Published in final edited form as:
Science. 2008 April 4; 320(5872): 97–100. doi:10.1126/science.1154040.

Selective blockade of microRNA processing by Lin-28

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Abstract

MicroRNAs (miRNAs) play critical roles in development, and dysregulation of miRNA expression has been observed in human malignancies. Recent evidence suggests that the processing of several primary miRNA transcripts (pri-miRNAs) is blocked post-transcriptionally in embryonic stem (ES) cells, embryonal carcinoma (EC) cells, and primary tumors. Here we show that Lin-28, a developmentally regulated RNA-binding protein, selectively blocks the processing of pri-let-7 miRNAs in embryonic cells. Using in vitro and in vivo studies, we demonstrate that Lin-28 is necessary and sufficient for blocking Microprocessor-mediated cleavage of pri-let-7 miRNAs. Our results identify Lin-28 as a negative regulator of miRNA biogenesis and suggest that Lin-28 may play a central role in blocking miRNA-mediated differentiation in stem cells and certain cancers.

MicroRNAs (miRNAs) constitute a large family of short, noncoding RNAs that post-transcriptionally repress gene expression in metazoans. Mature miRNAs are produced from primary miRNA transcripts (pri-miRNAs) through sequential cleavages by the Microprocessor (1,2) and Dicer (3,4) enzyme complexes to release pre-miRNA and mature miRNA species, respectively. Post-transcriptional control of miRNA expression has been reported to occur in a tissue-specific (5) and developmentally-regulated fashion (6-8). The processing of several pri-miRNAs is blocked in embryonic tissues, with activation of processing occurring only as development proceeds. In addition, it has been reported that certain pri-miRNAs are highly expressed in human(9) and mouse embryonic stem (ES) cells, mouse embryonal carcinoma (EC) cells, and human primary tumors; however, the corresponding mature species are not detectable(7). This suggests that there may be a post-transcriptional block in miRNA biogenesis, the mechanism of which has remained unknown. In ES and EC cells, the magnitude of the Microprocessor processing block is most dramatic for members of the let-7 family of miRNAs although it has been proposed that the processing of all miRNAs may be regulated at the Microprocessor step(7)

We observed that the pri-let-7g transcript is readily detectable in ES cells and remains at relatively constant levels over the course of differentiation into embryoid bodies (Fig. 1a). In contrast, mature let-7g is undetectable in undifferentiated ES cells but is strongly induced after day 10 of differentiation (Fig. 1b). A post-transcriptional induction of let-7g expression has also been reported during the differentiation of P19 EC cells with retinoic acid(7). We sought to understand the mechanism for the post-transcriptional block in miRNA processing in EC and ES cells. We first compared cell extracts from different cell types for their ability

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to inhibit Microprocessor-mediated cleavage of pri-miRNA substrates to the corresponding pre-miRNAs in vitro (Fig. 1c). Radiolabeled pri-miRNA substrates were preincubated with cell extract and subsequently subjected to processing by affinity-purified Microprocessor complex. Whereas extracts from undifferentiated P19 cells readily inhibited Microprocessor-mediated cleavage of pri-let-7g to pre-let-7g, cell extracts from differentiated mouse embryonic fibroblasts (MEF) did not inhibit cleavage. Thus, the cell-type specificity of the in vivo Microprocessor processing block is recapitulated in our in vitro assay.

We surmised that a protein factor or factors present in ES and EC cells might be inhibiting Microprocessor-mediated processing of pri-miRNAs, and we employed a biochemical approach to identify this factor. Electrophoretic gel mobility shift assays using a labeled pre-let-7g probe identified a specific band-shift present in P19 EC and ES extract but not with MEF extract (fig. S1). This suggested that pre-let-7g could be used as an effective affinity reagent for purification of the factor(s) responsible for the Microprocessor processing block. Pre-let-7g was conjugated to agarose beads and incubated with whole cell extract from P19 cells. The affinity eluate was subjected to SDS-polyacrylamide electrophoresis (PAGE) followed by colloidal staining. Bands were excised and subjected to mass spectroscopic sequencing in three segments (fig. S2). Sequencing revealed several RNA-binding proteins co-purifying with pre-let-7g. A number of these proteins were previously identified as members of a large Microprocessor-containing protein complex(2) (fig. S2).

One of the pre-let-7g-interacting proteins was the small, highly-conserved RNA-binding protein Lin-28. Lin-28 was an attractive candidate for the following reasons: 1) mutations within its RNA-binding domain have been shown to impair developmental timing regulation in C. elegans(10); 2) it is expressed specifically in undifferentiated P19 cells, mouse ES cells(11), and human ES cells(12), and down-regulated upon differentiation; and 3) a mammalian Lin-28 homolog, Lin-28B, is over-expressed in hepatocellular carcinoma, and over-expression of this gene promotes cancer cell proliferation in vitro(13); 4) it has been reported that Lin-28 is expressed in embryonic muscle, neurons, and epithelia in a stage-specific fashion, and Lin-28 is crucial for appropriate skeletal muscle differentiation(11); 5) Lin-28 was recently used with three other factors to reprogram human somatic fibroblasts to pluripotency(14).

We examined the kinetics of Lin-28 expression during embryoid body formation (Fig. 1d). Lin-28 is downregulated upon ES cell differentiation, with kinetics that are delayed relative to the known pluripotency factors Oct-4 and Nanog. This downregulation of Lin-28 temporally coincides with activation of pri-let-7 processing (Fig. 1d).

To explore the possibility that Lin-28 may regulate pri-let-7 processing, we confirmed that Lin-28 is capable of binding both pre-let-7g and pri-let-7g in a co-sedimentation assay (fig. S3). We then tested the ability of Lin-28 to functionally block pri-miRNA processing in vitro. We observed that a Flag-immunoprecipitate containing Flag-Lin-28 potently inhibited the processing of both pri-let-7a and pri-let-7g in vitro. Flag-immunoprecipitates containing the control RNA-binding proteins Flag-hnRNPA1 and Flag-Msi-2 had no effect on pri-let-7a and pri-let-7g processing (Fig. 2a and 2b). Flag-Lin-28 immunoprecipitate did not impair the processing of pri-miR15a/16-1, demonstrating the selectivity of the miRNA processing block (Fig. 2c). We next purified bacterially-expressed His-Lin-28 (Fig. 2d) and tested this recombinant Lin-28 (rLin-28) for its ability to block pri-miRNA processing in vitro. rLin-28 inhibited the processing of both pri-let-7a and pri-let-7g (Fig. 2d). Therefore, Lin-28 is sufficient to inhibit miRNA processing at the Microprocessor step.

To determine whether Lin-28 is capable of blocking miRNA processing in vivo, four pri-miRNAs were introduced in either the presence or absence of mouse Lin-28 cDNA into 293T cells, a transformed human cell line that lacks Lin-28. In the absence of Lin-28, all ectopic pri-miRNAs were efficiently processed to their mature form (Fig. 3a and fig. S4). However, ectopic expression of Lin-28 completely blocked processing of both pri-let-7a and pri-let-7g, while processing of pri-miR-15a and pri-miR-122 was largely unaffected (Fig. 3a and fig. S4). Co-transfection of pri-let-7g and Lin-28 led to accumulation of pri-let-7g (Fig 3b), consistent with the notion that Lin-28 blocks miRNA processing at the Microprocessor step. We performed these co-transfection experiments with four control RNA binding proteins (YBX-1, Msi-2, hnRNPA1, and hnRNPL) to confirm that this block in processing of pri-let-7 miRNAs is specific to Lin-28 (Fig. 3c and fig. S5). Finally, to test whether Lin-28 is capable of blocking endogenous miRNA processing (as opposed to only blocking the processing of ectopically expressed pri-miRNAs), we transfected Lin-28 cDNA into 293T cells and measured levels of several mature miRNAs after 4 days by quantitative PCR. We observed decreased endogenous levels of mature let-7 family members; levels of endogenous mature miR-21 were unaffected (Fig. 3d). Decreased mature let-7g upon Lin-28 over-expression was accompanied by a corresponding increase in levels of pri-let-7g (Fig. 3e).

We sought to determine whether Lin-28 is an endogenous blocker of miRNA processing in embryonic cells. We used three different shRNA hairpins and a siRNA targeting Lin-28 to knock down endogenous Lin-28 in P19 EC cells (Fig. 4a) and ES cells (fig. S6). Knockdown of Lin-28 leads to an induction of mature let-7g in both P19 cells (Fig. 4b) and ES cells (fig. S4b), indicating that Lin-28 serves to inhibit miRNA processing in vivo (Fig. 4b). All let-7 family members tested were substantially upregulated upon knockdown of Lin-28, whereas levels of other miRNAs were unchanged (Fig. 4d and fig. S7). Induction of mature let-7 miRNAs occurs within 60 hours of Lin-28 knockdown, whereas let-7 miRNAs are normally induced only after 10 days of ES and P19 differentiation, when endogenous Lin-28 levels fall (Fig. 1a, and ref. (7)). Therefore, the induction we observe likely represents a direct effect of Lin-28 on pri-miRNA processing rather than an indirect consequence of cell differentiation. In support of this notion, we observed no decrease in levels of the pluripotency markers Oct-4 and Nanog upon knockdown of Lin-28 over the time course of our experiment (Fig. 4f). Furthermore, global miRNA profiling detected upregulation of only let-7 miRNAs upon Lin-28 knockdown, underscoring the specificity of Lin-28 in regulating let-7 miRNAs (fig. S7).

A Lin-28 homologue, Lin-28B, is overexpressed in human hepatocellular carcinoma as well as in several cancer cell lines(13). Two isoforms of Lin-28B, differing in their 5’ exons, have been reported. The short isoform (Lin-28B-S) preserves the two retroviral-type CCHC zinc-finger motifs also present in the long isoform (Lin-28B-L), but contains a truncated cold-shock domain. Lin-28B-L overexpression induces cancer-cell growth, while Lin-28B-S overexpression has no effect (13). We find that Lin-28B-L potently inhibits the processing of pri-let-7g (fig. S8) while Lin-28B-S does not. This suggests that the previously reported oncogenic properties of Lin-28B may be mediated, at least in part, through blockade of let-7 processing. Our results also suggest that Lin-28 and Lin-28B may require both the cold-shock domain and CCHC zinc-fingers for blocking activity. Interestingly, Lin-28 and Lin-28B are the only animal proteins to contain both of these domains(15).

Our results demonstrate that Lin-28 is necessary and sufficient for blockade of pri-miRNA processing of let-7 family members both in vitro and in vivo. There are several possible reasons why ES and EC cells possess a mechanism for post-transcriptional regulation of let-7 miRNA expression. First, post-transcriptional activation of miRNA processing would allow for rapid induction of several let-7 miRNAs by downregulation of a single factor.

Second, disruption of DGCR8, a dsRNA-binding protein and essential component of the Microprocessor complex, interferes with ES cell differentiation, suggesting that activation of miRNAs may be important for silencing the self-renewal machinery(16). It has been suggested that post-transcriptional control could prevent even small amounts of let-7 from being produced in ES and EC cells, tightly maintaining the undifferentiated state(7). Third, post-transcriptional control of miRNA expression could serve as a means for dissociating expression patterns of intronic miRNAs from expression patterns of their host transcripts.

The precise mechanism by which Lin-28 blocks miRNA processing as well as the range and determinants of its substrate selectivity are unknown. Our data suggest that Lin-28 has a preference for selectively blocking the processing of let-7 family pri-miRNAs at the Microprocessor step. However, we cannot rule out the possibility that Lin-28, alone or in concert with other factors, may block other pri-miRNAs in different physiological contexts. Others have reported that miRNA processing can also be regulated at the Dicer step, when pre-miRNAs are cleaved to their mature form (5,8). Additional factors may yet be discovered that post-transcriptionally regulate miRNA processing. Lin-28 is predominantly localized to the cytoplasm, although it can also be found in the nucleus(10,11); Lin-28B is translocated into the nucleus in a cell-cycle dependent fashion(13). Lin-28 may post-transcriptionally regulate miRNA processing in embryonic cells in a cell-cycle specific manner.

Recently, Lin-28 was used in conjunction with Nanog, Oct-4, and Sox2 to reprogram human fibroblasts to pluripotency(14). Our data thus suggest that modulating miRNA processing may contribute to the reprogramming of somatic cells to an embryonic state. Additionally, global inhibition of miRNA processing by knockdown of the Drosha component of the Microprocessor was shown to promote cellular transformation and tumorigenesis; this phenotype was found to be, in large part, due to loss of let-7 expression (17). Let-7 has been reported to play a tumor suppressor role in lung and breast cancer by repression of oncogenes such as Hmga2(18) and Ras(19,20). We suggest that disruption of let-7 processing by activation of Lin-28 could promote the oncogenic phenotype. Notably, several human primary tumors show a general lack of correlation between expression of pri-miRNAs and the corresponding mature species(7,21). This suggests that a block in miRNA processing may contribute to the low miRNA expression observed in many human cancers(22). Future study of Lin-28 promises to reveal how miRNA processing contributes to the dedifferentiation that accompanies both somatic cell reprogramming and oncogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Many thanks to H. Steen of the Proteomics Center at Children's Hospital Boston for expertise in the microcapillary HPLC/mass spectrometry. Thanks to L. Skeffiington and R. LaPierre for technical support. We thank Donald Bloch for providing Flag-YB-1 plasmid. RIG was supported by lab start-up funds from The Children's Hospital Boston and a grant from The Harvard Stem Cell Institute. GQD was supported by grants from the NIH and the NIH Director's Pioneer Award of the NIH Roadmap for Medical Research. G.Q.D. is a recipient of the Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

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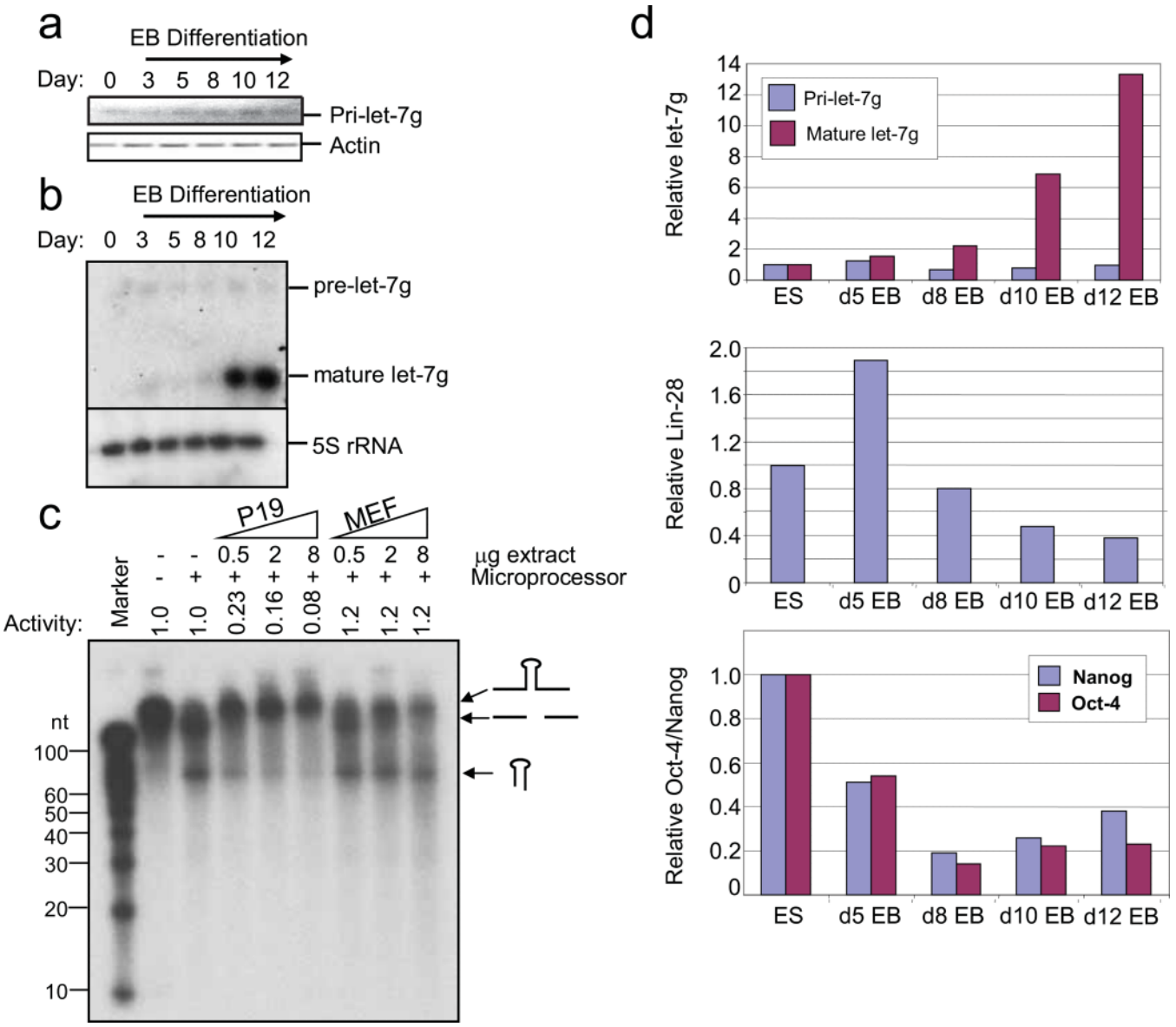

Figure 1. Post-transcriptional control of pri-let-7g processing

a, RT-PCR for pri-let-7g transcript (as described in ref. (7)) during ES differentiation to embryoid bodies. Actin serves as control**. b**, Northern blot showing post-transcriptional induction of mature let-7g during embryoid body formation 5S rRNA serves as loading control. **c**, *in vitro* pri-miRNA processing reaction using radiolabeled pri-let-7g as substrate. Pri-miRNA was pre-incubated with various amounts of P19 cell extract or mouse embryonic fibroblast (MEF) extract prior to processing reaction with Flag-Drosha immunoprecipitate, as described in Methods. The ratio of pre-miRNA to pri-miRNA was quantitated by densitometry and values were normalized to the Microprocessor only lane. **d**, qPCR analysis of gene expression during embryoid body formation of a feeder-free mouse ES line (J1 ES) . *Top Panel:* Pri-let-7g and mature let-7g; *Middle Panel:* Lin-28; *Bottom Panel:* pluripotency factors Oct-4 and Nanog.

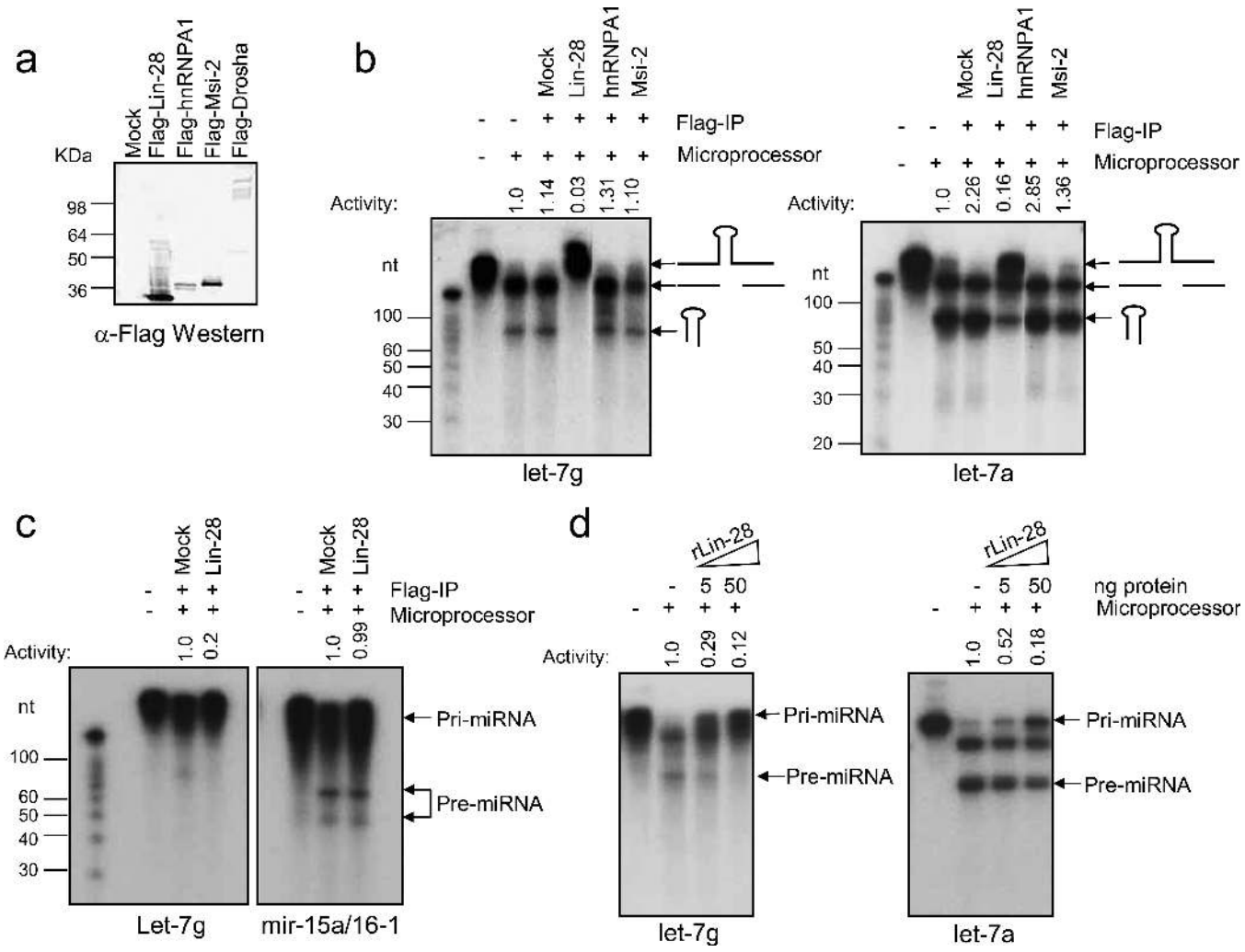

Figure 2. Lin-28 Inhibits pri-miRNA processing *in vitro*

a, α–Flag-Western to confirm expression of Flag-tagged proteins for use in *in vitro* assays. **b**, *in vitro* pri-miRNA processing reaction on pri-let-7g (left panel) and pri-let-7a (right panel) substrates in the presence of either Mock, Flag-Lin-28, Flag-hnRNPA1, or Flag-Msi-2 immunoprecipitate. Quantitation was normalized to the Microprocessor-only lane. **c**, *in vitro* pri-miRNA processing reaction on pri-let-7g (left panel) and pri-miR-15a/16-1 (right panel) substrates in the presence of either mock or Flag-Lin-28 immunoprecipitate and competitor tRNA. Quantitation was normalized to the Mock-IP lane. **d**, *in vitro* pri-miRNA processing reaction on pri-let-7g (left panel) and pri-let-7a (right panel) substrates in the presence of rHis-Lin-28. Quantitation was normalized to the Microprocessor-only lane.

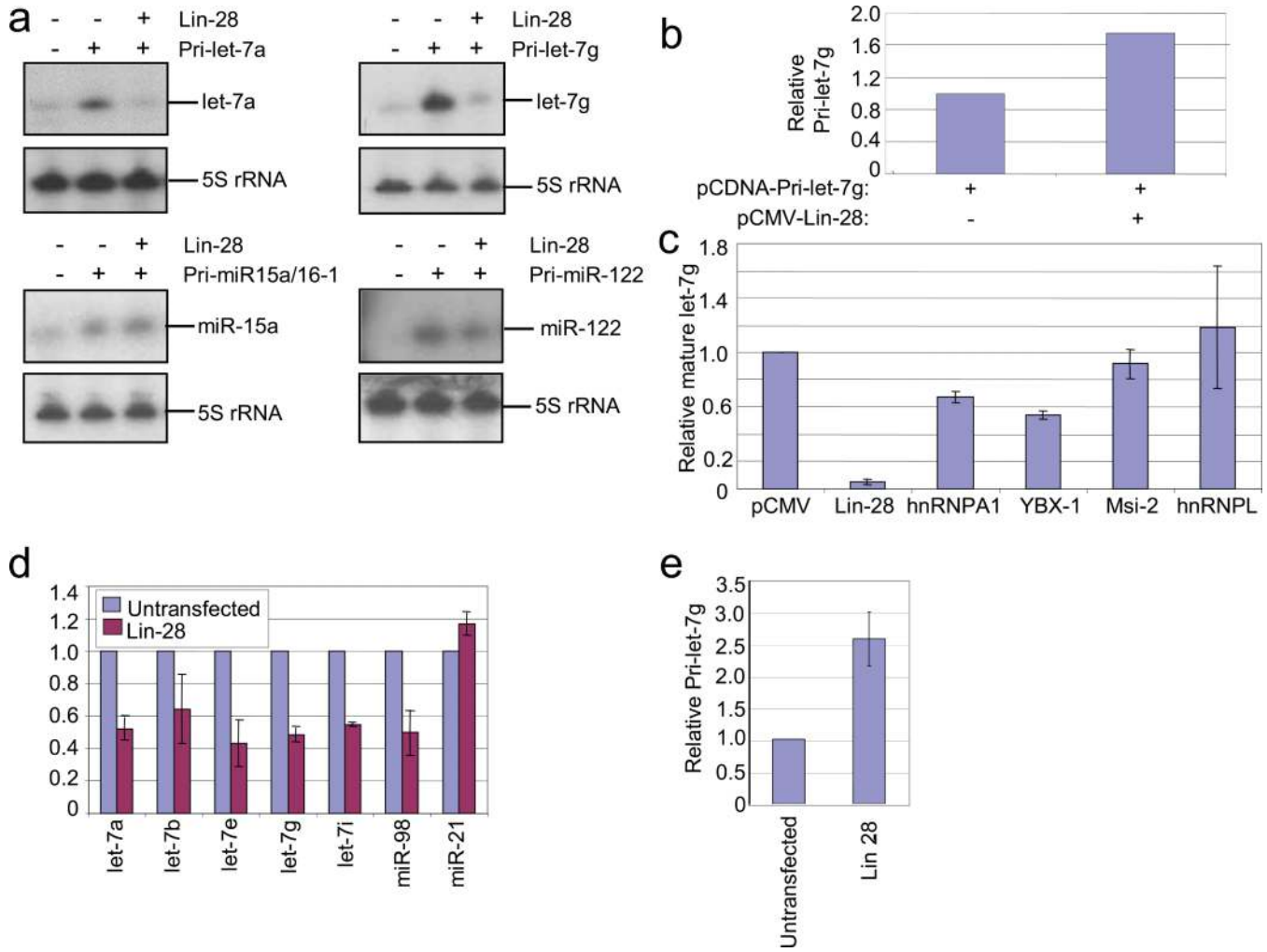

Figure 3. Ectopic expression of Lin-28 selectively inhibits pri-miRNA processing *in vivo*

a, In each panel, 293T cells were either untransfected (lane 1), co-transfected with the indicated pri-miRNA and 0.5 μg pCMV-Flag empty vector (lane 2), or co-transfected with the indicated pri-miRNA and 0.5 μg Flag-Lin-28 cDNA (lane 3). Total RNA was collected 40 h post-transfection and Northern blotted for the indicated miRNA. **b**, qPCR analysis of pri-let-7g levels for sample in a) (top right panel). **c**, Mature let-7g levels upon co-transfection of 293T cells with pri-let-7g and either pCMV-Flag, Flag-Lin-28, Flag-hnRNPA1, Flag-hnRNPL, Flag-YBX-1, or Flag-Msi-2 cDNAs, as measured by quantitative PCR. First, the amount of mature let-7g in each sample was calculated relative to untransfected control cells, then Flag-protein co-transfected samples were normalized to the corresponding pCMV-Flag co-transfected samples. **d**, qPCR showing changes in levels of endogenous mature miRNAs upon transfection of Flag-Lin-28 in 293T cells. **e**, qPCR showing accumulation of endogenous pri-let-7g upon transfection of Flag-Lin-28 in 293T cells. For **c-e**, values are given as average +/- S.E.M. from two or more independent transfections.

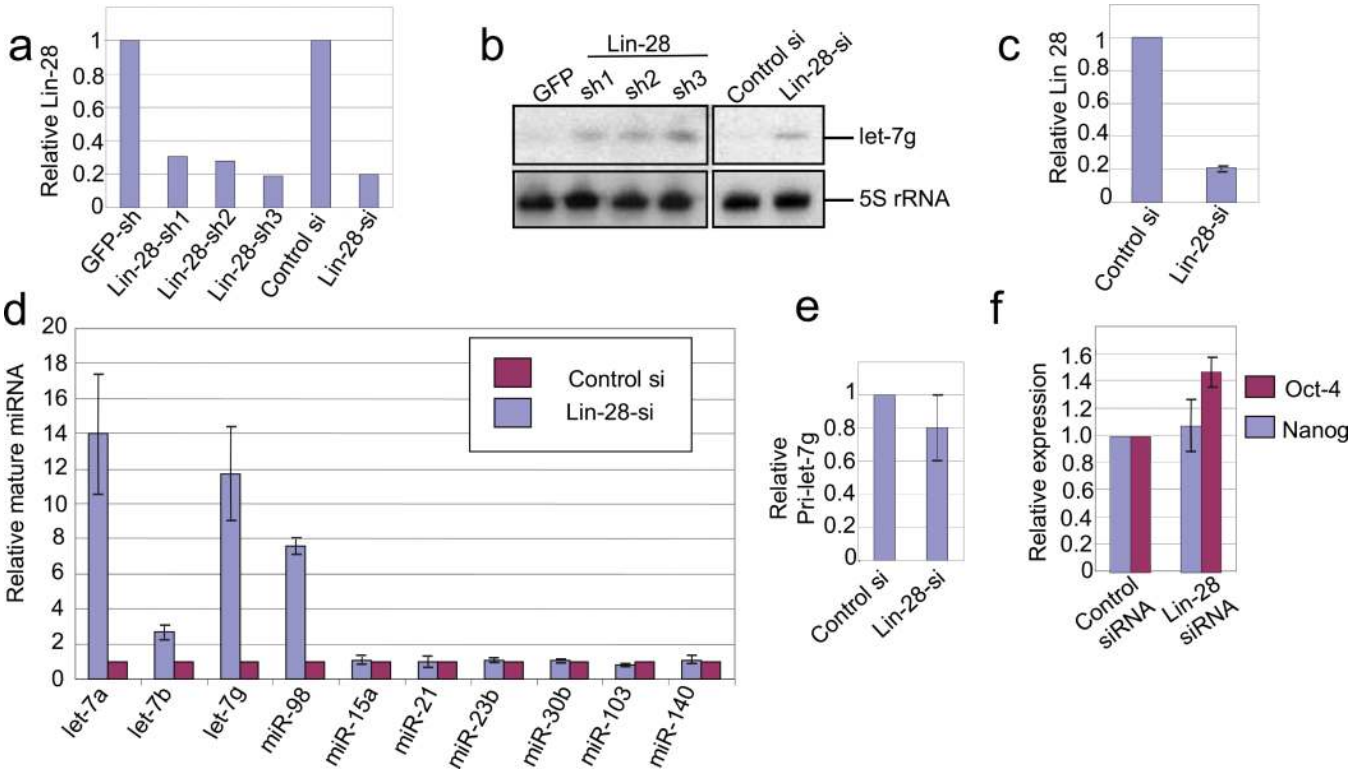

Figure 4. Knockdown of Lin-28 relieves the miRNA-processing block

P19 cells were transfected with control hairpin (GFPi), pLKO.1-shRNA hairpins targeting Lin-28, control siRNA (scrambled sequence), or Lin-28 siRNA. Total RNA was collected 60-hrs post-transfection for analysis. **a**, quantitative PCR analysis of Lin-28 expression, normalized to Lin-28 expression with control hairpin or control siRNA, for samples in **b**. **b**, Northern blot for mature let-7g. **c**, confirmation of Lin-28 knockdown using Lin28-SI2 on samples analyzed in **d**. Error bars represent S.E.M. with N=3. **d**, Changes in mature miRNA levels upon knockdown of Lin-28 as analyzed by quantitative PCR. Error bars represent S.E.M. with N=3. **e**, levels of pri-let-7g upon knockdown of Lin-28 in P19 cells. Error bars represent S.E.M. with N=3. **f**, levels of the pluripotency markers Oct-4 and Nanog in P19 cells transfected with either control siRNA or Lin28-SI2. Error bars represent S.E.M. with N=3.