of *LIN41* function. (A) *LIN41* ubiquitylates SHCBP1 to augment FGF signaling, increase proliferation rate, and inhibit premature differentiation in neural progenitor cells (J Chen et al. 2012). (B) *LIN41* ubiquitylates AGO2, inducing its degradation by the proteasome, leading to decreased miRNA activity (Rybak et al. 2009). (C) *LIN41* induces degradation and translational inhibition of target mRNAs such as *CDKN1a*, promoting rapid proliferation of ES cells (Chang et al. 2012; Loedige et al. 2012). See the text for details.

the NHL domain cannot (Loedige et al. 2012). Combined with the fact that NHL domains are structurally similar to the more widely studied WD40 propellers, which have been shown to mediate both protein–protein interaction (Xu and Min 2011) and RNA binding (Lau et al. 2009; Castello et al. 2012), it seems possible that the NHL domain could mediate specific, and possibly direct, binding of target mRNAs. A domain swap experiment with Trim32 supports a key role of the NHL repeats in determining target specificity: Trim32 containing the NHL repeats of LIN41, but not wild-type Trim32, represses luciferase reporters containing the 3′ UTRs of LIN41 targets, whereas LIN41 containing Trim32 NHL repeats is inactive (Loedige et al. 2012).

Is mRNA silencing a major activity of LIN41 in regulating cell differentiation and proliferation in development? In support of this notion, the cyclin-dependent kinase inhibitor *Cdkn1a* was identified as a LIN41 target in mES cells, where LIN41 promotes G1-to-S-phase transition and thus increases proliferation (Chang et al. 2012). Specifically, *Cdkn1a* levels were increased when LIN41 was depleted by siRNAs, and combined knockdown of LIN41 and *Cdkn1a* rescued the slow proliferation rate as well as the G1-phase accumulation of LIN41-deficient cells (Chang et al. 2012). These data are consistent with, although not a direct proof of, LIN41 regulating cell proliferation in these cells through repression of *Cdkn1a*.

Loedige et al. (2012) sought to obtain a more global picture of the effects of LIN41 on gene expression and found that ectopic expression of LIN41 in HEK293 cells caused the expression levels of >800 genes to change significantly, whereas knockdown of LIN41 in mES cells affected ~100 genes. The 3′ UTRs of selected dysregulated genes were sufficient to confer post-transcriptional regulation by LIN41 to a reporter gene, with targets thus validated including genes with cell cycle-promoting but also inhibitory roles. For instance, the retinoblastoma-like transcription factors Rb1 and Rb2 as well as E2F7 are inhibitors of the cell cycle; their repression might thus explain the increased proliferation rate observed in ES cells with high levels of LIN41. On the other hand, LIN41 also repressed positive regulators of the cell cycle such as cyclin D and cyclin E or Myb. The significance of these findings is currently not clear. It is also currently unknown which target mRNAs, if any, might mediate LIN41's effects on differentiation.

In addition to regulating mRNAs, mouse LIN41 was recently shown to bind to and stabilize Shc1p protein in mouse neural progenitor cells. Shc1p is a putative mediator of FGF signaling, and *Shc1p* knockdown impairs FGF signaling in neural progenitor cells (J Chen et al. 2012). FGF signaling, like LIN41 activity, promotes neural precursor proliferation and represses differentiation (Guillemot and Zimmer 2011). Moreover, neuroepithelial cells from LIN41-deficient mice have decreased activity of the FGF pathway in vivo and are hyporesponsive to FGF stimulation in vitro (J Chen et al. 2012). Although it remains to be formally demonstrated that LIN41 affects FGF signaling by stabilizing SHCBP1 in neural progenitor or other cells, it therefore seems possible that a major func-

tion of LIN41 in this developmental context is to promote FGF signaling to control cell proliferation and differentiation status.

Finally, as we discuss next, LIN41 has been shown to polyubiquitylate Argonaute (Ago), the core component of the miRNA-induced silencing complex (miRISC), which mediates mRNA degradation and translational repression by miRNAs. Since global impairment of miRNA activity has been shown in several examples to promote cell proliferation (Kumar et al. 2007; Hill et al. 2009; Melo et al. 2009, 2010), it is conceivable that ubiquitylation-dependent Ago degradation would mediate (some of) LIN41's effects on cell proliferation and differentiation.

LIN41 and miRNAs: a complex relationship

Several TRIM-NHL family members have been found to regulate gene expression by modulating the miRNA pathway, suggesting a model of how LIN41 could regulate targets such as *lin-29*. For instance, *C. elegans nhl-2* (Hammell et al. 2009), *Drosophila mei-P26* (Neumüller et al. 2008), and mouse *Trim32* (Schwamborn et al. 2009) influence miRNA activity either negatively or positively by interacting with Ago proteins. Intriguingly, a proteomic analysis identified LIN41 in immunoprecipitates of *C. elegans* Dicer, the enzyme that processes precursor miRNAs to mature miRNAs (Duchaine et al. 2006). However, this interaction has not been examined in great detail, and a functional relevance remains to be determined. In contrast, an interaction between LIN41 and Ago has been observed in various human and mouse cell lines. Specifically, several studies showed that LIN41 localizes at least in part to P-bodies, as assessed by immunofluorescence staining of endogenous LIN41 in KH2 ES cells (Chang et al. 2012) and embryonic carcinoma cells (Rybak et al. 2009) or staining tagged and overexpressed LIN41 in HEK293 (Loedige et al. 2012) and HeLa cells (Rybak et al. 2009). LIN41 not only resides in these sites of mRNA storage and degradation where Ago is also found (Kulkarni et al. 2010), but in fact physically interacts with Ago (Rybak et al. 2009; Chang et al. 2012; J Chen et al. 2012; Loedige et al. 2012), albeit in a largely RNA-dependent manner (Loedige et al. 2012).

Functionally, LIN41 was found to promote Ago ubiquitylation in vitro and in mouse embryonic carcinoma cells, where this resulted in proteasome-mediated Ago decay (Rybak et al. 2009). Repressive effects of LIN41 on Ago levels and on the function of several miRNAs have recently also been reported for HCC cell lines, although an involvement of Ago ubiquitylation was not examined in this study (YL Chen et al. 2012). Irrespective of mechanism, the observation of altered Ago levels raised the attractive hypothesis that LIN41, by antagonizing miRNA activity globally, could constitute a double-negative feedback loop with *let-7* to create a bistable switch at the crossroad between the stem cell state and differentiation. The notion also provided an alternative explanation to earlier genetic data, which had shown that LIN41 depletion in worms could suppress developmental phenotypes seen with reduced activity of core components of

the miRNA pathway (Grishok et al. 2001; Büssing et al. 2010). Since these phenotypes, vulval bursting and alae defects, are the same that are also found in *let-7lf* animals, it was originally assumed that the *let-7* defect was predominant in the miRNA pathway mutant animals and accordingly suppressed upon *LIN41* knockdown. The new data (Rybak et al. 2009) offered the alternative possibility that also in *C. elegans*, LIN41 could more directly modulate miRNA activity.

However, although ubiquitylation activity of LIN41 on Ago was confirmed in an independent study (Loedige et al. 2012), the functional consequences remain unclear. In particular, no changes in Ago2 levels or stability could be observed upon *LIN41* knockdown in mES cells (Chang et al. 2012) or *LIN41* overexpression in HEK293 cells (Loedige et al. 2012). Similarly, there was no difference in Ago2 levels between a wild-type and a *LIN41*-overexpressing neural progenitor cell line (J Chen et al. 2012). More importantly, neural progenitor cells of wild-type and *LIN41lf* mice had similar Ago2 levels and ubiquitylation patterns in vivo (J Chen et al. 2012).

Although it is unclear why the results in the earlier (Rybak et al. 2009) and later (Chang et al. 2012; J Chen et al. 2012; Loedige et al. 2012) studies differ, collectively, these data indicate that regulation of Ago2 levels and thus global miRNA activity through polyubiquitylation by LIN41 does not seem to be a general phenomenon. As both studies that observed Ago2 decline upon LIN41 overexpression used transformed cell lines (Rybak et al. 2009; YL Chen et al. 2012), different LIN41 cofactors, altered baseline Ago2 stability, or levels in malignant cells might explain the observed differences.

Regardless of the possibility that LIN41 may alter miRNA activity globally, several LIN41 targets are also under miRNA control, and it has been suggested that similar to NHL-2, LIN41 and miRNAs may collaborate in target repression. For instance, in mES cells, *Cdkn1a* is regulated by both miR-302 and LIN41 via its 3' UTR. Consistent with LIN41 activity depending on miR-302 activity and vice versa, a reporter containing a 59-nucleotide (nt) fragment of the *Cdkn1a* 3' UTR that contained a validated miR-302-binding site was equally desilenced whether LIN41 and miR-302 were inactivated individually or jointly (Chang et al. 2012). Moreover, whereas *LIN41* overexpression in wild-type ES cells promoted their proliferation, this effect was abrogated in *Dgcr8* knockout cells, which lack most miRNAs (Chang et al. 2012). In contrast, examination of endogenous *Cdkn1a* mRNA showed that joint depletion of *LIN41* and miR-302 additively stabilized its levels, which would argue against their cooperation (Chang et al. 2012). However, *Cdkn1a* protein levels were not examined in this experiment, whereas the reporter assay determined luciferase activity, which would integrate both mRNA and protein level changes. An intriguing but speculative possibility could be that LIN41 and miR-302 activities on mRNA levels are independent, whereas further repression of protein accumulation might require a cooperative mechanism.

In contrast to the findings on *Cdkn1a* regulation, examination of a larger set of LIN41 targets revealed that

many of these remained unchanged upon Ago depletion (Loedige et al. 2012), suggesting that LIN41 can silence them independently of miRNA activity or can at least tolerate compromised miRNA activity. Moreover, ectopic expression of both *miR-302* and *LIN41* in HEK293 cells additively silenced an *E2F7* reporter (Loedige et al. 2012). Although the latter experiment leaves open the possibility that LIN41 and miRNAs cooperate under physiological conditions with presumably lower endogenous expression levels, these results show that LIN41 does not, per se, require miRNAs to achieve mRNA silencing. However, with the emerging view of 3' UTRs as platforms for regulation through RNA-binding proteins and miRNAs, we predict that more examples of cooperation—and, indeed, antagonism—between LIN41 and miRNAs will emerge, with the rules of interaction depending on the sequence or architecture of individual 3' UTRs.

An enigmatic RING domain: To ubiquitylate or not to ubiquitylate?

Ubiquitylation is a post-translational protein modification that not only results in proteasome-mediated degradation, but also alters protein function in many different ways (Komander and Rape 2012). It is catalyzed by a multisubunit enzyme complex, which obtains its substrate specificity from its E3 subunit. A large class of these E3 ligase subunits contains a characteristic RING domain, which is also found in the TRIM domain of most LIN41 proteins. Accordingly, and as an alternative to LIN41 functioning as a translational repressor, it was suggested to destabilize target proteins such as LIN-29 through polyubiquitylation (Slack et al. 2000).

However, a few observations seemed to argue against ubiquitylation as a major molecular activity of LIN41 in developmental processes. First, the putative *Drosophila* LIN41 *dappled/wech* lacks a RING domain (O'Farrell et al. 2008). Second, although screens for heterochronic mutants and *let-7lf* suppressors identified many *LIN41lf* and null alleles, no point mutations affecting the RING domain were identified (Slack et al. 2000). In contrast, several point mutations affected the NHL domain. Finally, LIN41 function in hypodermal and male tail development is highly dosage-sensitive (Del Rio-Albrechtsen et al. 2006), which would be unexpected if its major role was catalytic.

Nonetheless, ubiquitylation activity of LIN41 has now been reported in several studies. LIN41 can autoubiquitylate in vitro and in cells (Rybak et al. 2009; Loedige et al. 2012), and it has been speculated that this may be used as an autoregulatory mechanism in vivo (Del Rio-Albrechtsen et al. 2006), although experimental evidence for this is currently lacking. A second validated target is Ago, although, as discussed above, the consequences of Ago ubiquitylation are unclear, with Ago destabilization not observed in most instances.

Recently, J Chen et al. (2012) identified *Shc-binding protein 1* (*Shcbp1*) as a possible mediator of LIN41's functions in regulating mouse neural development, as discussed in an earlier section. Strikingly, *Shcbp1* is ubiquity-

lated in vivo and binds to LIN41, as determined by yeast two-hybrid and coimmunoprecipitation analysis of tagged, ectopically expressed proteins in HEK293 cells. Somewhat counterintuitively, Shcblp levels are reduced in embryonic neuroepithelium from *LIN41lf* mice, and conversely, *LIN41* overexpression in HEK293T cells increases the stability, and thus levels of, Shcblp. It is, however, unclear whether stabilization and ubiquitylation of Shcblp are linked. This is because expression in HEK293T cells of a RING domain-less LIN41, which is inactive in promoting autoubiquitylation, continues to promote significant, albeit reduced, ubiquitylation of Shcblp and stabilizes Shcblp to almost the same degree as does wild-type LIN41. Thus, it will be important to identify the sites on Shcblp that are targets of ubiquitylation to test whether Shcblp mutated to prevent ubiquitylation is destabilized. Similarly, it will be interesting to determine the ubiquitylation status of endogenous Shcblp, whose levels decline in sync with those of LIN41 during mouse embryonic brain development.

Taken together, it is now well documented that LIN41 has E3 ligase activity. However, it is still unclear in which contexts and to what extent ubiquitylation contributes to LIN41 function, including effects on LIN41 targets that are still elusive. Furthermore, a major question is whether the ubiquitylating activity of LIN41 represents a function separate from translational repression. Possibly, the RING and other domains of LIN41 jointly induce mRNA degradation and translational repression, as recently observed with another RING domain protein and translational repressor, MEX-3C (Cano et al. 2012).

Outlook and conclusion

Converging results from research in vertebrates and invertebrates have now helped to generate a picture of LIN41 as a key effector of the *let-7* miRNA pathway that promotes cell proliferation and inhibits differentiation to control various developmental processes. The emerging fact that LIN41 is itself a translational repressor further increases the sophistication of the *LIN28/let-7/LIN41* network architecture: *let-7* directly represses cell cycle genes and thus proliferation (Johnson et al. 2007). To promote differentiation, it further down-regulates transcription factors that are important for progenitor states (Großhans et al. 2005; Melton et al. 2010). At the same time, repression of *LIN41* by *let-7* derepresses cell cycle inhibitors, which provides a further break on proliferation. Finally, differentiation-inducing factors are also likely to be released from LIN41-mediated repression, although their identities need to be revealed. This dual function—repression of direct targets and liberation of *LIN41* targets from repression—might be important to achieve robust changes in gene expression and thus cell fates.

Despite the evident importance of LIN41 as an effector of *let-7*, we predict that *LIN41* may also emerge as a critical node in developmental events not controlled by *let-7*. The *lin-28* heterochronic gene has at least three distinct molecular activities in mammalian cells, including some not requiring *let-7* (Polesskaya et al. 2007;

Rybak et al. 2008; Viswanathan et al. 2008; Cho et al. 2012), and by analogy, *LIN41* might influence development by several distinct mechanisms. The widespread expression and complex biological output of *LIN41*, together with its intricate domain architecture, also favor such a scenario, as they suggest that the activity of *LIN41* might be modulated at various levels. Nonetheless, how such modulation occurs and how LIN41 promotes translational silencing and mRNA degradation mechanistically remains to be determined. Moreover, although there is now little doubt that LIN41 can support ubiquitylation, the physiological significance of this activity requires further study. We speculate that the E3 ligase activity of LIN41—or (given the activity of overexpressed, RING-less LIN41) of a LIN41-binding partner—has more specific functions in particular processes. Examination of the rescue of *LIN41lf* mouse phenotypes by a ubiquitylation-deficient *LIN41* transgene may offer one way forward.

Finally, the *Drosophila LIN41 dappled/wech* is required for a functional link between integrins and the cytoskeleton—and thus muscle attachment to the body wall—by virtue of its interaction with Talin and the integrin-linked kinase ILK (Loer et al. 2008). Although it remains to be determined whether the canonical, RING finger-containing LIN41 proteins in other animals share this function, it is intriguing that endogenous TRIM71 in adult mice is also detected at sarcomeric Z disks of adult muscles, where it colocalizes with ILK (Loer et al. 2008).

At any rate, the recent exciting progress on the developmental, cellular, and molecular functions of LIN41 has clearly demonstrated that, beyond serving as a model miRNA target, LIN41 is a worthy subject of scientific examination in its own right.

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LIN-41/TRIM71: emancipation of a miRNA target

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