LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in Caenorhabditis elegans

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Abstract

The let-7 microRNA (miRNA) is an ultraconserved regulator of stem cell differentiation and developmental timing, and a candidate tumour suppressor. Here we show that LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 processing in C. elegans. We demonstrate that lin-28 is necessary and sufficient to block let-7 activity in vivo; LIN-28 directly binds let-7 pre-miRNA to prevent Dicer processing. Moreover, we have identified a poly(U) polymerase, PUP-2, which regulates the stability of LIN-28 blockaded let-7 pre-miRNA, and contributes to lin-28 dependent regulation of let-7 during development. We show that PUP-2 and LIN-28 interact directly, and that LIN-28 stimulates uridylation of let-7 pre-miRNA by PUP-2 in vitro. Our results demonstrate that LIN-28 and let-7 form an ancient regulatory switch, conserved from nematodes to humans, and provide insight into the mechanism of LIN-28 action in vivo. Uridylation by a PUP-2 orthologue might regulate let-7 and additional miRNAs in other species. Given the roles of Lin28 and let-7 in stem cell and cancer biology, we propose such poly(U) polymerases are potential therapeutic targets.

Small RNAs regulate gene expression in many eukaryotes including plants, animals and fungi. miRNAs are endogenous short RNAs that modulate gene expression by blocking translation and/or destabilizing target mRNAs. In animals miRNAs are transcribed as long precursors (pri-miRNAs), which are processed in the nucleus by the RNase III enzyme complex Drosha-Pasha/DGCR8 to form ~80 nt pre-miRNAs, or are derived directly from introns. pre-miRNAs are exported from the nucleus and processed by the RNase III enzyme Dicer, and incorporated into an Argonaute-containing RNA-induced silencing complex (RISC). The first identified miRNAs, the products of the C. elegans genes lin-4 and let-7, control cell fates during larval development. When either lin-4 or let-7 is inactivated, specific epithelial cells fail to differentiate and undergo additional divisions. lin-4 acts during early larval development, regulating lin-14 and lin-28 mRNAs. let-7 acts during late larval development, regulating lin-41, hbl-1, daf-12 andpha-4 mRNAs. As such, the time of appearance of these miRNAs must be tightly controlled. In C. elegans

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N.J.L., S.B. and E.A.M. conceived the original project. N.J.L. carried out all experiments unless stated otherwise. J.A. carried out northern blotting, uridylase assays and microarray experiments. H.L.L., J.A., A.B. carried out RNA mobility shift assays. K.J.M., J.A. carried out some of the in vitro binding assays. N.J.L., J.A. and E.A.M. wrote the manuscript.
and other animals the expression of let-7 is developmentally regulated, but the mechanisms underlying this regulation remain unknown\(^\text{13}\). Post-transcriptional regulation of specific miRNAs has recently been uncovered\(^\text{14}\). let-7 biogenesis is blocked by Lin28 at either the Drosha\(^\text{15, 16}\) or Dicer\(^\text{17, 18}\) step in mammalian cell culture. Lin28 is a conserved RNA-binding protein, which in mammals controls stem cell lineages and inhibits let-7 miRNA processing \textit{in vitro}\(^\text{15, 16, 17, 18, 19}\). However, the mechanism and \textit{in vivo} significance of this activity are unclear.

**RESULTS**

**An \textit{in vivo} assay of let-7 miRNA function reveals developmental regulation**

To study the mechanism of miRNA action \textit{in vivo}, we established a quantitative miRNA reporter assay based on let-7 in \textit{C. elegans} (Fig 1a, b and Supplementary Methods). We generated two transgenes comprising the promoter of myo-2, the coding sequences of either GFP or mCherry and the 3′ UTR of either lin-41 or unc-54 (myo-2::gfp::lin-41 and \textit{myo-2::mcherry::unc-54}; hereafter referred to as the \textit{let-7 sensor}; Fig. 1a). The myo-2 promoter confers expression exclusively in the pharyngeal muscle, the food pump of \textit{C. elegans}\(^\text{20}\), and \textit{lin-41} is a genetically identified target of the let-7 miRNA\(^\text{9}\), whereas the \textit{unc-54} 3′ UTR is not known to be regulated by any miRNA. Transgenic animals carrying an intrachromosomal array of the \textit{let-7 sensor} expressed both GFP and mCherry strongly throughout larval development (Fig. 1c). As expected, a transgene expressing \textit{let-7} (\textit{myo-2::let-7}) silenced GFP, but not mCherry (Fig. 1d). Surprisingly, this effect was developmentally regulated; inhibition of GFP is markedly stronger in adults than L1 larvae (Fig. 1d). As the \textit{let-7} transgene does not contain the \textit{let-7} promoter, this regulation must occur post-transcriptionally. In addition to the qualitative analysis of fluorescent protein expression using microscopy we quantified the activity of the \textit{let-7 sensor} using flow cytometry of whole animals. We used a COPAS Biosort instrument to quantify GFP and mCherry expression along the body axis of thousands of individual animals at different stages during development. \textit{let-7 sensor} silencing was least efficient at the L1 larval stage, and reached maximal efficiency during L3 (Supplementary Methods and Supplementary Fig. 1). This correlates with the temporal expression pattern of \textit{let-7}, which begins to accumulate during the L3 stage\(^\text{21}\). Since \textit{let-7} is being driven by a promoter active at all stages, but appears active only at later larval stages, these data may reflect a mechanism that post-transcriptionally regulates \textit{let-7} during development.

**LIN-28 regulates pre-let-7 processing**

Next we carried out forward genetic and RNAi screens to identify factors regulating \textit{let-7} activity \textit{in vivo}. Knockdown of \textit{lin-28} by RNAi resulted in reduced GFP at L1 and L2 stages, in a manner dependent on the \textit{myo-2::let-7} transgene (Supplementary Fig. 2a, b and data not shown). We confirmed these results using a \textit{lin-28} loss-of-function mutant (Fig. 1e and data not shown). Mutations in \textit{lin-46} completely suppress the developmental timing defect of \textit{lin-28} mutants\(^\text{22}\), but do not restore developmental regulation of \textit{let-7} (Fig. 1e). Thus deregulation of \textit{let-7} activity in \textit{lin-28} mutants is not an indirect consequence of developmental timing defects. Other heterochronic genes, including \textit{lin-14} and \textit{lin-42} did not affect the \textit{let-7 sensor} (Supplementary Fig. 2c and data not shown). Next we tested if LIN-28 was sufficient to inhibit \textit{let-7} activity. Ectopic expression of LIN-28 in the pharynx from an extrachromosomal array resulted in inhibition of \textit{let-7} in adults, which do not normally express LIN-28\(^\text{8}\) (Fig. 1f). Mosaic expression of the extrachromosomal array within the pharynx indicated that LIN-28 acts cell autonomously. We concluded that LIN-28 is required and sufficient to inhibit \textit{let-7} activity in \textit{C. elegans}. 

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We then used miRNA microarrays and northern blotting to confirm that LIN-28 regulates endogenous let-7 accumulation in L2 larvae (Supplementary Data 1; Supplementary Fig. 3)\textsuperscript{23}. Further, expression of other let-7 family members was not increased in lin-28 mutants, whereas three unrelated miRNAs, including the developmentally regulated miRNA miR-85, showed increased expression in lin-28 mutant L2s (Supplementary Fig. 3a,b).

Whether Lin28 regulates let-7 processing at the Drosha\textsuperscript{15,16} or Dicer\textsuperscript{17,18} step in mammalian cells is unresolved. We addressed this in vivo in C. elegans. We used northern blotting and qRT-PCR to compare expression of let-7 and its processing intermediates from the myo-2::let-7 transgene in otherwise wild-type and lin-28 mutant L2 larvae. lin-28 mutants expressed higher levels of let-7 than wild type, indicating increased processing efficiency; this was accompanied by a slight reduction in the level of pre-let-7, and no change in pri-let-7 levels; these data are consistent with increased efficiency of Dicer-mediated processing (Supplementary Fig. 3c,d). We obtained similar results for endogenous let-7, although levels of pri-let-7 were decreased in lin-28 mutants, suggesting an indirect effect on the let-7 promoter (Supplementary Fig. 3e,f). Interestingly, miR-85 also appears to be regulated at the Dicer step in a lin-28-dependent fashion (Supplementary Fig. 3b,g). Consistent with these findings a functional LIN-28-GFP translational fusion is localised in the cytoplasm (Supplementary Fig. 4a and data not shown). Taken together, these data suggest that LIN-28 blocks Dicer-mediated processing of let-7 and possibly other developmentally regulated miRNAs.

Next, we tested whether LIN-28 directly interacts with pre-let-7. We performed pull-down assays using streptavidin beads and biotinylated pre-let-7. LIN-28-GFP from transgenic worm extracts was retained on streptavidin beads if the synthetic pre-let-7 RNA was biotinylated, but not using a non-biotinylated control (Supplementary Fig. 4b). We tested whether this interaction was direct by native gel mobility shift assay. pre-let-7 and GST-LIN-28 interact with an estimated $K_d$ of 2 $\mu$M (Supplementary Fig. 4c and data not shown). We conclude that LIN-28 binds pre-let-7 to prevent Dicer processing. Experiments in mammalian cells suggested that the loop of the pre-let-7 hairpin is required for the interaction with Lin28\textsuperscript{15,24}. However, the pre-let-7 loop is not conserved in C. elegans. We therefore tested a number of pre-let-7 loop mutants in vivo using the let-7 sensor. We found that the pre-let-7 loop is not required for the normal developmental regulation of let-7 activity (see Supplementary Methods and Supplementary Fig. 5).

**PUP-2 regulates pre-let-7 processing in a lin-28 dependent fashion**

Our results so far were consistent with a LIN-28 blockade of pre-let-7 processing, but we were puzzled that pre-let-7 accumulation in L2 larvae differed little in wild-type compared to lin-28 mutant animals (Supplementary Fig. 3). We reasoned that LIN-28 might target pre-let-7 for degradation. Recent work by Kim and colleagues demonstrated that Lin28 promotes pre-let-7 uridylation and subsequent degradation in mammalian cell lines, although the enzyme(s) involved are unknown\textsuperscript{18}. We inspected published high-throughput sequencing data of C. elegans small RNA libraries\textsuperscript{25,26}, and found frequent modification of the 3’ end of let-7 with 1 or 2 untemplated uracil residues (C. elegans let-7 resides on the 5’ arm of the hairpin; Fig. 2a). These species are likely to arise from Dicer processing of partially uridylated intermediates, and indicate in vivo uridylation of let-7. Therefore we carried out an RNAi screen against 15 potential poly(U) polymerases (Supplementary Table 1) assaying let-7 and pre-let-7 abundance in myo-2::let-7 transgenic L2 larvae. RNAi against pup-2 resulted in increased pre-let-7 levels (Fig. 2b,c; $P = 7.5 \times 10^{-5}$), and a small but significant increase in mature let-7 levels (Fig. 2b,d; $P = 0.029$). This effect is specific to pup-2, no other poly(U) polymerases, including cid-1, a potential paralogue, had this effect\textsuperscript{27} (Fig. 2c,d). These data suggest that PUP-2 uridylation targets pre-let-7 for degradation, and is required for maximally efficient blockade of let-7 processing by LIN-28.
We reasoned that LIN-28 might target uridylation of pre-let-7 by PUP-2, leading to degradation of the uridylated pre-let-7 and turnover of LIN-28/pre-let-7 complexes, ensuring efficient LIN-28 function. We examined the effect of pup-2 RNAi in situations where pre-let-7 is released from LIN-28 blockade. The effect of pup-2 RNAi on pre- and mature let-7 levels is abolished at the L4 stage (Fig. 2c,d $P = 1 \times 10^{-5}$ and $P = 0.01$ respectively). Further, L2 larvae exposed to both pup-2 and lin-28 RNAi show significantly reduced accumulation of pre-let-7 (Fig. 2c $P = 0.0003$). These effects are not due to reduced RNAi against pup-2 (Supplementary Fig. 6a). In contrast, the effect of lin-28 RNAi on mature let-7 levels is not altered in lin-28, pup-2 double RNAi L2 larvae (Fig. 2d $P = 0.2$). From these data we concluded that PUP-2 post-transcriptionally regulates let-7 in a LIN-28-dependent fashion.

PUP-2 contributes to LIN-28-dependent regulation of let-7 during development

Next we sought to determine if PUP-2 is required for regulation of let-7 during development. Misregulation of let-7 results in altered timing of larval development, defects in differentiation of a hypodermal stem cell lineage required for the formation of adult-specific lateral alae, and defects in vulval morphogenesis. Lateral seam cells differentiate and fuse into a syncytium in wild-type adults, but this fusion is defective if pup-2 or lin-28 is knocked down, consistent with a role in regulating let-7 (Fig. 2e, Supplementary Table 2). pup-2 RNAi in a lin-28 null mutant background does not increase seam cell fusion defects suggesting this activity of pup-2 is lin-28 dependent (Table 1). Next we tested whether pup-2 genetically interacts with let-7. let-7(n2853ts) animals show reduced let-7 expression and temperature sensitive vulval bursting. At 15°C, vulval bursting of let-7(n2853ts) animals was suppressed by pup-2 RNAi, whereas lin-28 RNAi suppressed vulval bursting at both 15°C and 20°C (Table 1). Weaker suppression of let-7(n2853ts) by pup-2 compared to lin-28 is consistent with a role for pup-2 as a lin-28 modifier. Taken together with the effect of pup-2 on let-7 processing, these data indicate pup-2 ensures efficient activity of lin-28 by targeting blockaded pre-let-7 molecules for destruction. This might occur via LIN-28 dependent uridylyl-transferase activity of PUP-2 on pre-let-7; we sought to test this hypothesis in vitro.

PUP-2 uridylates pre-let-7 in a LIN-28 dependent fashion in vitro

We expressed HA-FLAG tagged PUP-2 and SBP tagged LIN-28 in a HEK293T human embryonic kidney cell line. Immunoprecipitation of HA-FLAG-PUP2 using anti-FLAG antibodies specifically co-precipitated SBP-LIN-28 (Fig. 3a). This interaction was in absence of C. elegans pre-let-7 and likely direct. Indeed, the addition of excess exogenous pre-let-7 to the cell extract did not enhance the interaction. We also confirmed this interaction in GST pull-down experiments. In vitro translated PUP-2 directly interacts with GST-LIN-28 (Fig. 3b). PUP-2 was previously shown to polyuridylate an artificially tethered RNA in Xenopus oocytes, but was inactive without tethering. Therefore, we tested if LIN-28 might be able to recruit PUP-2 to mediate pre-let-7 uridylation (Fig. 3c). We incubated anti-FLAG immunoprecipitates from cell extracts expressing HA-FLAG-PUP2 and/or SBP-LIN-28 with radiolabeled pre-let-7 and radiolabelled UTP. We find that HA-FLAG-PUP2 uridylated pre-let-7 only in the presence of SBP-LIN-28 (Fig. 3c). We also confirmed LIN-28 dependent uridylation of pre-let-7 by PUP-2 in vitro (Supplementary Fig. 6b). Finally, we attempted to identify in vivo uridylated pre-let-7 directly by cloning, but we were unable to do so. We conclude that rapid degradation of uridylated pre-let-7 prevents accumulation of these species in vivo, as has been postulated in human cell lines.
DISCUSSION

We have developed a quantitative assay of let-7 miRNA function in C. elegans. This assay is highly sensitive and amenable to high-throughput experiments. We have isolated new mutants in known miRNA pathway components through mutagenesis screens using this assay; analysis of novel miRNA function defective mutants should provide insights into the miRNA mechanism. In addition, this assay could be modified to study post-transcriptional regulation or target specificity of other miRNAs.

Here we demonstrate that LIN-28 regulates C. elegans pre-let-7 (see Supplementary Fig. 7a for a model). These results provide a molecular basis for the genetic link between lin-28 and let-7 in controlling developmental timing. In C. elegans this pathway determines the behaviour of epithelial stem cells. In mammals let-7 and Lin28 might regulate primordial germ cell differentiation and other stem cell lineages. Therefore, the specific interaction of a structured RNA (pre-let-7) with a protein (Lin28) constitutes an ultraconserved switch regulating stem cell differentiation. The let-7/lin-28 switch might be as conserved as let-7 itself. For example, pre-let-7 processing is developmentally regulated in the sea urchin Strongylocentrotus purpuratus, which also expresses a Lin28 orthologue (data not shown).

Our finding that the terminal loop of pre-let-7 is dispensable for regulation by LIN-28 is at odds with two previous studies, but is consistent with competition experiments carried out by Rybak et al. Our approach has been to assess let-7 function in vivo, whereas previous work was based on in vitro interaction studies. All 22 nucleotides of mature let-7 are conserved in bilateria, whereas for many other miRNAs only the “seed” sequence (nucleotides 2 to 8) appears to be under evolutionary constraint. In contrast, there is little sequence similarity in the terminal loops of let-7 in different species. It is therefore tempting to speculate that nucleotides corresponding to mature let-7 contribute to LIN-28 recognition. Similar RNA-protein interactions might impose evolutionary constraint on the sequences of other ultraconserved miRNAs.

Here we show that LIN-28 recruits the poly(U) polymerase PUP-2 to uridylate C. elegans pre-let-7. We speculate that mammalian PUP-2 orthologues might similarly regulate let-7 in stem cells (Supplementary Fig. 7b). Indeed, the mouse Tut4/Zcchc11 uridyl transferase regulates let-7 in embryonic stem cells. Let-7 is a candidate tumour suppressor and LIN28 is a potential proto-oncprotein. Therefore TUT4/ZCCHC11 might be an important novel target for anti-cancer therapy. Our data suggest that miRNAs are regulated through pre-miRNA sequestration and uridylation-dependent pre-miRNA degradation. This situation appears to be analogous to two-step regulation of the activity of proteins through sequestration and targeted degradation, for example in the case of cadherin. Uridylation-dependent degradation of RNA has been observed previously and U tails have been shown to recruit either 5′ to 3′ or 3′ to 5′ exonucleases. High-throughput sequencing suggests additional miRNAs and/or pre-miRNAs are subject to uridylation (data not shown), so regulation in this way may be widespread. Further uncovering the mechanisms underlying this pathway will be of great interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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TO_GATEWAY_TEV_SBP and pDEST-3FLAG 3HA vectors, Eric Moss (University of Medicine and Dentistry of New Jersey, NJ) for anti-LIN-28 antibody, and Marv Wickens (Department of Biochemistry, University of Wisconsin, MA) for PUP-2 cDNA. We thank Richard Gregory and Narry Kim for sharing unpublished data. N.J.L. and K.J.M. were supported by a PhD studentship from the Wellcome Trust (UK). J.A. and A.B. were supported by grants from the Biotechnology and Biological Sciences Research Council (UK). H.L.L. was supported by a PhD studentship from Cancer Research UK. This work was supported by a Cancer Research UK Programme Grants to E.A.M. and S.B. and core funding to the Wellcome Trust/Cancer Research UK Gurdon Institute provided by the Wellcome Trust and Cancer Research UK.

Appendix

Online Methods

Nematode culture and strains

We grew *C. elegans* under standard conditions at 20 °C\(^37\). The food source used was *E. coli* strain HB101 (*Caenorhabditis Genetics Center, University of Minnesota, Twin Cities, MN, USA*). We used bleaching followed by starvation-induced L1 arrest to generate synchronized cultures. The wild-type strain was var. Bristol N2\(^38\). Additional strains used are listed in Supplementary Table 3.

DNA constructs and transgenics

We generated DNA vectors using the Multisite Gateway Three-Fragment vector construction kit (Invitrogen; Supplementary Data 2). We performed site directed mutagenesis using PCR and mutagenic primers (Supplementary Data 2). All constructs were confirmed by sequencing. To generate transgenic animals, we performed germline transformations as described\(^39\). Injection mixes contained 2-10 ng \(\mu\text{l}^{-1}\) of vector, 5-10 ng \(\mu\text{l}^{-1}\) of marker, and Invitrogen 1 kb ladder to a final concentration of 100 ng \(\mu\text{l}^{-1}\) DNA (see Supplementary Methods for details). We integrated array transgenes via X-ray irradiation as described\(^40\). We generated single copy transgenes by transposase mediated integration (mosSCI) as described\(^41\).

Microscopy

We carried out differential interference contrast (DIC) and fluorescence imaging using standard methods\(^42\) and using an AxioImager A1 upright microscope (Zeiss, Jena, Germany). We captured images using an ORCA-ER digital camera (Hamamatsu, Hamamatsu, Japan) and processed images using OpenLabs 4.0 software (Improvision, Coventry, UK). For analysis of *let-7 sensor* transgene expression, we imaged all animals under identical conditions. We performed confocal microscopy using an Olympus FluoView FV1000 upright microscope using 63x objective magnification.

Analyses with the COPAS Biosort instrument

We used a COPAS Biosort instrument (Union Biometrica, Holliston, MA, USA) to simultaneously measure length (time of flight), absorbance (extinction), and fluorescence. We optimized fluorophore detection for simultaneous detection of GFP and mCherry. We used a multiline solid state argon laser for excitation (488nm GFP and 561nm mCherry), and detected emission by appropriate PMTs after passing through band pass filters (510/23nm GFP and 615/45nm mCherry). We harvested animals from plates and washed in M9 buffer\(^37\) prior to sorting. We determined length and absorbance for each larval stage using synchronised wild-type populations. We then generated gates to isolate animals of specific developmental stages from mixed populations (Supplementary Fig. 1a).
RNA interference assays

We obtained RNAi clones from genome-wide RNAi libraries\textsuperscript{43,44,45}. We generated additional RNAi constructs by subcloning of an appropriate genomic DNA fragment into pDEST-L4440\textsuperscript{45,46} (Supplementary Data 2). We confirmed all RNAi constructs by sequencing. For experiments using \textit{let-7} sensor and \textit{myo-2::let-7} transgenes we performed RNAi by feeding as described using the \textit{eri-1(mg366)} RNAi hypersensitive genetic background\textsuperscript{47}. For COPAS Biosort analysis, we plated 10-50 L1 larvae on 90 mm RNAi plates, and analysed animals once the oldest progeny reached the L3 larval stage. For harvest and RNA extraction we plated \textasciitilde3,000 L1 larvae per RNAi plate and grew animals to adulthood prior to bleaching. After synchronisation by starvation, we plated the progeny onto fresh RNAi plates and grew to the desired stage before harvesting. We performed RNAi by injection as described\textsuperscript{48}. We analyzed phenotypes on progeny laid 24-48 hrs post-injection.

Phenotypic analysis of seam cell development

We performed RNAi by injection into strains carrying seam cell marker transgenes \textit{wIs51} and \textit{mjIs15}.

Vulval bursting assay

\textit{let-7(n2853ts)} embryos were added to RNAi plates by bleaching gravid adults, and grown at 15 °C. Non-burst adults were then transferred to fresh RNAi plates and temperature-shifted as required. L4 progeny were picked to fresh RNAi plates (15-25 animals per plate), and vulval bursting was scored after 48 hrs.

RNA Extraction

For total RNA isolation we harvested animals from plates by washing with M9\textsuperscript{37}. We pelleted and froze animals in liquid nitrogen and dissolved pellets in 10 volumes of Trizol reagent (Invitrogen, Carlsbad, CA, USA). We extracted total RNA was from Trizol reagent according to the manufacturer’s protocol.

miRNA microarray analysis

We performed miRNA microarrays using custom DNA oligonucleotide arrays as described previously\textsuperscript{49,50}. Data analysis was as described\textsuperscript{50}. To compare miRNA expression in wild-type and \textit{lin-28} mutant L2 larvae we isolated and size selected total RNA from synchronized animals to 18-26 nt using polyacrylamide gel electrophoresis. The small RNA fraction was 3′ end-labelled using T4 RNA ligase (Fermentas UK, York, UK). \textit{C. elegans} miRNA microarrays were based on miRBase release 8.0\textsuperscript{51,52}. We performed all experiments in triplicates. For microarray probe information and primary microarray data see Supplementary Data 1.

Northern blotting

We performed northern blotting as described\textsuperscript{25,53}, with the following modifications. We used 5-20 \( \mu \)g total RNA, or small RNA fraction (miRvana, Ambion) isolated from \textasciitilde200 \( \mu \)g total RNA. For developmental expression profiles (Supplementary Fig. 3a) we carried out 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Perbio Science, Erembodegem, Belgium) crosslinking reactions for 2 hrs at 60 °C. Otherwise blots were UV-crosslinked. We modified northern hybridisations as follows; membranes were pre-hybridised at 40 °C for 4 hrs in hybridisation buffer (0.36 M Na\textsubscript{2}HPO\textsubscript{4}, 0.14 M NaH\textsubscript{2}PO\textsubscript{4}, 7% (v/v) SDS and 1 mg of sheared, denatured salmon sperm DNA) and hybridised at 40 °C overnight using 20 pmole of \( \gamma\textsuperscript{32}P\)-ATP-radiolabelled DNA oligonucleotide probes (Supplementary Data 2). After hybridisation, we washed membranes twice with 0.5 \( x \)SSC,
0.1% (v/v) SDS at 40 °C for 10 min and once with 0.1× SSC, 0.1% (v/v) SDS at 40 °C for 5 min. We detected radioactivity by phosphoimager (GE Healthcare, Amersham, UK). We quantified band intensity using ImageQuant software (GE Healthcare).

**Real-time RT-PCR**

We performed RT-PCR as described\(^\text{25}\), using the standard curve method. Primers used are listed in Supplementary Data 2.

**pre-let-7 pull-down**

For these experiments we generated a strain carrying a rescuing lin-28::gfp translational fusion transgene (mosSCI integrated) in a lin-28(n719) mutant background. We prepared protein extracts from starvation synchronised L1 larvae. We cleared lysates against streptavidin Dynabeads (Invitrogen) for 30 min at 4 °C in PD buffer [18 mM HEPES-KOH pH 7.9, 10% (v/v) glycerol, 40 mM KCl, 2 mM MgCl\(_2\), 10 mM DTT, 100 μM ZnSO\(_4\), 1x Proteinase Inhibitor Cocktail (PIC; Roche)]. Dynabeads were blocked with 15 μg yeast tRNA for 1 h at 4 °C in PD buffer before addition of 100 pmol synthetic 5′ biotinylated pre-let-7 (Microsynth, Balgach, Switzerland) for pull-down, or unmodified synthetic pre-let-7 for control reactions, and incubated for 1 hr at room temperature. We added pre-blocked Dynabeads to the binding reaction and incubated for 1 h at room temperature. We washed beads 3 times in PD buffer. We analyzed bound proteins by western blotting with primary mouse anti-GFP (Clontech JL-8; 1:1000) and secondary HRP-conjugated anti-mouse (Dakocytomation P0450; 1:10,000), or rat anti-tubulin (Chemicon international MAB1684, 1:1000) and secondary HRP-conjugated mouse anti-rat (GE Healthcare NA9310; 1:10,000).

**Recombinant protein expression**

We obtained LIN-28 cDNA (F02E9.2b) from the ORFeome library\(^\text{45}\). We subcloned cDNAs into pDEST-GEX-2TK (Gateway cassette inserted at SmaI site in pGEX-2TK), or pDEST-MAL. We expressed and purified recombinant proteins as described\(^\text{25}, \text{54}\).

**GST-pull-down**

We used PUP-2 cDNA in pDEST14 (Invitrogen) to produce \(^{35}\)S-methionine-radiolabelled protein by in vitro transcription-translation using a TNT T7 coupled reticulocyte lysate kit (Promega). We performed pull-downs were performed using GST-LIN-28 as described\(^\text{54}\).

**Pre-let-7 transcription**

We performed in vitro transcription reactions in a volume of 20 μl with 0.5 mM of each NTP, 40 mM Tris pH 7.9, 12 mM MgCl\(_2\), 2 mM spermidine, 20 mM DTT, 1 mM NaCl, 100 U T7 RNA polymerase (Roche, Basel, Switzerland), and 1U Rnasin (Promega, Madison WI, USA). We incubated reactions for 1 hr at 37 °C, prior to phenol/chloroform extraction and ethanol precipitation. We transcribed radiolabeled RNA for electrophoretic mobility shift assays with α-\(^{32}\)P-UTP to a specific activity of approximately 6,000 cpm/fmol.

**Immunoprecipitation**

We cloned LIN-28 cDNA into pcDNA5/FRT/TO_GATEWAY_TEV_SBP. We cloned PUP-2 cDNA into pDEST-3FLAG 3HA. We performed immunoprecipitation assays as described previously\(^\text{18}\). Briefly, we transfected HEK293T cells with pHA-FLAG-PUP-2 and/or pLIN-28-SBP. After 48 h we collected cells in cold lysis buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris (pH 8.0), 1% (v/v) Triton X-100), sonicated for 4 min on ice and centrifuged for 10 min. We incubated 50 μl of the supernatant was with 5 μl of pre-washed anti-FLAG antibody, conjugated to agarose beads (Sigma) and incubated for 2 hr at 4°C. We washed agarose-beads twice with lysis buffer and twice with buffer D. For in vitro
uridylation, we incubated agarose beads in a 30 μl reaction containing 3.2 mM of MgCl₂, 1 mM of DTT and 0.25 mM of rUTP and 5'-end-labeled pre-miRNA of 1 × 10⁴-1 × 10⁵ cpm, for 20 min at 37 °C. We purified RNA by Trizol extraction and isopropanol precipitation. We analyzed reactions in a 12% urea polyacrylamide gel.

**In vitro uridylation assays**

We performed in vitro uridylation assays in 30 μl reactions containing 1.5 μg of in vitro transcribed pre-let-7 in 10 mM Tris pH 7.5, 30 mM KCl, 1 mM DTT, 10 mM MnCl₂, 2 mM MgCl₂, 0.25 mM UTP, 1 μl of RNaseOut and 0.01 Mbq α-32P-UTP. We added 1 μg of recombinant MBP-PUP-2 and increasing amounts of recombinant GST-LIN-28 to a maximum of 10 μg. We incubated reaction mixtures at 30 °C for 30 min. We purified RNA by phenol/chloroform extraction and ethanol precipitation. We analyzed reactions in a 6% urea polyacrylamide gel. We used 2U of *S. pombe* CID1 poly(U) polymerase (NEB, Ipswich, MA, USA) as a positive control. We detected radioactivity by phosphoimager (GE Healthcare).

**Electrophoretic mobility shift assay**

We carried out binding reactions in a total volume of 20 μl containing 50,000 cpm of radiolabelled RNA, 30 μg tRNA, 1 μl RNaseOut (40 unit/μl, Invitrogen), 50 mM Tris pH 7.6, 100 mM NaCl, 0.07 % (v/v) β-mercaptoethanol, 5 mM MgOAc₂ and increasing amounts of recombinant GST-LIN-28 to a maximum of 10 μM. We incubated the reactions at room temperature for 45 min, followed by analysis using 5% native polyacrylamide gel electrophoresis. We detected radioactivity by phosphoimager (GE Healthcare).

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Figure 1. A quantitative assay reveals post-transcriptional regulation of the let-7 miRNA by lin-28
(a,b) Schematic of the pharynx based assay of let-7 activity. (c) Fluorescence images of animals carrying the let-7 sensor transgene at L1 larval and adult stages. Both GFP and mCherry are strongly expressed.
(d) Fluorescence images of animals carrying both the let-7 sensor and myo-2::let-7 transgenes at L1 larval and adult stages. GFP is specifically and robustly downregulated in adults, but not in L1 larvae. Scale bar shows 20 μm.
(e) Fluorescence images showing that lin-28 mutants downregulate let-7 sensor GFP at the L1 stage in a myo-2::let-7 dependent fashion. This effect is not reversed in a lin-46 mutant background. Scale bar shows 20 μm.
(f) Fluorescence images showing that a myo-2::lin-28::unc-54 transgene is sufficient to block let-7 activity in adults carrying let-7 sensor and myo-2::let-7 transgenes. Scale bar shows 20 μm.
**Figure 2. pup-2 regulates let-7 processing in a lin-28-dependent fashion**

a *let-7* is uridylated *in vivo*. Frequency of unmodified and modified *let-7* molecules identified by high-throughput sequencing.

b Representative northern blot showing *pup-2*-dependent regulation of pre-*let-7*. 5 μg of total RNA from control, *lin-28* (RNAi), and *pup-2* (RNAi) *myo-2::let-7* L2 larvae was loaded. U6 was used as a loading control.

c,d Quantification of relative pre-*let-7* (c), and *let-7* (d) abundance in *lin-28* (RNAi), *pup-2* (RNAi), and *cid-1* (RNAi) *myo-2::let-7* L2 and L4 larvae from northern blotting experiments. Mean fold change relative to empty vector control samples is shown. *P*-values from Students’ *t*-tests indicated; *n* = 4. Error bars show standard error of the mean.

e Fluorescence image showing the seam cell defect observed in *pup-2* (RNAi) adults. A DLG-1-mCherry fusion marks seam cell boundaries. Upper panel: wild-type with continuous seam. Lower panel: *pup-2* (RNAi) with incompletely fused seam. Arrows indicate sites of failed fusion. Scale bar shows 20 μm.
Figure 3. LIN-28 interacts with PUP-2 and promotes uridylation of pre-let-7 by PUP-2

a Co-Immunoprecipitation of PUP-2 and LIN-28 expressed in HEK293T cells.

b GST pull-down assay demonstrating a direct interaction of GST-LIN-28 and PUP-2 in vitro.

c In vitro uridylation assay showing that PUP-2 uridylates pre-let-7 in a LIN-28 dependent fashion.
### Table 1

Genetic interactions of *pup-2* and *lin-28* with *let-7* in vulval development

| genotype                          | % burst at 20°C (n) | % burst at 15°C (n) |
|-----------------------------------|---------------------|---------------------|
| *let-7(n2853); empty vector RNAi*| 97 (119)            | 47 (86)             |
| *let-7(n2853); lin-28(RNAi)*      | 16 (115)            | 0 (28)              |
| *let-7(n2853); pup-2(RNAi)*       | 97 (120)            | 28 (114)            |

RNAi by feeding.