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microRNA activity is suppressed in mouse oocytes

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Summary

MicroRNAs (miRNAs) are small endogenous RNAs, which typically imperfectly base-pair with 3′ UTRs and mediate translational repression and mRNA degradation. Dicer, an RNase III generating small RNAs in the miRNA and RNAi pathways, is essential for meiotic maturation of mouse oocytes. We found that 3′UTRs of transcripts up-regulated in *Dicer1*$^{-/-}$ oocytes are not enriched in miRNA binding sites implicating a weak impact of miRNAs on the maternal transcriptome. Therefore, we tested the ability of endogenous miRNAs to mediate RNA-like cleavage or translational repression of reporter mRNAs. In contrast to somatic cells, endogenous miRNAs in fully-grown GV oocytes poorly repressed translation of mRNA reporters whereas their RNAi-like activity was much less affected. In addition, reporter mRNA carrying let-7-binding sites failed to localize to P-body-like structures in oocytes. Our data suggest that normal miRNA function is down-regulated during oocyte development and this idea is further supported by normal meiotic maturation of oocytes lacking *Dgcr8*, which is required for the miRNA but not the RNAi pathway [Suh et al.]. We propose that suppression of miRNA function during oocyte growth is an early event in reprogramming gene expression during the transition of a differentiated oocyte into pluripotent blastomeres of the embryo.

Keywords

miRNA; endo-siRNA; P-body; maternal mRNA; oocyte; mRNA stability; mRNA degradation; translational arrest

Results and Discussion

Minimal impact of miRNAs on mouse oocyte transcriptome

The eight 5′-terminal nucleotides of a miRNA form a “seed”, which hybridizes nearly perfectly with the target mRNA and nucleates the miRNA-mRNA interaction [1]. Whereas enrichment of motifs complementary to seeds of highly active miRNAs has been observed in 3′ UTRs of mRNAs whose relative abundance is increased (hereafter referred to as up-regulated) upon depletion of *Dicer1* [2–4], transcriptome analysis of *Dicer1*$^{-/-}$ metaphase II (MII) eggs did not identify any miRNA-related motifs [5]. Because transcriptome remodeling during meiosis

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[6] could mask up-regulation of primary miRNA targets, we performed an analysis of fully-grown germinal vesicle-intact (GV) *Dicer1*$^{-/-}$ oocytes. Microarray profiling revealed a comparable number of up-regulated (489; P-value <0.001) and down-regulated (628, P-value <0.001) transcripts (Figure 1A). The magnitude of these changes was ~5 times smaller when compared to other studies of *Dicer1*-depleted mammalian cells [3,4]. In fact, the loss of *Dicer1* in the oocyte causes a transcriptome change comparable to the effect of a single miRNA in embryonic stem (ES) cells (Figure 1A) [4].

We searched for heptamer motifs enriched in 3′ UTRs of transcripts that were up-regulated in the *Dicer1*$^{-/-}$ oocytes and that could explain the mRNA expression changes. One of the four motifs most significantly enriched (Table S1) was complementary to the seed of miR-1195 (GAACUCA, Figure 1B). This motif, however, is likely not associated with miRNA function because miR-1195 was absent in deep sequencing of small RNAs from mouse oocytes [7]. Likewise, none of the predicted miR-1195 targets in the miRBase [8] was up-regulated in the *Dicer1*$^{-/-}$ oocytes. Sylamer [9], an alternative approach to analyze miRNA signals in 3′ UTRs, showed that none of the high-scoring motifs and none of the top five miRNA-related heptamers (Figure S1) match seed regions of miRNAs with a cloning frequency in oocytes >0.1%.

We also examined motifs related to abundant miRNAs in transcriptomes of *Dicer1*$^{-/-}$ oocytes and ES cells. These motifs, which were selected based on deep sequencing data [7,10], represent binding sites for more than half of all miRNAs cloned from these cells (Table S2). Interestingly, none of the motifs (including those for the let-7 family, which represents ~30% of maternal miRNAs [7,11]) showed any enrichment or any statistical bias in 3′UTRs of transcripts up-regulated in *Dicer1*$^{-/-}$ oocytes. This contrasts with *Dicer1*$^{-/-}$ ES cells, where the most significant motifs match a family of highly abundant miRNAs (~25% of cloned miRNAs [10]) and several motifs corresponding to other abundant miRNAs also showed enrichment and deviation from the statistical background (Figure 1C, Table S3).

Our data suggest limited miRNA-associated mRNA degradation in the oocyte and do not support the notion that miRNAs extensively modulate gene expression in oocytes [11,12]. Our analysis of 3′UTRs of transcripts up-regulated in *Dicer1*$^{-/-}$ oocytes does not provide evidence that the up-regulation is associated with miRNA function via seed-mediated interaction with 3′UTRs. Likewise, we observed no significant enrichment of miRNA-associated motifs in 3′ UTRs of intrinsically unstable mRNAs [13] and mRNAs degraded during meiosis [6]. Although miRNA binding sites were associated with specific transcript isoforms during meiotic mRNA degradation [14], it is unclear whether this observation reflects miRNA effects. It is possible that none of the maternal miRNAs is functionally dominant, and therefore none generates a strong signal, but this does not explain the low number of up-regulated transcripts in *Dicer1*$^{-/-}$ oocytes. Alternatively, miRNA-mediated mRNA degradation is not robust and the transcriptome change reflect the loss of endogenous small interfering RNAs (endo-siRNAs). We found that 42 of 489 up-regulated but only 6 of the 628 down-regulated transcripts in *Dicer1*$^{-/-}$ oocytes perfectly base-pair (Table S4) with endo-siRNAs [15]. Because RNAi-like cleavage by small RNAs requires less than complete base-pairing and can occur without a perfect seed complementarity [16], it is plausible that inhibition of the RNAi pathway is the major cause of transcriptome changes in *Dicer1*$^{-/-}$ oocytes.

The idea that low activity of miRNA-mediated mRNA degradation is responsible for the absence of an miRNA signature in *Dicer1*$^{-/-}$ oocytes is supported by Suh et al. [Suh et al.], who analyzed the maternal loss of *Dgcr8*, a component of the Microprocessor complex involved in miRNA biogenesis. *Dgcr8*$^{-/-}$ oocytes show the same depletion of miRNAs like *Dicer1*$^{-/-}$ oocytes, yet the transcriptome of *Dgcr8*$^{-/-}$ oocytes is more similar to the wild-type and mice with *Dgcr8*$^{-/-}$ oocytes are fertile, showing no meiotic spindle defects reported for *Dicer1*$^{-/-}$ and *Ago2*$^{-/-}$ oocytes. Therefore, the sterile phenotype of *Dicer1*$^{-/-}$ oocytes [5,11] is likely due

to mis-regulation of genes controlled by endo-siRNAs [7]. If this is true, the “slicer” activity of AGO2 is indispensable for meiotic maturation.

Endogenous miRNAs poorly repress cognate mRNAs

To understand the function of maternal miRNAs, we used three sets of reporter mRNAs carrying binding sites for the endogenous miRNAs let-7a and miR-30c. Let-7 is the most abundant miRNA family in the oocyte (~30% of maternal miRNAs [7,11,15]). The miR-30 family is less abundant; it represents ~8% of maternal miRNAs as suggested by RT-PCR [11]. The deep-sequencing data suggest a lower abundance (~2.4% [7]), but such estimates are prone to errors [17].

To assess let-7 activity during oocyte growth and meiotic maturation, we used firefly luciferase reporters (Figure 2A) carrying a lin-41 fragment with two natural bulged let-7 binding sites (FL-2xlet-7), which were mutated in the control (FL-control) [18]. Because fully-grown GV oocytes and MII eggs are transcriptionally quiescent, we microinjected *in vitro* synthesized mRNAs instead of plasmid reporters. First, we compared let-7-mediated repression of FL-2xlet-7 mRNA microinjected into meiotically incompetent oocytes with repression of the FL-2xlet-7 plasmid or synthetic FL-2xlet-7 mRNA transfected into NIH 3T3 cells. FL-2xlet-7 expression was reduced by ~40% relative to FL-control in oocytes (Figure 2B). Although this was less than repression of FL-2xlet-7 reporters in NIH 3T3 cells (~50%, Figure 2B), it showed that reporter mRNA is repressed by endogenous let-7 in small, growing oocytes.

When FL-2xlet-7 mRNA was microinjected into fully-grown GV oocytes, we observed inefficient let-7 repression, which was found also upon meiotic maturation (Figure 2C). This was unlikely due to insufficient amounts of endogenous let-7 miRNA because delivering the FL-2xlet-7 mRNA with a 50 molar excess of let-7a miRNA did not, in contrast to NIH 3T3 cells, improve reporter repression (Figure S2A). Likewise, 50 molar excess of let-7a antagomir did not increase FL-2xlet-7 expression in oocytes but did in NIH 3T3 cells (Figure S2B).

To explore further let-7 function in oocytes, we obtained another set of reporters (Figure 3A), which contained three bulged let-7 sites (RL-3xB let-7) or a single perfectly complementary let-7 site (RL-1xP let-7) downstream of the *Renilla* luciferase coding sequence [19]. These two reporters are repressed to the same extent in different cell lines but by different mechanisms [3]. The RL-1xP let-7 is cleaved by AGO2 loaded with let-7 in the middle of the duplex. The bulged sites of RL-3xB let-7 mediate translational repression and subsequent mRNA degradation, presumably as a consequence of relocation of the repressed mRNA to P-bodies, centers of mRNA metabolism [3]. To extend the analysis to other miRNAs, we produced a similar set of reporters for miR-30c (Figure 3A and Supplementary material).

Our results showed that repression of all miRNA targeted reporters was reduced during oocyte growth (Figure 3B-D) despite a 3- and 5-fold increase in the amount of miR-30 and let-7, respectively, during oocyte growth [11]. This repression was presumably miRNA-mediated because reporters harboring mutated miRNA binding sites (RL-3xM let-7 and RL-4xM miR-30) were not repressed (Figure 3B-D). Repression of perfectly complementary reporters was always significantly greater than that of their bulged versions, contrasting with data from cell lines where bulged reporters were either significantly better (Figure S3) or equally well [3]. This finding suggests that RNAi-like cleavage by miRNAs loaded on the AGO2-RISC complex is less affected during oocyte growth than translational repression, which is typical for most natural mammalian miRNA targets. Target site accessibility probably partially influences reduced repression of all reporters; our data show that siRNAs target 3′UTR sequences less efficiently in the oocyte when compared to somatic cells or siRNAs targeting the coding sequence (Figure S4).

The miR-30 reporter was consistently better repressed than the let-7 reporter. This finding was unexpected because let-7 family constitutes ~30% of maternal miRNAs whereas miR-30 mRNAs are several times less abundant [7,11]. An additional miR-30 binding site in the bulged miR-30 reporter could explain its better repression relative to the bulged let-7 reporter. However, this cannot explain differences between RL-1xP let-7 and RL-1xP miR-30 reporters. This difference may stem from secondary structures of miRNA binding sites or may reflect yet unknown let-7-specific regulation.

Repression of the RL-4xB miR-30 reporter could involve miRNA-mediated translational repression or miRNA-mediated mRNA degradation or a combination of both. Thus, we microinjected fully-grown GV oocytes with the RL-4xB miR-30 reporter and assayed for luciferase activity and the relative amount of *Luc* mRNA (Figure 4A and B). Whereas RL-1xP miR-30 mRNA was reduced at protein and mRNA levels as expected, RL-4xM miR-30 luciferase activity was reduced ~50% whereas there was negligible reduction in the amount of *Luc* mRNA. This observation suggests that the remaining miRNA-mediated translational repression is uncoupled from mRNA degradation in fully-grown GV oocytes. Therefore, we tested whether miRNA-targeted mRNAs localize to P-bodies, cytoplasmic foci involved in miRNA-mediated mRNA degradation [18, 19]. We visualized let-7-targeted and non-targeted mRNAs using a MS2-YFP binding strategy [18]. Whereas the let-7-targeted and non-targeted reporters were uniformly distributed in the oocyte cytoplasm, only the reporter harboring functional let-7 miRNA binding sites was targeted to P-bodies in NIH 3T3 cells (Figure 4C). This result is consistent with the loss of P-bodies during oocyte growth (Flemr et al., manuscript under revision).

Taken together, our data present a puzzling paradox: although mouse oocytes produce abundant RISC-loaded miRNAs their mRNA targets are poorly repressed. Uncoupling the loaded RISC from translational repression, however, may be an elegant solution for selective inhibition of the miRNA pathway in the oocyte because the RNAi and miRNA pathways have common components, e.g., Dicer and AGO2. Reducing miRNA activity during oocyte growth may have two roles. First, the low activity of miRNA-mediated mRNA degradation, perhaps linked to the absence of P-bodies, may contribute to mRNA stability and accumulation in growing oocytes. Second, down-regulation of the miRNA pathway may be required for oocyte-to-zygote transition. Abundant maternal miRNAs, such as let-7, are found in somatic cells [20]. Efficient reprogramming of somatic cells into pluripotent stem cells requires large remodeling of miRNA expression, including down-regulation of “somatic” miRNAs like let-7 (reviewed in [21]). Therefore, reducing miRNA activity may be associated with acquisition of developmental competence and miRNAs may not be required until the zygotic genome activation is completed and the pluripotency program, which controls also miRNA expression [22], is established. From this perspective, suppression of maternal miRNAs function during oocyte growth may be the first event in reprogramming the differentiated oocyte into pluripotent blastomeres of the embryo.

Experimental Procedures

Animals and oocytes

Fully-grown GV *Dicer1*$^{-/-}$ oocytes were obtained from 3A8 *Dicer1* conditional mice as previously described [5]. Meiotically incompetent oocytes, fully grown, GV-intact cumulus-enclosed oocytes and MII eggs were collected, microinjected, and cultured as described [23–26]. All animal experiments were approved by the Institutional Animal Use and Care Committee and were consistent with National Institutes of Health guidelines. A more detailed overview is provided in Supplemental Experimental Procedures.

mRNA microarray analysis

RNA was isolated from 25 fully-grown GV-intact mouse oocytes and amplified as previously described [27,28]. Oocytes for each sample were collected from an individual mouse and four samples were generated for each group. Biotinylated cRNA was fragmented and hybridized to the Affymetrix MOE430 v2 chip, which contains ~45,000 probe sets. All arrays yielded hybridization signals of comparable intensity and quality. Original CEL files were processed and 3′UTR heptamer analysis was performed as described previously [3,4]. A detailed overview of bioinformatic analyses is provided in Supplemental Experimental Procedures.

Reporter mRNA preparation and microinjection

Meiotically incompetent oocytes and fully-grown GV oocytes were injected as described [26]. The same concentration of reporter mRNA was achieved in both stages by microinjecting incompetent oocytes with ~1.7 pl and fully-grown GV oocytes with three times that amount (i.e., ~5 pl) because the volume of the meiotically incompetent oocytes used in these studies is about 1/3 of the fully-grown GV oocyte. Five pl contained ~10^5 molecules of the reporter. Reporter mRNAs were microinjected at following concentrations: FL-2xlet-7 & FL-Control reporter cRNA for let-7 at 0.2 μg/μl with spiked *Renilla* luciferase mRNA at 0.05 μg/μl; RL-C, RL-1xP, RL-3xB, RL-3xM for let-7 reporter at 0.05 μg/μl with spiked firefly luciferase mRNA at 0.05 μg/μl; RL-C, RL-1xP, RL-4xB, RL-4xM for miR-30 reporter at 0.05 μg/μl with spiked firefly luciferase mRNA at 0.05 μg/μl; let-7 reporter with 12xMS2-YFP binding sites and MS2-YFP at 1 μg/μl each; Let-7 mimic or antagonist at 50:1 molar ratio to FL-2xlet-7 reporter mRNA. After microinjection, oocytes were cultured overnight in CZB containing 2.5 μM milrinone (to maintain meiotic arrest of meiotically competent oocytes) or CZB without milrinone (for meiotically incompetent oocytes) in an atmosphere of 5% CO_2 in air at 37°C before they were processed for RT-PCR analysis, luciferase assay or immunocytochemistry. A detailed description of analysis of microinjected oocytes is provided in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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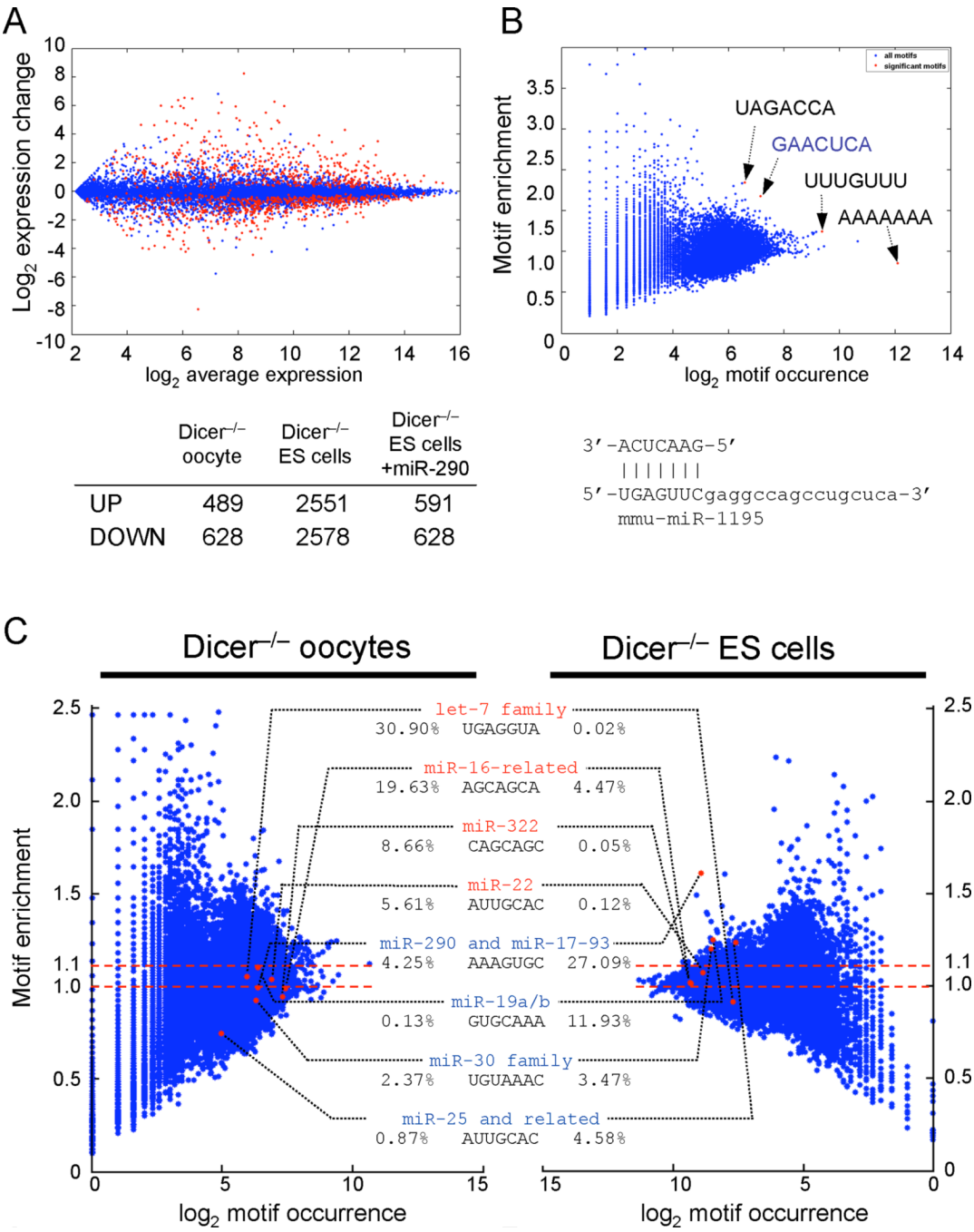

	Dicer−/− oocyte	Dicer−/− ES cells	Dicer−/− ES cells +miR-290
UP	489	2551	591
DOWN	628	2578	628

Figure 1. Transcriptome analysis of *Dicer1*$^{-/-}$ oocytes

(**A**) M [$\log_2$(fold change)] vs. A [average $\log_2$(expression level)] plot for the *Dicer1* *Dicer1*$^{+/+}$ fully-grown GV oocytes. Each dot represents a transcript. Significant expression changes (p-value < 0.001 computed from four replicate experiments) are shown in red. (**B**) 7-mer motif analysis of up-regulated transcripts. The motifs whose frequency in the 3′-UTRs of up-regulated transcripts is significantly different from the frequency in the entire set of 3′-UTRs are shown in red (see also Experimental Procedures). One of the significantly enriched motifs is complementary to the positions 1–7 of the miR-1195. (**C**) Comparison of 7-mer motif analyses of *Dicer1*$^{-/-}$ oocytes (left panel) and ES cells (right panel, horizontally inverted); most relevant motifs complementary to seeds of most abundant miRNAs in both cell types are

highlighted. The most abundant miRNAs in the oocyte and ES cells are shown in red and blue font, respectively. Note that none of the motifs corresponding to abundant maternal miRNAs is enriched more than 1.1 times in 3′ UTRs of transcripts up-regulated in *Dicer1*$^{-/-}$ oocytes whereas all four motifs corresponding to miRNAs abundant in ES cells are enriched in *Dicer1*$^{-/-}$ ES cells. Posterior probability analysis shows a high significance (1.000) only for the GCACUUU motif. However, posterior probability for the other three motifs corresponding to ES cell miRNAs was one to three orders of magnitude higher than all other motifs, which scored within the statistical background (~ 10^{-5}, Table S3). Abundance (%) of miRNAs related to individual motifs in both cell types is indicated next to each motif. Dashed lines mark 1.0 and 1.1-fold motif enrichment.

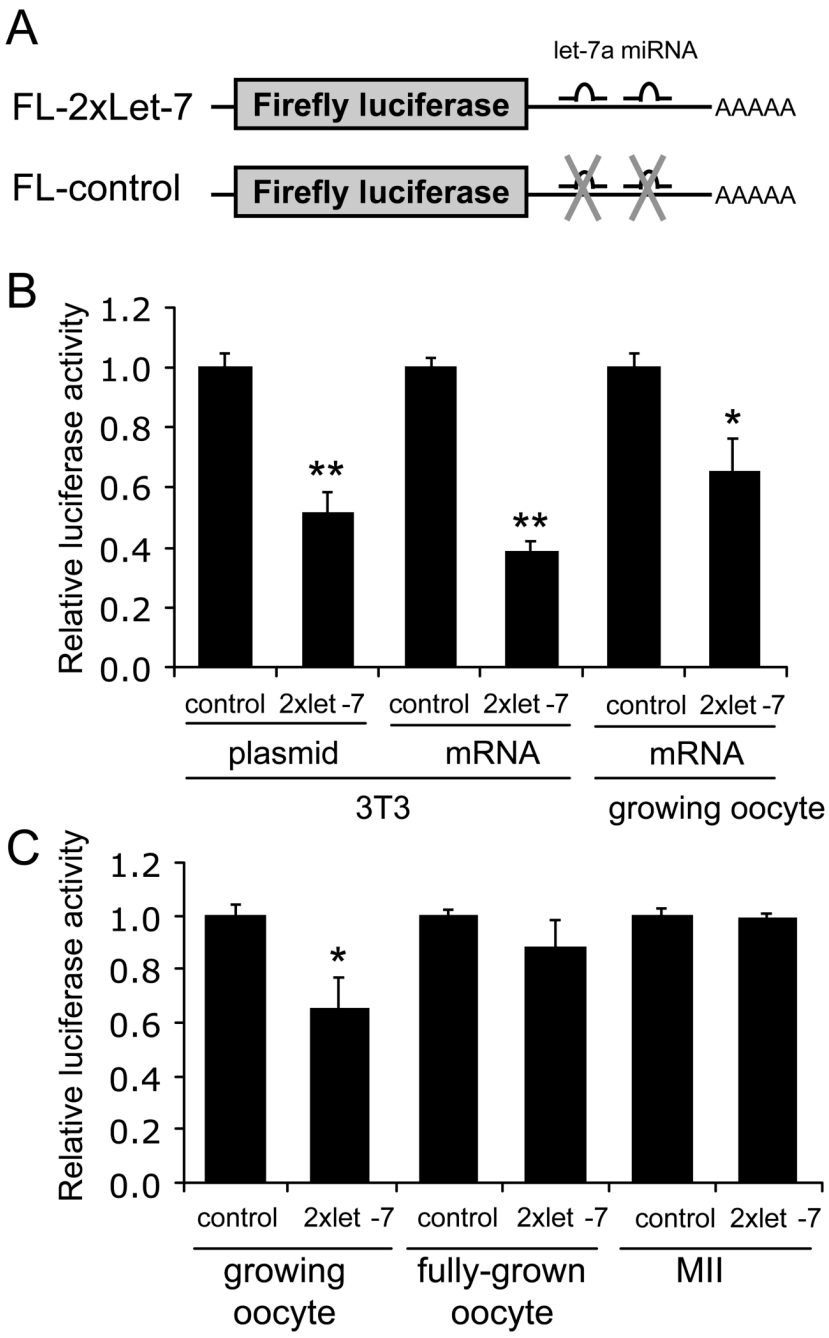

Figure 2. FL-2xlet-7 reporter analysis

(**A**) Schematic drawing of reporters used in the experiments presented in Figure 2. (**B**) Relative firefly luciferase reporter activity in NIH 3T3 cells and growing oocytes. NIH 3T3 cells were transfected with reporter plasmids or mRNAs and small, growing oocytes obtained from 13-day-old mice were microinjected with reporter mRNAs as described in Experimental Procedures. Firefly luciferase reporter activities were normalized to the co-injected *Renilla* luciferase control and are shown relative to FL control, which was set to one. The experiment was performed three times and similar results were obtained in each case. Shown are data (mean ± SEM) from one experiment. (**C**) Relative firefly luciferase reporter activity in growing oocytes obtained from 13-day-old mice, fully-grown GV oocytes, and oocytes matured to MII.

Data are presented as the mean ± SEM from six independent experiments. In this and subsequent figures, including supplemental figures, * and ** indicate that the difference compared to the control (ANOVA) is significant, with a p value <0.05 and 0.01, respectively.

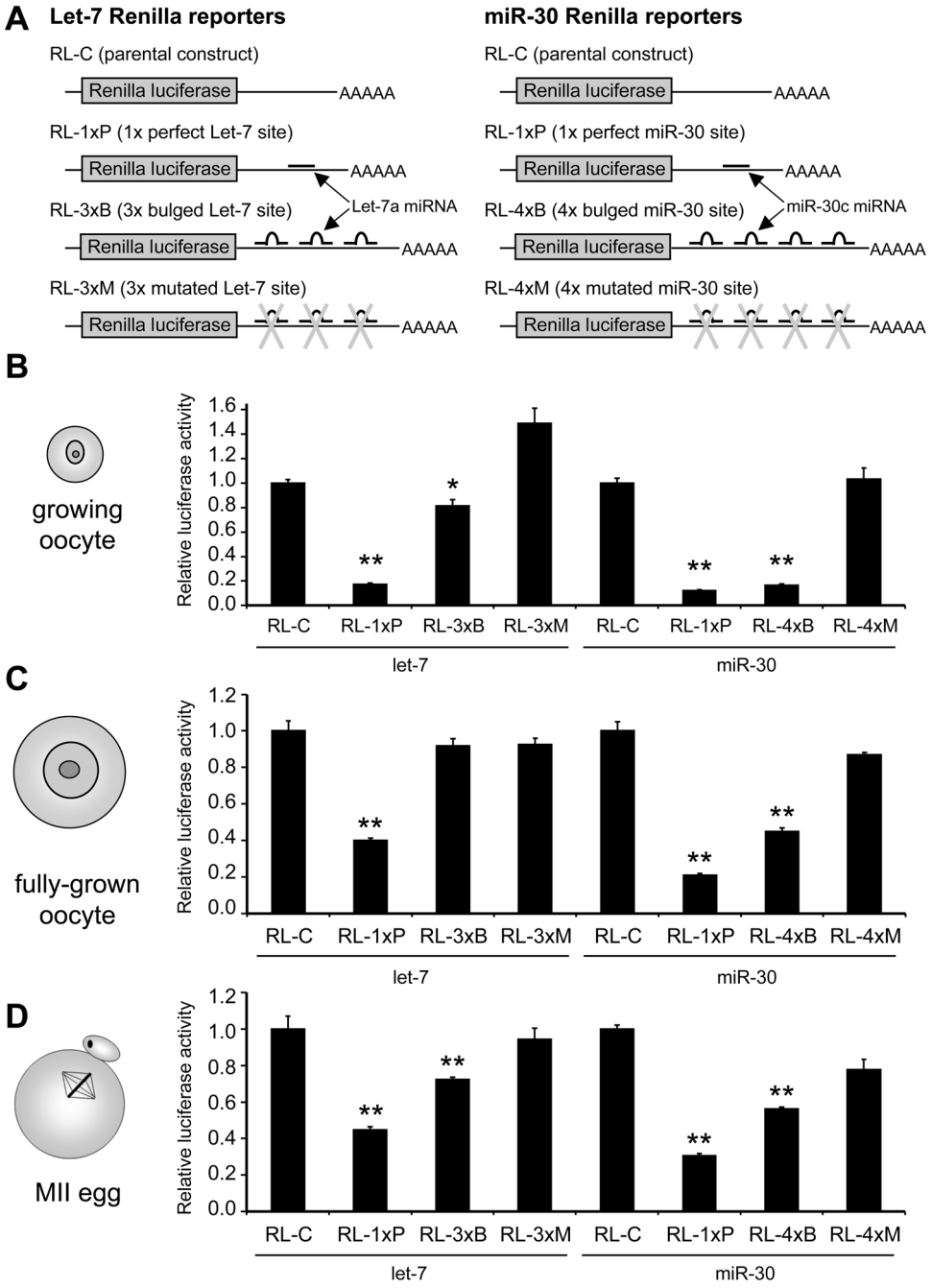

Figure 3. RL let-7 and miR-30 reporter analysis

(**A**) Schematic drawing of reporters used in experiments presented in Figure 3. (**B–D**) Relative *Renilla* luciferase reporter activities in growing oocytes (**B**), fully-grown GV oocytes (**C**), and metaphase II eggs (**D**). *In vitro*-produced reporter mRNAs were microinjected as described in Experimental Procedures. *Renilla* luciferase reporter activities were normalized to co-injected firefly luciferase control and are shown relative to RL-C control, which was set to one for each studied miRNA. The experiment was performed three times and similar results were obtained in each case. Shown are data (mean ± SEM) from one experiment.

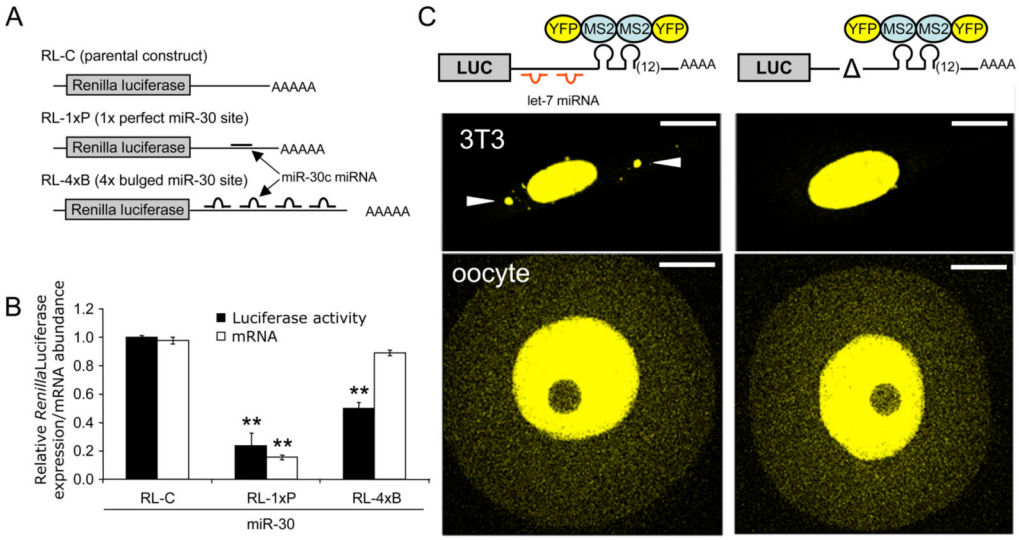

Figure 4. Repressed bulged luciferase transcripts are not degraded and do not localize to P-bodies

(**A**) Schematic of the miR-30 reporters is shown. (**B**) Oocytes were microinjected with miR-30 reporter mRNAs shown in panel A, and after one day of culture the relative reporter mRNA abundance was measured by qRT-PCR, and reporter mRNA translation efficiency was monitored by the dual luciferase assay. *Renilla* luciferase reporter activities were normalized to co-injected firefly luciferase control and are shown relative to RL-C control, which was set to one. The experiment was performed three times and similar results were obtained in each case. Shown are data (mean ± SEM) from one experiment. (**C**) mRNA harboring a let-7-binding sequence fails to localize to P-bodies in oocytes. Schematic depiction of reporters bound and not bound by endogenous let-7 is shown on the top of the figure. Below are confocal images showing cytoplasmic localization of corresponding reporter mRNAs. NIH 3T3 cells (upper images) were transfected with the corresponding reporter plasmids and fully-grown GV oocytes (lower images) were microinjected with *in vitro* transcribed mRNAs as described in Experimental Procedures. Co-transfected (co-injected) YFP-MS2 fusion protein containing NLS is retained in the cytoplasm upon binding to reporter transcripts, thus visualizing their localization [18]. White arrowheads depict P-bodies visualized by let-7 targeted reporter mRNA in NIH 3T3 cells. Scale bars represent 20 μm.