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A LIN28-dependent structural change of pre-let-7g directly inhibits Dicer processing

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Abstract

Several recent studies have provided evidence that LIN28, a cytoplasmic RNA-binding protein, inhibits the biogenesis of members of the let-7 microRNA (miRNA) family at the Dicer step in both mammals and *C. elegans*. However, the precise mechanism of inhibition is still poorly understood. Here we report on an *in vitro* study, which combined RNase footprinting, gel shift binding assays and processing assays, to investigate the molecular basis and function of the interaction between the native let-7g precursor (pre-let-7g) and LIN28. We have mapped the structure of pre-let-7g and identified regions of the terminal loop of pre-let-7g that physically interact with LIN28. We have also identified a conformational change upon LIN28 binding that results in the unwinding of an otherwise double-stranded region at the Dicer-processing site of pre-let-7g. Furthermore, we showed that a mutant pre-let-7g that displays an open upper stem inhibited pre-let-7g Dicer processing to the same extent as LIN28. The data supports a mechanism by which LIN28 can directly inhibit let-7g biogenesis at the Dicer processing step.

MicroRNAs (miRNAs) are small single-stranded ~22 nucleotide RNAs that play important roles in post-transcriptional gene regulation (1-2). MiRNAs are the products of a multistep-processing pathway that begins with the transcription of a long primary transcript referred to as the pri-miRNA. The pri-miRNA transcript is then cleaved in the nucleus by the RNase III enzyme complex Drosha-Pasha/DGCR8 which releases a ~80 nucleotide RNA hairpin referred to as the pre-miRNA. The pre-miRNA is subsequently exported from the nucleus to the cytoplasm where it is processed by a second RNase III enzyme, called Dicer, to release a small RNA duplex. One strand of this duplex, the miRNA, is incorporated into a RNA-induced silencing complex (RISC) and directs the RISC complex to target messenger RNA (mRNA) molecules triggering their degradation and/or translational repression. miRNA expression can be regulated at both transcriptional and post-transcriptional levels (3).

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SUPPORTING INFORMATION AVAILABLE Additional experimental details, data analysis and control binding experiments can be found in the supporting information. This material is available free of charge via the internet at <http://pubs.acs.org>.

miRNAs were first characterised in *Caenorhabditis elegans* (*C. elegans*) as regulators of developmental timing. The miRNAs let-7 and lin-4 were shown to be essential in the control of temporal cell fates during larval development (4). Since then, hundreds of miRNAs have been identified, not only in *C. elegans*, but also in plants and animals, including mammals. These miRNAs are involved in a variety of physiological processes, such as development, apoptosis, and carcinogenesis (5-6).

Both the sequence and temporal expression patterns of the let-7 miRNAs are conserved from *C. elegans* to mammals (7-8). In mammals there are ten members of the let-7 miRNA family. The let-7 miRNA family are thought to control stem cell differentiation and developmental timing (8). In addition, the let-7 miRNAs also function as tumor suppressors through the silencing of key oncogenes, including RAS, HMGA2 and c-MYC (9-11).

Recently the expression of specific members of the let-7 family have been shown to be post-transcriptionally regulated by LIN28, a cytoplasmic RNA binding protein conserved in mammals and *C. elegans* (12-15). LIN28 protein contains two RNA-binding domains: a cold shock domain (CSD) and a retroviral-type CCHC zinc finger domain (ZFD). In mammals, LIN28 is expressed in embryonic stem cells (ESC) and developing tissues (16). LIN28 has been shown to block the biogenesis of let-7 miRNAs at both the Drosha step (13-14) and the Dicer step in mammalian cells (12, 17). This regulation is mediated *via* a direct interaction between the LIN28 protein and the let-7 precursor RNA. In mammals, mutagenesis and competitor studies have been used to investigate the regions of the let-7 precursor that is required for LIN28 binding and processing inhibition. These studies have produced conflicting evidence as to the importance of the pre-let-7 terminal loop for LIN28 mediated inhibition (12, 13, 18).

Undifferentiated, highly aggressive, radio-resistant tumors have been associated with reduced-expression of specific miRNA let-7 family members (19-20) and it has been proposed that this could be due to the up regulation of LIN28 (21). LIN28 activation occurs in many different tumor types with a frequency of 15 %, all of which are poorly differentiated tumors carrying the worst prognosis (22). Elucidation of the molecular and mechanistic details of how LIN28 inhibits the processing of let-7 miRNAs could be essential to further understand how its misregulation can contribute to the most severe cancers, and its potential as a new therapeutic target.

Here we describe the use of *in vitro* RNA structural mapping experiments to study the interaction of LIN28 with pre-let-7g, one of the most responsive let-7 family members to LIN28 over expression and knockdown studies (13), at the molecular level, and Dicer processing assays to establish its biological function.

MATERIALS AND METHODS

In vitro Transcription using α -³²P

A reaction mixture of dsDNA oligonucleotides (T7 promoter, 1 μ g/ μ L (Table S1 of Supporting Information), NTPs (1 μ L, 5 M CTP, GTP, ATP & 1M UTP), 10 X transcription buffer (Ambion, 1 μ L) and uridine 5' triphosphate, [α -³²P] (Perkin Elmer®) was prepared. RNA polymerase (20 U) was added and the resulting mixture heated (40 °C, 1 h). TE (pH 8, 80 μ L) was added and the RNA was purified by phenol:chloroform extraction followed by a MicroSpin™ G-50 Column (Ambion) and finally precipitated with ethanol. The extent of α -³²P incorporation, the counts per minute (cpms), was measured using a LS 6500 multi-purpose scintillation counter (Beckman Coulter™).

Electromobility Shift Assay (EMSA)

In vitro transcribed α - ^{32}P -RNA (2.5×10^4 cpm) was incubated with increasing amounts of recombinant LIN28 for 45 min at RT in a binding buffer containing Tris-HCl pH 7.6 (50 mM), NaCl (100 mM), β -mercaptoethanol (0.07 %), $\text{Mg}(\text{OAc})_2$ (1 mM), and total yeast RNA (12.5 μg). Glycerol (50 %, 2 μL) was added and Protein/RNA complex band shifts were observed using a 5% non-denaturing PAGE. The band intensities of three independent experiments were quantified using imageQuantTM software and used to calculate the average proportion of complex formed at each concentration of LIN28 (Fraction Bound). Dissociation constant (K_d) values were derived from data point fitting with Origin 7.5 (OriginLab), according to the hyperbolic function: $B = B_{\text{max}}[\text{protein}]/([\text{protein}] + K_d)$, where B is the average proportion of complex, B_{max} is the maximum of complex formed, and [protein] is the total concentration of protein.

Enzymatic Probing and Footprinting

5'-[^{32}P]-labeled synthetic RNA (Table S2 of Supporting Information) was partially digested with a combination of RNase T1, A, V1 or 1 (Ambion) in the presence and absence of LIN28. Labeled RNA ($\pm$ LIN28) was mixed with 0.1 U RNase T1, 0.1 U RNase V1, 0.05 U RNase A or 0.5 U RNase I with 5 μg total yeast RNA in reaction buffer [10 mM Tris pH 7, 100 mM KCl, 10 mM MgCl_2] and incubated at RT for 15 min (A, T1, V1) or 37 °C for 3 mins (1). The cleavage reactions were stopped by ethanol precipitation. The RNA was purified by phenol:chloroform extraction followed by overnight precipitation. For an alkaline ladder, 5'-[^{32}P]-labeled pre-miRNA was hydrolyzed in 4 μL alkaline solution [50 mM Sodium Carbonate [$\text{NaHCO}_3/\text{Na}_2\text{CO}_3$] pH 9.2, 1 mM EDTA] by heating at 90 °C for 7.5 min with 1 μg carrier total yeast RNA. For the RNase T1 sequencing ladder 5'-[^{32}P]-labeled pre-miRNA was heat at 50 °C for 5 mins in denaturing buffer [20 mM sodium citrate pH 5, 1 mM EDTA, 7 M urea]. RNase T1 (0.5 U) was then added and the reaction incubated for a further 15 mins at 50 °C. Digested products were resolved by a 20 % denaturing (8 M urea) PAGE and visualized by autoradiography. Cleavage bands were quantified using imageQuantTM software and normalized against the total lane radioactivity.

Recombinant Protein Expression

LIN28 (Table S3 of Supporting Information) and GST were subcloned into pDEST-GEX-2TK (Gateway cassette inserted at SmaI site in pGEX-2TK). The recombinant proteins were expressed and purified as described previously (15).

Dicer Processing Assay

Each reaction contained 10 ng of end labeled 5'-[^{32}P]-pre-miRNA (Table S2 of Supporting Information), 0.1 U of Dicer (Invitrogen) in 10 μL of 1X Dicer buffer (Invitrogen). For the protection assay, LIN28 (2 μM) was incubated with the labeled RNA at room temperature for 45 min before Dicer was added. After incubation at 37 °C for 5 mins, reactions were stopped by the addition of 2X sample buffer (98% formamide, 10 mM EDTA and 0.1% bromophenol blue) and put on ice. The RNA was resolved on a 20 % denaturing gel and visualized by autoradiography. Cleavage bands were quantified from three independent experiments using imageQuantTM software. To quantify the data in Figure 5b, we used 'relative Dicer processing efficiency' which is defined as the product intensity divided by total (products and full-length substrate) intensity.

RESULTS

The loop region of human pre-let-7g is the sole region interacting directly with human LIN28

We first used enzymatic footprinting experiments with ribonuclease (RNase) enzymes to investigate the secondary structure of human pre-let-7g RNA.

A range of different RNase enzymes with alternate sequence and/or structural specificities was chosen. We employed RNase T1, that preferentially cleaves single-stranded RNA after guanine residues; RNase A, that cleaves single-stranded RNA after the pyrimidines cytosine or uridine; and RNase V1, that is a non-sequence specific double-stranded RNA cleaving enzyme. Overall, the enzymatic digestion of pre-let-7g yielded a cleavage profile that is in good agreement with one of the high-energy secondary structures predicted by the RNA folding software mFOLD (Figure 1a and 1b). An exception was C41, a residue in the tetraloop (A40 to C43) whose susceptibility to RNase A cleavage was lower than expected (Figure 1a, Expanded Image, lane 4). It is possible that the close proximity of the bases in this constrained small loop favoured stacking interactions that confer some double stranded characteristics. In contrast, the adjacent residue C42, also in the tetraloop, was highly cleaved by RNase A (Figure 1a, Expanded Image, lane 4). In general, the cleavage patterns produced by RNase V1, RNases A and T1 were distinct (Figure 1a, lane 3, 4, 5, 1b). In cases where common cleavage sites were observed, such as U23 (compare lane 4 and 5 in Figure 1a), it is likely that these residues either have a stacked conformation hence have characteristics that are between the single-stranded and double-stranded conformations, or there could be dynamic interconversion between such states. Notably, U23 is located at the junction between a stem and a bulge (Figure 1a, lane 5) and could possibly be involved in stacking interactions with the terminal residues of the helical stem.

We also performed an Electromobility Shift Assay (EMSA) to show that a glutathione S-transferase (GST)-tagged recombinant human LIN28 (LIN28) binds directly to pre-let-7g with a K_d value of $0.88 \pm 0.22 \mu\text{M}$ (Figure S1a and S1b in Supporting Information), in good agreement with a previous study using a histidine-tagged mouse LIN28 ($K_d = 2.1 \mu\text{M}$) (18). The GST tag alone did not bind to pre-let-7g (Figure S1c in Supporting Information).

Next, we carried out RNase T1 and RNase A enzymatic footprinting of pre-let-7g in the presence of LIN28. Enzymatic footprinting can be used to identify protein binding sites and also RNA conformational changes that are induced upon protein binding. Concentrations of protein were set at about ($1 \mu\text{M}$) and above ($3 \mu\text{M}$) the K_d value determined by the EMSA. The footprinting pattern of the terminal loop of pre-let-7g significantly changed upon LIN28 binding. When probing with RNase T1 (Figure 2a, compare lane 6 and 7 (LIN28 present) to lane 5 (LIN28 absent)), we observed a high degree of protection from enzymatic cleavage at the internal loop residues G24, G26, G27 and G28, and the bulge residue G34. Protection to a lower extent was also observed at residues, G45 and G50. When probing with RNase A (Figure 2b, compare lane 6 and 7 (LIN28 present) to lane 5 (LIN28 absent)), we observed a high degree of protection from enzymatic cleavage at i) the internal loop/bulge residues U23, U31, ii) the tetraloop residue C42 and iii) the Watson-Crick base-paired residues U33, U36 and C48.

The protection pattern observed in the presence of LIN28 when using both the RNase T1 and RNase A probe was consistent (RNase A protected residues were generally in close proximity to RNase T1 protected residues) and together identified several regions, spread across the terminal loop, which interacted with LIN28.

We next sought to determine if the pre-let-7g terminal loop, in the absence of the characteristic precursor stem, interacted with LIN28 in the same manner as the full-length pre-let-7g, indicating that the pre-let-7g terminal loop alone is the sole requirement for the pre-let-7g-LIN28 interaction. Using a truncated form of pre-let-7g (tpre-let-7g), consisting of the terminal loop region with a 5-bp flanking stem, we found by EMSA that LIN28 binds to tpre-let-7g with a K_d value of $1.1 \pm 0.24 \mu\text{M}$ (Figure S2 in Supporting Information), consistent with a previous study using mouse LIN28 (18). Furthermore, on RNase T1 and RNase A footprinting we found LIN28 interacted with tpre-let-7g in a similar manner to that observed for the terminal loop in full-length pre-let-7g (Figure 3b, compare 3a to 2c). This suggests that no other region beyond the terminal loop of pre-let-7g is required for LIN28 binding, in agreement with previous reports that showed that the terminal loop is necessary and sufficient for interaction with LIN28 (12, 13, 14, 23).

LIN28 binding induces a conformational change at the Dicer cleavage site of the pre-let-7g

In addition to the protection pattern described above, further enzymatic probing experiments utilizing the sequence-independent single stranded RNase I also identified regions of increased cleavage susceptibility in the presence of LIN28. In particular, a double-stranded region (U21/U22) and the adjacent stacked base (U23) in the upper stem of pre-let-7g showed increased single-stranded character by about 2 to 3-fold in the presence of LIN28 (Figure 4a, compare lane 5 and 6 to lane 4, quantification can be found in Figure S3 of Supporting Information). To a lower extent, other residues in the pre-let-7g terminal loop were also shown to have increased susceptibility to RNase I (such as U29-U31 and G45-A47) (Figure 4a, compare lanes 5 and 6 to lane 4). Overall our observations indicate that on LIN28 binding, conformational changes occur in the terminal loop of pre-let-7g. The conformational changes observed at residues U21-U23 predominantly result in the unwinding of the upper stem region of pre-let-7g. Interestingly this upper stem region comprises the Dicer cleavage site (U22), and the double stranded character of the upper stem region is thought to be essential for let-7 maturation by Dicer (24-26).

Opening of the upper stem of pre-let-7g inhibits Dicer processing to the same extent than LIN28

To evaluate whether the LIN28-induced opening of the upper-stem of pre-let-7g could affect Dicer processing, wild type pre-let-7g (WTpre-let-7g), WTpre-let-7g in the presence of LIN28 and a pre-let-7g mutant with an open upper stem (MUTpre-let-7g) were tested for Dicer cleavage (Figure 5a and b). using an *in vitro* Dicer processing assay (12). MUTpre-let-7g was created by mutating G20, U21 and U22 in the upper stem of WT pre-let-7g to A20, A21 and A22 (Figure 5c). RNaseV1 footprinting confirmed that MUTpre-let-7g was single stranded at the Dicer-processing site (Figure S4 in Supporting Information).

In the presence of Dicer we observed a reduction in the amount of WTpre-let-7g and the appearance of an additional ~20-nucleotide product corresponding to the mature let-7g (Figure 5a, Lane 4). On addition of LIN28 the intensity of the mature let-7g band reduced, indicating that LIN28 inhibits Dicer processing of WTpre-let-7g *in vitro* (Figure 5a, Lane 6). In the absence of Dicer, LIN28 had no effect on WTpre-let-7g (Figure 5a, Lane 7). For MUTpre-let-7g, the pre-let-7g mutant with an open upper stem, we found that the intensity of the mature let-7g band was greatly reduced, suggesting that the open upper stem of MUTpre-let-7g affects Dicer processing (Figure 5a, Lane 5). Remarkably, we found that both the addition of LIN28 and the use of the open upper stem mutant resulted in a similar decrease, by about 65%, in Dicer processing efficiency as compared to the wild-type pre-let-7g (Figure 5b).

DISCUSSION

Using a combination of binding and enzymatic footprinting assays, we have shown that LIN28 binds to the terminal loop region of native pre-let-7g. Furthermore, we have mapped the residues of the pre-let-7g terminal loop that interact with LIN28. Overall, this data demonstrates that LIN28 binds directly to the terminal loop of pre-let-7g, in agreement with previous reports that showed that the terminal loop is necessary and sufficient for interaction with LIN28 (12, 13, 14, 23). Mutagenesis studies previously indicated that some of these residues are involved in TUT4/LIN28-mediated let-7 inhibition (G50) (27), the binding of LIN28 to pre-let-7g (C42) (18) and LIN28-inhibition of let-7g processing at the Drosha step (G50) (13)). In addition, we have also identified a GGG motif (G26, G27 and G28) in the bulge of the pre-let-7g terminal loop that is substantially protected against nuclease cleavage upon LIN28 binding. Interestingly, this GGG motif has been demonstrated to be essential for the KH-type splicing regulatory protein (KSRP)-mediated activation of the let-7 family maturation at both the Dicer and Drosha steps during differentiation of mammalian cells (28).

We have shown that upon LIN28 binding a conformational change occurs in the upper stem region of pre-let-7g. This conformational change results in the opening of the helix at the Dicer-processing site of pre-let-7g. In the absence of LIN28, Dicer cleaves pre-let-7g at the 5' U22 and the 3' A57 residues, releasing a dsRNA intermediate containing the 22-nucleotide mature let-7g (29). As Dicer only cleaves dsRNA, we hypothesized that the unwinding of the upper stem region of pre-let-7g on LIN28 binding has the potential to interfere with the Dicer processing of pre-let-7g. We performed a Dicer processing assay using a mutant pre-let-7g with an open upper stem at the Dicer processing site. We found that opening of the upper stem of pre-let-7g decreases Dicer processing efficiency to the same extent (about 65%) than LIN28. Based on these results we propose a model for LIN28 inhibition of let-7g biogenesis at the Dicer step, in which LIN28 binds to pre-let-7g at the terminal loop and induces a conformational change that predominately opens the helical structure of the upper stem region, reducing Dicer processing efficiency.

Several reports have demonstrated that LIN28 is able to recruit a pre-let-7 terminal uridylyl transferase (TUTase) (17, 23, 27). The resulting 3' polyU-tail is thought to provide a signal for pre-let-7 degradation that decreases let-7 expression. Our model for a direct inhibition of pre-let-7g Dicer processing by LIN28 might be a parallel mechanism to the recruitment of an uridylylase to modify the pre-let-7 3' end.

In conclusion, we have provided data to characterise the interaction between LIN28 and pre-let-7g. On the basis of these studies we propose that LIN28 could inhibit let-7g biogenesis at the Dicer step *via* a mechanism involving a LIN28 dependant conformational change at the Dicer processing site of pre-let-7g.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Enzymatic structural probing of pre-let-7g with RNase T1, RNase A & RNase V1. (a) Representative autoradiogram of the enzymatic structural probing. Lane 1: pre-let-7g, Lane 2a/b: pre-let-7g hydrolysis ladder, Lane 3: pre-let-7g & RNase T1, Lane 4: pre-let-7g & RNase A, Lane 5: pre-let-7g & RNase V1. Right: expansion of lanes 3-5. ds: double stranded. ss: single stranded. (b) Secondary structure of pre-let-7g based on the data from the enzymatic structural probing experiments. The coloured triangles indicate the extent of nuclease-induced cleavage at various sites. The purple and red coloured bonds represent Watson-Crick base pairing between adenine and uracil, and guanine and cytosine respectively. The green bonds represent non-Watson-Crick base pairing between guanine and uracil.

Figure 2.

Enzymatic structural probing of pre-let-7g in the presence of LIN28. (a) Representative autoradiogram of the RNase T1 footprinting of pre-let-7g in the presence LIN28. Lane 1: pre-let-7g, Lane 2: pre-let-7g hydrolysis ladder, Lane 3: pre-let-7g & RNase T1 sequencing ladder, Lane 4: pre-let-7g & LIN28 (3 μM), Lane 5: pre-let-7g & RNase T1, Lane 6: pre-let-7g, RNase T1 & LIN28 (1 μM), Lane 7: pre-let-7g, RNase T1 & LIN28 (3 μM) (b) Representative autoradiogram of the RNase A footprinting of pre-let-7g in the presence LIN28. Lane 1: pre-let-7g hydrolysis ladder, Lane 2: pre-let-7g, Lane 3: pre-let-7g & LIN28 (3 μM), Lane 4: pre-let-7g & RNase T1 sequencing ladder, Lane 5: pre-let-7g & RNase A, Lane 6: pre-let-7g, RNase A & LIN28 (1 μM), Lane 7: pre-let-7g, RNase A & LIN28 (3

μM). Filled blue circles represent highly reduced RNase cleavage and blue circles represent minor reductions in RNase cleavage. (d): denaturing conditions. (c) Secondary structure of pre-let-7g summarizing the experimental data from the footprinting experiments in the presence of LIN28.

Figure 3.

Enzymatic probing of tpre-let-7g in the absence and presence of LIN28. (a) Secondary structure of tpre-let-7g summarizing the experimental data from the LIN28 footprinting analysis. Filled blue circles represent highly reduced RNase cleavage, blue circles represent minor reductions in RNase cleavage and triangles represent increased cleavage. (b). Representative autoradiogram of the enzymatic footprinting of tpre-let-7g in the absence and presence of LIN28. Lane 1: tpre-let-7g hydrolysis ladder, Lane 2: tpre-let-7g, Lane 3: tpre-let-7g & LIN28 (3 μ M), Lane 4: tpre-let-7g & RNase T1 sequencing ladder, Lane 5: tpre-let-7g & RNase T1, Lane 6: tpre-let-7g, RNase T1 & LIN28 (3 μ M), Lane 7: tpre-let-7g & RNase A. Lane 8: tpre-let-7g, RNase A & LIN28 (3 μ M).

Figure 4.

RNase 1 structural probing of pre-let-7g in the presence of LIN28. (a) Representative autoradiogram of the RNase 1 footprinting of pre-let-7g in the presence LIN28. Lane 1: pre-let-7g hydrolysis ladder (exposure reduced relative to full gel), Lane 2: pre-let-7g, Lane 3: pre-let-7g & LIN28 (3 μM), Lane 4: pre-let-7g & RNase 1, Lane 5: pre-let-7g, RNase 1 & LIN28 (1 μM), Lane 6: pre-let-7g, RNase 1 & LIN28 (3 μM), Lane 7: RNase T1 ladder (b) Secondary structure of pre-let-7g summarizing the experimental data from the RNase 1 footprinting experiments in the presence of LIN28.

Figure 5.

Dicer processing assay (a) Representative autoradiogram of the Dicer processing assay of 5'-end-labeled wild-type pre-let-7g (WT) and mutant pre-let-7 (MUT). Dicer cleavage was allowed to proceed for 5 min. The extent of Dicer processing was quantified by calculating the 'relative Dicer processing efficiency' which is defined as the product intensity divided by total (products and full-length substrate) intensity. Lane 1: ladder, Lane 2: WT pre-let-7g, Lane 3: MUT pre-let-7g, Lane 4: WT pre-let-7g and Dicer (0.1 U), Lane 5: MUT pre-let-7g and Dicer (0.1 U), Lane 6: WT pre-let-7g, Dicer (0.1 U) and LIN28 (2 μ M), Lane 7: WT pre-let-7g and LIN28 (2 μ M). (b) Relative Dicer processing efficiency. Results were normalized relative to the Dicer processing efficiency obtained for the wild-type pre-let-7g alone, which was taken as 100%. Error bars represent the standard deviation of three independent experiments. (c) Secondary structure of the stem mutant pre-let-7g. Mutations to the wild-type pre-let-7g in the upper stem are marked with asterisks.