Summary of the final achievements towards objectives and details for each task

Summary:

MicroRNAs (miRNAs) constitute a large class of genes that control diverse biological processes. Animal miRNAs repress their targets through an antisense mechanism, where they base-pair imperfectly with their target mRNAs, promoting translational repression and target degradation[1]. Recent studies have linked over- and underexpression of miRNAs to various human diseases, particularly cancers, where miRNAs can function as tumour suppressors and oncogenes[2, 3]. Accordingly, miRNA expression is a highly regulated process under physiological conditions that is controlled at the level of transcription and, post-transcriptionally, at various steps of miRNA maturation[4]. Such tight regulation helps to maintain constant steady-state levels of some miRNAs during animal development, and dynamic expression patterns of others[5-7].

As RNA concentrations are generally a function of biogenesis and turnover, it is possible that active miRNA degradation can also modulate miRNA accumulation, providing an additional layer of regulation of miRNA activity. Thus understanding of miRNA turnover would not only provide new insights into the miRNA metabolism circuit but might also open up new avenues towards unravelling of pathological states associated with miRNA dysregulation. **My first objective was to identify a miRNA turnover machinery/machinery component(s) employing genetics and the second being biochemical characterization of miRNA turnover and its machinery/machinery component(s).**

Based on my results obtained during the last 24 months (1st March 2008-28th February 2010) I report here that alongside regulation at the level of biogenesis, functional miRNA homeostasis is further regulated by degradation of mature miRNAs *in vivo*, mediated by the XRN-2; a 5'→3' exoribonuclease of *C. elegans,* which was identified through a genetic screen. The XRN-2-dependent miRNA turnover can be recapitulated in larval lysates, where processing of pre-*let-7* by Dicer, unannealing of the *let-7* duplex, and loading into Argonaute proteins are coupled processes that precede degradation of the mature miRNA. Unexpectedly, although Argonaute:miRNA complexes are highly salt-resistant, larval lysate promotes efficient release of the miRNA, exposing it to degradation by XRN-2. Release and degradation can both be blocked by addition of miRNA target RNA. My results thus suggest the presence of an additional layer of regulation of miRNA activity that might be important for rapid changes of miRNA expression profiles during developmental transitions, and maintenance of miRNA steady-state concentrations.

Details of each task:

Objective I

Depletion of the *xrn-2* exoribonuclease increases *let-7* miRNA levels and activity *in vivo*

The *let-7* miRNA regulates stem cell fates in animals and functions as a human tumor suppressor gene[8]. In *C. elegans*, the temperature-sensitive *let-7(n2853)* allele causes vulval bursting phenotype at the larval-to-adult transition when animals are grown at the restrictive temperature, 25°C. This allele is characterized by a single point mutation towards the 5' end of the mature miRNA, impairing its binding to target mRNAs[9, 10]. However, the expression level of this miRNA is also moderately decreased compared to its wild-type counterpart[9, 11]. As target site mutations compensatory to the *let-7(n2853)* point mutation restore target gene repression in a

let-7(n2853) background only partially, this reduction appears functionally relevant[10]. We therefore hypothesized that increased abundance of the mutant *let-7* and/or its sequence-related 'sister' miRNAs *mir-48, mir-84*, and *mir-241* (ref. 12) might be sufficient to downregulate some of its targets, thus partially suppressing the vulval bursting of *let-7(n2853)* animals. Genes involved in miRNA turnover would be strong candidates for suppressor activity.

An RNAi-based screen of genes encoded on *C. elegans* chromosome I had previously identified suppressors of the *let-7(n2853)* mutation, including known and novel *let-7* target genes[13]. Some of the suppressors were not *let-7* targets, indicating their involvement by other means. Encouraged by this finding, we tested diverse nucleases for their ability to suppress *let-7*-associated lethality when depleted through RNAi by feeding, initiated on synchronized L1 stage larvae. We found that *xrn-2*, the *C. elegans* orthologue of the yeast 5'→3' exoribonuclease Rat1p, was a particular potent suppressor of vulval bursting, with >95 % of animals surviving (Table 1). By contrast, depletion of the *C. elegans* siRNase; *eri-1* (ref. 14), and *C. elegans* homologues of Rex1p through Rex4p 3'→5' exoribonucleases (Table 1), regulators of miRNA stability in plants[15], did not suppress *let-7(n2853)* lethality.

To confirm that depletion of *xrn-2* affected *let-7* RNA levels, we examined RNA from late L4 stage *let-7(n2853)* worms, exposed to either *xrn-2(RNAi*) or mock (i.e., empty vector) RNAi. Consistent with a function in turnover of mature *let-7* and/or its precursors, northern blot analysis revealed that mature *let-7* levels were increased substantially upon *xrn-2* depletion (Fig. 1a). In yeast, the Xrn2p/Rat1p exoribonuclease frequently functions redundantly with its paralogue Xrn1p[16, 17], but depletion of *C. elegans xrn-1* neither substantially suppressed the *let-7* mutation nor resulted in *let-7* miRNA accumulation Table 1; Fig. 1a). This finding supports a major role of XRN-2 but not XRN-1 in determining mutant *let-7* RNA accumulation, although a minor function of *xrn-1* remains possible.

Yeast Xrn2p/Rat1p functions in quality control of mature tRNA, selectively removing tRNAs that have been incompletely modified[18]. However, the effects of *C. elegans xrn-2* depletion were not restricted to the mutant *let-7* miRNA, but were also seen for wild-type *let-7*, as well as other miRNAs, unrelated in sequence (Fig. 1a and data not shown), arguing against a miRNA 'quality control' turnover system and supporting a broader function of XRN-2 in miRNA homeostasis.

Yeast Xrn2p/Rat1p is involved in the processing of rRNA and snoRNA precursors[16]. Moreover, *Arabidopsis* has three homologues of Xrn2p/Rat1p, among which XRN2 and XRN3 act as scavenger enzymes that degrade the loop sequence of the miRNA precursor[19]. Therefore, we investigated whether *C. elegans* XRN-2 might act during miRNA biogenesis to affect pri-miRNA or pre-miRNA levels, either in addition to, or instead of, acting on mature miRNAs. However, although depletion of the pre-miRNA processing enzyme Dicer permits ready detection of accumulated pre-miRNA[20, 21] (see below), *xrn-2(RNAi)* did not (Fig. 1a), arguing against a major role of XRN-2 in pre-miRNA turnover. To test this possibility directly, we examined expression of pre-*mir-60*, which is detectable by northern blotting[22]. Also in this case we observed that *xrn-2(RNAi)* did not increase pre-miRNA levels relative to the control situation (Fig. 1b). We also measured the levels of pri-*let-7* and pri-*mir-77*

primary miRNAs by RT-qPCR and again found no change in their levels in *xrn-2(RNAi)* relative to control worms (Fig. 1c). We conclude that depletion of *xrn-2* preferentially, possibly exclusively, affects accumulation of mature miRNAs. To our knowledge, this is the first time that XRN-2 has been implicated in the turnover of mature, functional RNA species.

We noticed that some of the *let-7(n2853); xrn-2(RNAi)* double mutant animals displayed moulting defects, as reported previously for *xrn-2(RNAi)* single mutant animals[23]. However, the penetrance of this phenotype was low (~30%), suggesting that it did not account for the almost complete suppression of the *let-7(n2853)*-associated vulval bursting phenotype. Some of the *let-7(n2853); xrn-2(RNAi)* animals also displayed defects in vulval morphogenesis, i.e., the vulva did not close during the late L4/young adult stage (Fig. 2a). Vulval formation appeared to be delayed, rather than terminated, as most *let-7(n2853); xrn-2(RNAi)* adult animals ultimately developed fully closed vulvae and still did not burst. Moreover, *xrn-2* depletion not only suppressed the bursting phenotype, but also permitted generation of adult alae (Fig. 2b), a cuticular structure whose formation depends on *let-7* function[9]. Although these observations indicate that suppression of *let-7* bursting by *xrn-2* depletion is direct and specific, we sought to confirm that *xrn-2(RNAi)* enhances *let-7* activity by examining the levels of *daf-12* and *lin-41* mRNAs. These two mRNAs are targets of *let-7* (ref. 24, 25) and loss of *let-7* activity increases their transcript levels[11, 26] (Fig 1). Depletion of *xrn-2* decreased the levels of these mRNAs comparable to what was seen in the wild-type animals (Fig. 1d), demonstrating a molecular basis for specific suppression of *let-7(n2853)* by *xrn-2(RNAi)*. These data thus confirm that XRN-2 modulates *let-7* activity, rather than acting as a 'scavenger' enzyme that clears away inactive miRNA.

Objective II

XRN-2 is a functional component of a miRNA turnover machinery *in vitro*

To examine miRNA turnover biochemically, we developed an *in vitro* system using larval lysates and radiolabelled synthetic or *in vitro* transcribed miRNAs. Initially, pCp labelling was used to block the 3' terminal hydroxyl group of synthetic *let-7*, precluding the activity of 3'→5' exonucleases on the substrate. In wild-type worm lysate the substrate was converted to mononucleotides without the production of any visible intermediates both at 25°C, the physiological temperature, and 37°C (Fig. 3a). As the latter temperature yielded more product, and for technical convenience, we performed all subsequent *in vitro* reactions at 37°C.

A 5' labelled synthetic *let-7* and an internally labelled *let-7 in vitro* transcript having free 3' hydroxyl-groups were similarly degraded when exposed to the lysate (Fig. 3b). Thin-layer chromatography identified the product as nucleotide-5'-monophosphate when internally labelled *let-7* was used as substrate (Fig. 3c), revealing a hydrolytic mode of degradation as expected of 5'→3' exonucleases[27], although 3'→5' hydrolytic exonucleases present in the lysate might contribute to this pattern. Nuclease activity was largely sequence-independent, as four other synthetic miRNAs were similarly degraded (data not shown). Nonetheless, the degradative activity was distinct from that seen for other small RNAs bearing a free 3'-hydroxyl group, such as tRNA, for which we observed an array of bands of sizes greater than

the substrate and a final product that was a few nucleotides long (Fig. 3d), presumably reflecting the well-documented degradation by the exosome following adenylation by TRAMP[28, 29].

To examine whether the exonuclease activity depended on XRN-2, we prepared lysate from worms exposed to *xrn-2(RNAi)*. Under these conditions, miRNA degradation was significantly reduced relative to a control RNAi lysate (Fig. 3e). By contrast, and mirroring the *in vivo* situation, depletion of *xrn-1* did not affect miRNA turnover (Fig. 3f). We confirmed that the effect of reducing *xrn-2* was specific, as add-back of bacterially expressed, recombinant GST-tagged XRN-2, but not GST alone, restored miRNA turnover (Fig. 3e, compare lanes 4 and 5). Of note, bacterial cells are thought to be largely devoid of endogenous 5'→3' exoribonucleases, with the only known bacterial activity residing in a 66 kDa protein, the *B. subtilis* endoribonuclease/exoribonuclease RNase J1 (ref. 30). Our use of gel purified and renatured GST-XRN-2 (~140 kDa) thus excluded contamination by 5'→3' exoribonuclease activity of bacterial origin. Consistent with the known preference of 5'→3' exoribonucleases for a 5' phosphate on its substrate[31], miRNA turnover was also decreased when non-phosphorylated substrate was used (Fig. 3g). From these results we can attribute the exonuclease activity acting in the lysates on miRNAs to XRN-2.

Pre-miRNA processing and mature miRNA turnover are coupled

The *in vivo* assays had indicated that XRN-2 specifically affected accumulation of the mature miRNA, but not its precursors. To confirm this *in vitro*, we generated radiolabelled, 5' mono-phosphorylated pre-*let-7* by *in vitro* transcription, and incubated it with the lysate. This substrate was converted into several products including mononucleotides (Fig. 4b), which is the sole product formed in a mature miRNA turnover assay. To rule out nonspecific degradation of pre-*let-7*, we repeated the assay with *dcr-1(RNAi)* extract, and observed stabilization of the pre-*let-7* substrate (Fig. 4c), as we did for the endogenous pre-*let-7 in vivo* (Fig. 4d). Thus, product formation in the control lysate depended on an upstream processing activity, i.e. the dicing activity of Dicer

When pre-*let-7* was incubated in *xrn-2(RNAi)* lysate, the pre-*let-7* substrate still disappeared. This is in accord with our *in vivo* data, which suggested that *xrn-2* does not degrade pre-*let-7*. However, a band co-migrating with a synthetic mature *let-7* accumulated in the *xrn-2(RNAi)*, but not the mock RNAi lysate (Fig. 4e). We identified this band as the mature *let-7* by performing a scaled-up assay with cold substrate and subjecting the extracted RNA to northern analysis (Fig. 4f). In this experiment, lysates were pre-treated with micrococcal nuclease (MN) to exclude the possibility of detecting endogenous RNAs in northern analysis. Taken together, these findings indicate that the assay faithfully recapitulates pre-*let-7* cleavage by Dicer and that processing by Dicer is a prerequisite for miRNA degradation by XRN-2.

One possible interpretation of our results is that XRN-2 can dislodge the mature, single stranded miRNA from the guide-passenger strand RNA duplex that Dicer generates from the pre-miRNA (Fig. 4a, e). However, we find that a synthetic guide:passenger duplex remains stable when incubated with lysate (Fig. 4g),

suggesting that degradation should occur at a step downstream of cleavage, i.e., during or after separation of the two strands. Indeed, extraction of RNA under non-denaturing conditions followed by native gel analysis directly demonstrated that the mature *let-7* generated by pre-*let-7* processing in *xrn-2(RNAi)* lysates was single stranded (Fig. 4h). Strikingly, the *in vitro* system thus appears to recapitulate several steps in miRNA biogenesis and turnover, i.e., pre-miRNA processing by Dicer, unannealing of the guide-passenger duplex, and degradation of the single stranded miRNA.

Target-mediated stabilization of RISC-bound miRNA

The observation that single-stranded mature miRNA was subject to turnover in our assay, whereas a guide:passenger duplex was not, suggested that target binding might modulate miRNA stability. To test this possibility, we supplemented the lysates with *in vitro* transcribed *let-7* target RNA[32], i.e., a luciferase coding sequence fused to a 3' artificial UTR containing three *let-7* binding sites or control transcripts with mutated *let-7* binding sites or lacking the 3' UTR entirely (Fig. 5a). Under these conditions, the transcript with the 3' UTR containing the *let-7* binding sites, but not the two control transcripts, efficiently prevented mature *let-7* miRNA degradation (Fig. 5b). Importantly, the controls exclude the possibility that excess exogenous RNA simply quenched RNase activity. Northern blot of a scaled-up assay using cold substrate confirmed accumulation of mature *let-7* under these conditions (Fig. 5c). We conclude that miRNA targets can modulate the extent of mature miRNA degradation *in vitro*.

It remained possible that the target RNA sequestered the 'naked' mature miRNA into a duplex that was inaccessible to degradation, similar to the passenger-guide duplex. As the majority of cellular miRNA is thought to be in association with Argonaute proteins in miRNA induced silencing complexes (miRISC), such a scenario would have little physiological relevance. We therefore wished to test whether mature miRNA that was produced through *in vitro* dicing became associated with Argonaute. We prepared lysates from transgenic worms expressing GFP-tagged versions of both of the *C. elegans* miRNA argonautes, ALG-1 and ALG-2 (subsequently named GFP/AGO)[33]. Following incubation with radiolabelled *pre-let-7*, and immunoprecipitation of GFP/AGO, co-immunoprecipitated RNA was extracted, resolved on a gel, and subjected to autoradiography. No radiolabelled RNA, precursor or mature, was detected in GFP/AGO immmunoprecipitates from control lysates lacking miRNA target, consistent with the complete degradation of the radiolabelled substrate in the lysate. However, addition of the *let-7* target RNA permitted not only accumulation of the mature miRNA in the lysate (Fig. 5d, top panel), but also its co-immunoprecipitation with GFP/AGO (Fig. 5d, middle panel) demonstrating incorporation of the mature miRNA into miRISC.

***let-7* is released from miRISC prior to degradation**

Depletion of *xrn-2* caused substantial accumulation of mature *let-7* generated from pre-*let-7* by GFP/AGO larval lysates (Fig. 5d, top panel). However, modest amounts of mature *let-7* co-immunoprecipitated with GFP/AGO (Fig. 5d, middle panel) in the absence of target RNA, whereas abundant *let-7* remained in the post-immunoprecipitation (IP) supernatant (Fig. 5d, bottom panel). These data support the notion that, *in vitro*, miRNAs are dislodged from ALG-1/2 through a mechanism that

is partially dependent on XRN-2, and modulated by the target RNA binding status of the miRNA. Indeed, as both the miRNA 5' and 3' ends are thought to be directly bound by Argonaute[34], they would be inaccessible to exoribonucleases while residing in RISC. The fact that not only the levels of endogenous miRNAs but also *let-7* activity are increased in *xrn-2(RNAi)* animals, further suggests that release and degradation steps are tightly coupled *in vivo*.

Although these data support the notion that miRNAs can be released from RISC, this result is unexpected as related human siRNA:AGO complexes have been shown to be highly stable[35]. To demonstrate miRNA release from AGO directly, we investigated the association of GFP/AGO with endogenous miRNA. To this end, we immunoprecipitated GFP/AGO and incubated the protein at 37°C for 15 min, while bead bound, with either our assay buffer (AB), AB supplemented with KCl to a final concentration of 0.6 M, or the same amount of the lysate from which GFP/AGO had been immunoprecipitated. After recovery, RNA was extracted and probed for presence of endogenous, mature *let-7* by northern blot analysis. Addition of neither buffer nor high salt diminished *let-7* binding relative to the control, consistent with the reported stability of human AGO-siRNA complexes[35] (Fig. 6a, compare lanes 1, 2, and 3). By contrast, incubation with wild-type larval lysate resulted in a strong loss of *let-7* signal, consistent with its removal from the ALG-1/-2 complexes (Fig. 6a, lane 4).

Finally, we immunoprecipitated GFP/AGO from larval lysate either immediately or following 15 minutes of incubation at the worm physiological temperature, 25°C. Again, consistent with a miRNA release factor acting on RISC, *let-7* levels were decreased in immunoprecipitate obtained after the incubation step relative to the pre-incubation immunoprecipitate (Fig. 6b, compare lane1 and 2). As the levels and integrity of GFP/AGO are not altered under these conditions (Fig. 6b), proteolytic degradation of GFP/AGO does not appear to mediate this release.

Discussion

We have shown here that XRN-2 is required for miRNA turnover *in vivo* and *in vitro*, and that it can modulate the activity of miRNAs *in vivo*. Thus, miRNA degradation contributes to miRNA homeostasis, potentially helping to prevent detrimental overexpression of miRNAs associated with many diseases[2]. We find that mature miRNA biogenesis and turnover are coordinated *in vitro*, and this is consistent with a complementary role towards the maintenance of miRNA steady-state concentrations.

We have also observed that miRNA targets can stabilize their cognate miRNAs *in vitro*, potentially permitting coordination of miRNA levels with abundance of their targets. Under conditions of reduced target abundance, such a mechanism would make Argonaute available for loading of other miRNAs, facilitating its reuse. Additionally, when miRNA silencing is relieved or prevented by antagonists such as HuR or Dnd1 (ref. 36, 37), increased degradation of unoccupied miRNA might provide a mechanism that enhances desilencing by preventing the miRISC from re-binding its released target, thus restricting cycles of alternate silencing and desilencing.

Although XRN-2 plays a crucial role in miRNA turnover under our experimental conditions, additional RNases might contribute to this process.

Moreover, specialized regulatory proteins might guide the differential turnover of different miRNAs using the same core machinery. The latter scenario appears particularly attractive given that developmental transitions such as embryonic stem cell differentiation are frequently accompanied by substantial alterations in expression profiles of specifc miRNAs. Alongside transcriptional downregulation, upregulation of miRNA turnover pathways could be one of the mechanisms facilitating such transitions.

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Figure legends

Fig.1. Depletion of *xrn-2* increases mature miRNA levels and activity *in vivo*

a, b, Northern blotting of RNA from *let-7(n2853)* worms exposed to the indicated RNAi. *xrn-2(RNAi)* leads to the accumulation of mature *let-7* comparable to the levels in wild-type (*N2*) worms. *mir-85* and *mir-77* also accumulate relative to empty vector RNAi (control) but (b) pre-*mir-60* remains unchanged. $tRNA^{Gly}$ serves as loading control.

c, Examination of total RNA by RT-qPCR analysis demonstrates that x*rn-2(RNAi)* does not cause an increase of pri-*let-7* or pri-*mir-77* levels relative to control.

d, RT-qPCR shows that the levels of *lin-41* and *daf-12* mRNAs, two *let-7* targets, are elevated in *let-7(n2853)* relative to *N2* and *let-7(n2853)*; *xrn-2(RNAi)* animals

All experiments in this and subsequent figures used synchronized L4 stage animals. Results in C and D are averages of three independent experiments +/- standard error of mean.

Fig. 2. x*rn-2 (RNAi) exerts* suppression of bursting phenotype of *let-7(n2853)* worms

a, Synchronized L1 larvae of *let-7(n2853)* worms were placed either on empty vector RNAi plates (control) or *xrn-2(RNAi)* plates and grown at 25°C to young adult stage. Whereas worms growing on control plates (upper panel, at ~38 hrs) burst through their vulva, *xrn-*

2(RNAi); let-7(n2853) animals do not (lower panel, at ~40 hrs). Arrow points to the vulva, arrowhead to distal gonadal tip, indicating correct young adult stage of an animal with delayed vulval closure. **b,** Top panel shows a worm, at 52 hrs, with closed, protruding but intact vulva (arrow); the distal tip of one gonadal arm is indicated by an arrowhead. Lower panel shows development of continuous alae (indicated by arrowheads) in that same worm. Alae are visible even in animals with delayed vulval closure (not shown).

Fig. 3. XRN-2 is required for miRNA degradation *in vitro*.

Radiolabelled mature *let-7* miRNA was incubated with lysate from *N2* worms, the reaction products were analyzed by urea PAGE unless indicated otherwise.

a, Fate of 3'-pCp end labelled and blocked synthetic *let-7* exposed to lysate at two different temperatures: 25°C, worm physiological temperature; 37°C, conventional biochemical assay temperature. An identical, single product (mononucleotide) is obtained at both the temperatures, although 37°C incubation yielded more product. All subsequent *in vitro* assays were performed at 37°C.

b, Incubation of 5'-end labelled synthetic *let-7* with lysate also yields mononucleotides.

c, Thin layer chromatographic analysis of the assay performed with internally α-^{32}P-UTP labelled *let-7* and lysate. 5'-UMP co-migrated with the assay product (shown by arrowhead on top right), whereas 5'-UDP migrated much slower.

d, 5'-end labelled yeast tRNA^{Phe} was incubated with lysate. An array of bands (vertical bar on the right) migrating slower than the input RNA and a final product of a few nucleotides length (arrowhead on the left) were obtained.

e, Incubation of 3'-end labelled and blocked synthetic *let-7* (lane 1) with control lysate (lane 2), *xrn-2(RNAi)* lysate (lane 3), *xrn-2(RNAi)* lysate supplemented with recombinant GST-XRN-2 (lane 4), *xrn-2(RNAi)* lysate supplemented with GST only (lane 5) shows that XRN-2 is required for miRNA turnover.

f, *xrn-1(RNAi)* lysate and control RNAi lysates support degradation of 3'-end labelled and blocked synthetic *let-7* equally efficiently.

g, 5'-end phosphorylation is required for efficient degradation of a 3'-end labelled and blocked synthetic *let-7* RNA in larval lysate.

Fig. 4. Coordination of *in vitro* miRNA processing and turnover.

*In vitro a*ssays were performed with α-^{32}P-UTP labelled, *in vitro* transcribed pre-*let-7* and *N2* worm lysate, unless indicated otherwise.

a, Schematic representation of the stepwise processing of a pre-miRNA stem-loop into the single stranded mature miRNA.

b, Fate of radiolabelled pre-*let-7* incubated with lysate. The products are shown with arrowheads on the left; some relevant sizes are indicated on the right.

c, Pre-*let-7* is stabilized in *dcr-1(RNAi)* relative to control RNAi lysates.

d, Northern blot confirms accumulation of endogenous pre-*let-7* in *dcr-1(RNAi)* animals *in vivo*, confirming efficient dicer depletion. tRNA ^{Gly} levels serve as loading control.

e, Pre-*let-7* assayed with control and *xrn-2(RNAi)* lysate. Left side arrowheads point to different reaction products, which include one band corresponding to mature *let-7* in size.

f, A 20-fold scaled-up pre-*let-7* turnover assay was performed with cold substrate and micrococcal nuclease (MN) treated lysates as indicated. The recovered products were subjected to northern probing for *let-7*, revealing accumulation of mature *let-7* (arrowhead).

g, 5'-end labelled *let-7* guide RNA annealed to the passenger strand was incubated with lysate and subsequently analyzed on a native gel, demonstrating its stability. 5'-end labelled single stranded (ss) *let-7* migrates faster than double stranded (ds) 5'-labelled guide-passenger duplex.

h, Native gel analysis of products obtained from a pre *let-7* turnover assay shows that the band accumulating in *xrn-2(RNAi)* lysates co-migrates with single stranded mature *let-7.* Asterisk points to a conformer of pre-*let-7*.

Fig. 5. Target-mediated stabilization of mature miRNA

a, Schematic representation of Renilla luciferase (RL) reporter mRNAs, containing synthetic 3'UTRs with i) three functional *let-7* binding sites, ii) three mutated sites and iii) lacking a 3' UTR.

b, Pre-*let-7* assay with radiolabelled substrate and *N2* lysate in absence or presence of three different mRNAs as indicated reveals accumulation of a band corresponding to mature *let-7* in size (arrowhead on the right).

c, The assay as in (b) was scaled-up 20 fold using cold substrates and MN-treated lysates, eliminating endogenous RNAs. Recovered RNA was subjected to northern probing, demonstrating accumulation of mature *let-7* in the presence of its target RNA.

d, Pre-*let-7* turnover assay (top panel) was performed as above except control and *xrn-2* kd lysates were used from a GFP*::*AGO expressing strain. Middle panel shows the immunoprecipitates from the corresponding top panel reactions using α-GFP antibody, and the lower panel shows the recovered material (electrophoresed on a separate gel) from the post-immunoprecipitation supernatant (sup).

Fig. 6. Release of miRNA from miRISC

a, Equal amounts of immunoprecipitated miRISC argonaute (GFP/AGO) proteins were subjected, while bead-bound, to either no treatment (lane 1), or incubated at 37^{o}C for 15 min with assay buffer (AB, lane 2), AB + high salt (0.6 M KCl; lane 3), or the same amount of lysate from which the miRISC argonautes (used in the reaction) have been immunoprecipitated (lane 4). After recovery each reaction was split into two halves. One half was subjected to RNA extraction followed by northern probing with α-*let-7* probe (upper panel), and the other (lower panel) was subjected to western blotting using α-GFP antibody.

b, Immunoprecipitation was performed as above from lysate either directly or after incubation at 25°C for 15 min. One half of the immunoprecipitate was subjected to northern probing

(upper panel) after RNA extraction, the corresponding other halves (lower panel) were subjected to western blotting as above. Lane 3 corresponds to a no-antibody control.

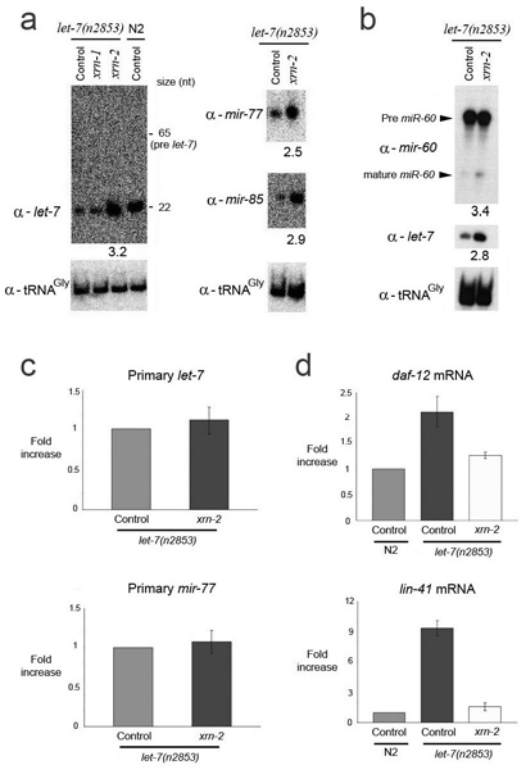

Fig. 1

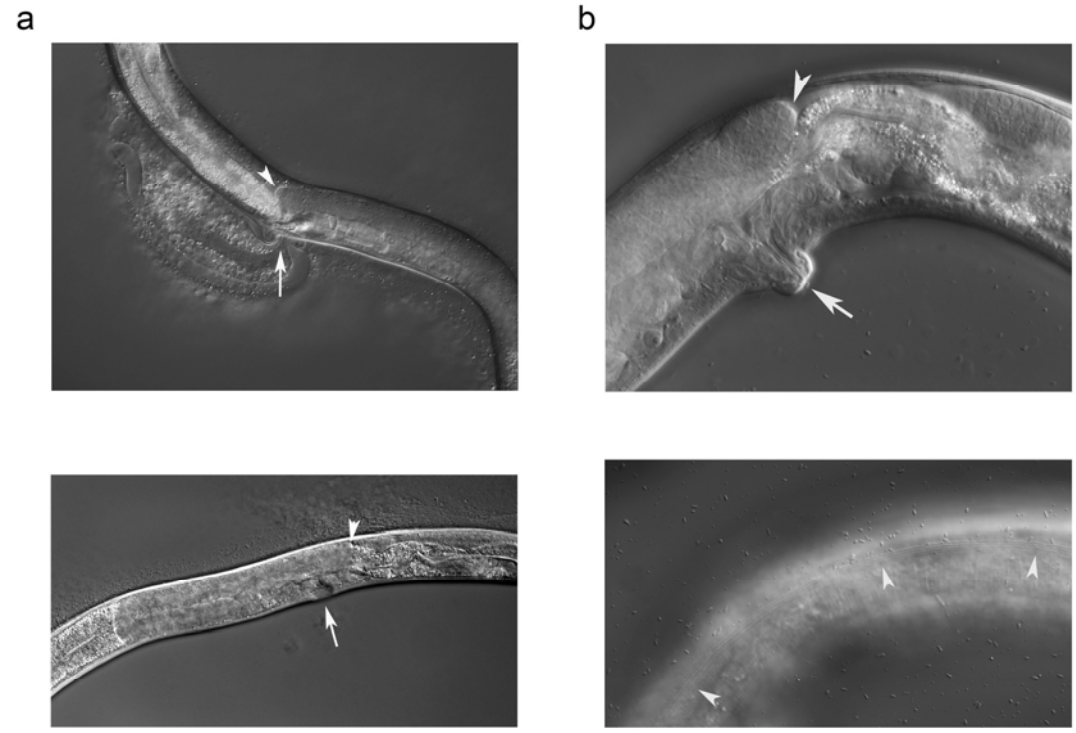
a
b

Fig. 2

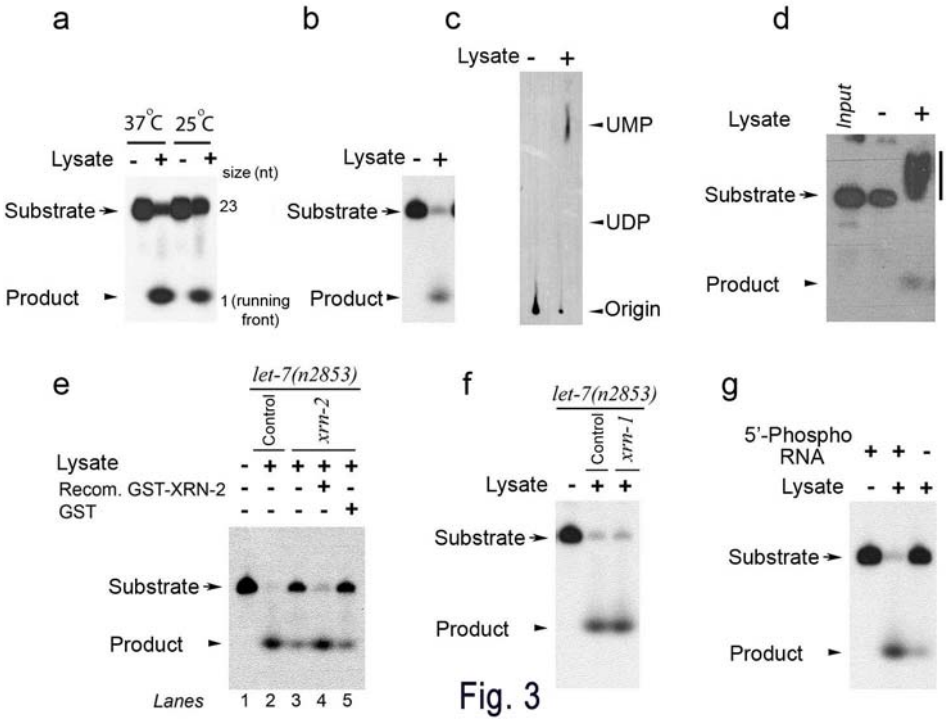
a
37°C 25°C
Lysate - + - +
size (nt)
Substrate
23
Product
1 (running front)
b
Lysate - +
Substrate
Product
c
Lysate - +
UMP
UDP
Origin
d
Lysate
Input
- +
Substrate
Product
e
let-7(n2853)
Control
xrn-2
Lysate - + + + +
Recom. GST-XRN-2 - - - + -
GST - - - - +
Substrate
Product
Lanes 1 2 3 4 5
f
let-7(n2853)
Control
xrn-1
Lysate - + +
Substrate
Product
g
5'-Phospho RNA + + -
Lysate - + +
Substrate
Product

Fig. 3

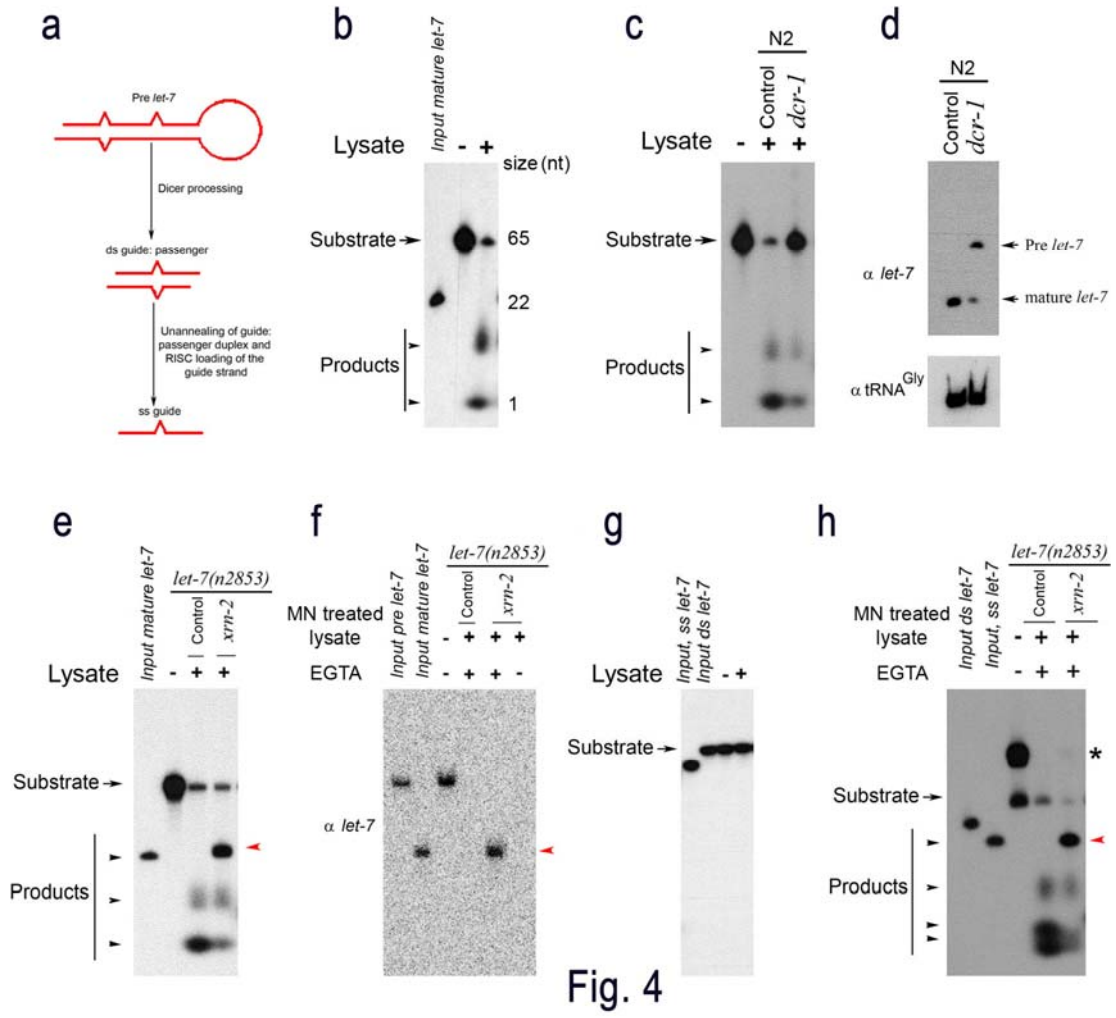
a
Pre let-7
Dicer processing
ds guide: passenger
Unannealing of guide: passenger duplex and RISC loading of the guide strand
ss guide
b
Input mature let-7
Lysate
- +
size (nt)
Substrate
65
22
Products
1
c
N2
Control
dcr-1
Lysate
- + +
Substrate
Products
d
N2
Control
dcr-1
Pre let-7
α let-7
mature let-7
α tRNAGly
e
Input mature let-7
let-7(n2853)
Control
xrn-2
Lysate
- + +
Substrate
Products
f
Input pre let-7
Input mature let-7
let-7(n2853)
Control
xrn-2
MN treated lysate
EGTA
- + + +
- + + -
α let-7
g
Input, ss let-7
Input ds let-7
Lysate
- +
Substrate
h
Input ds let-7
Input, ss let-7
let-7(n2853)
Control
xrn-2
MN treated lysate
EGTA
- + +
- + +
*
Substrate
Products

Fig. 4

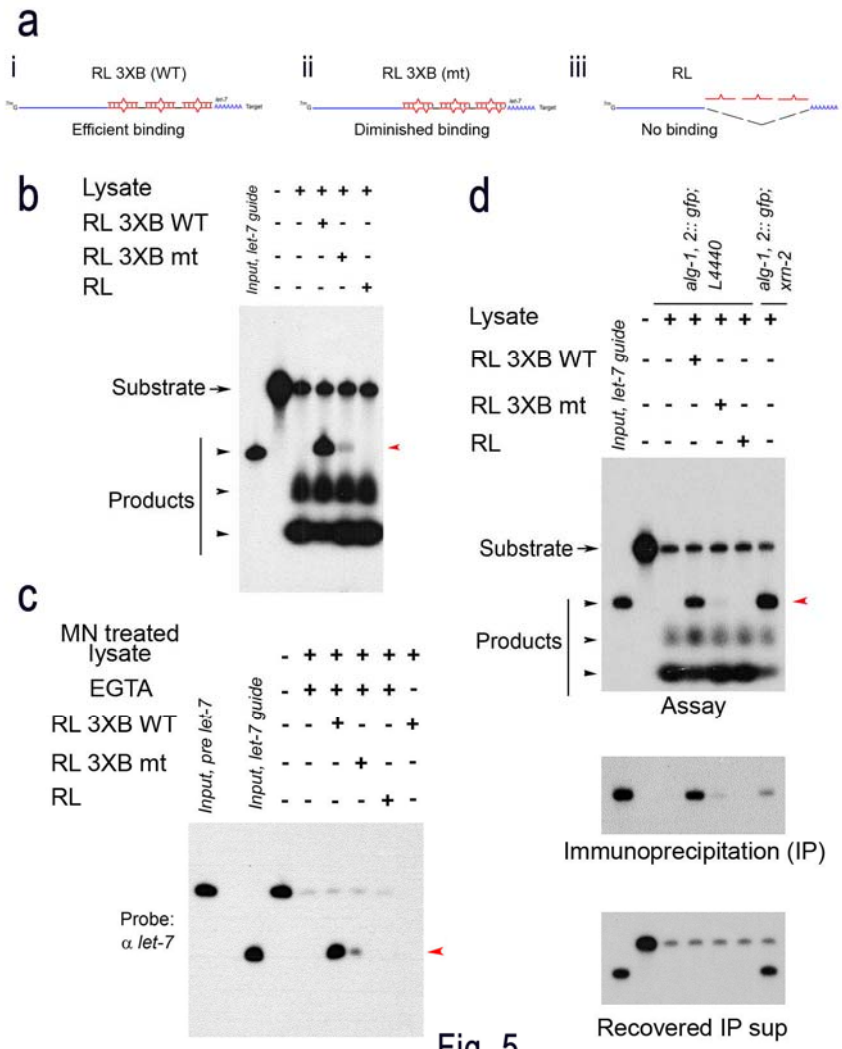
a
i
RL 3XB (WT)
Efficient binding
ii
RL 3XB (mt)
Diminished binding
iii
RL
No binding
b
Lysate
RL 3XB WT
RL 3XB mt
RL
Input, let-7 guide
Substrate
Products
c
MN treated lysate
EGTA
RL 3XB WT
RL 3XB mt
RL
Input, pre let-7
Input, let-7 guide
Probe:
α let-7
d
alg-1, 2:: gfp; L4440
alg-1, 2:: gfp; xrn-2
Lysate
RL 3XB WT
RL 3XB mt
RL
Input, let-7 guide
Substrate
Products
Assay
Immunoprecipitation (IP)
Recovered IP sup

Fig. 5

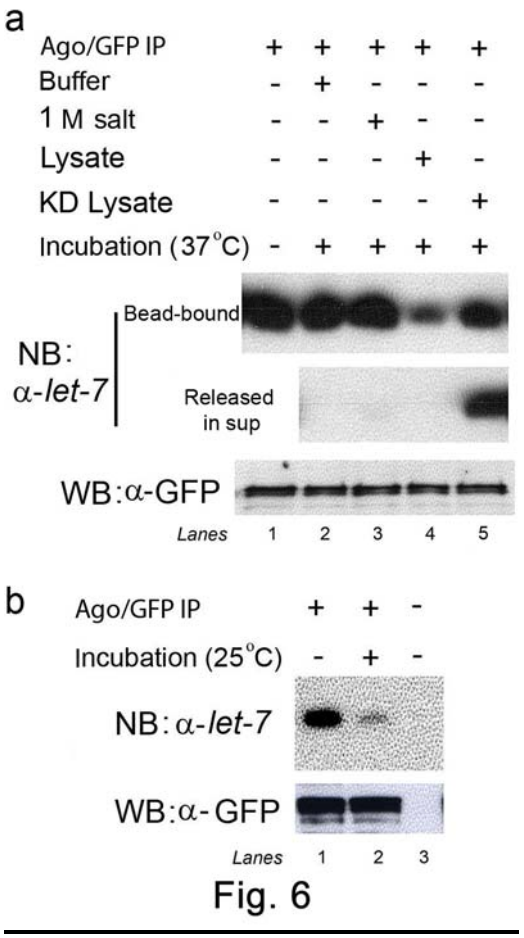

Fig. 6

Table 1. **Suppression of *let-7(n2853)* bursting phenotype**

Gene (yeast homologue and/or comment)	%survival at 60 hrs [a]
xrn-1 (5'-3' exoribonuclease)	15
xrn-2 (Rat1, 5'-3' exoribonuclease)	98
eri-1 (*C. elegans* siRNase)	5
C05C8.5 (Rex1)	0
C08B6.8/Oligoribonuclease (Rex2)	5
pqe-1(Rex3)	0
Y56A3A.33 (Rex3)	0
Y17G7B.12 (Rex4)	0
L4440 (empty vector)	0

[a] Average of three independent experiments performed with 100 or more animals.