

## Genomes &amp; Developmental Control

## Select microRNAs are essential for early development in the sea urchin

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## ABSTRACT

microRNAs (miRNAs) are small noncoding RNAs that mediate post-transcriptional gene regulation and have emerged as essential regulators of many developmental events. The transcriptional network during early embryogenesis of the purple sea urchin, *Strongylocentrotus purpuratus*, is well described and can serve as an excellent model to test functional contributions of miRNAs in embryogenesis. We examined the loss of function phenotypes of major components of the miRNA biogenesis pathway. Inhibition of de novo synthesis of Drosha and Dicer in the embryo led to consistent developmental defects, a failure to gastrulate, and embryonic lethality, including changes in the steady state levels of transcription factors and signaling molecules involved in germ layer specification. We annotated and profiled small RNA expression from the ovary and several early embryonic stages by deep sequencing followed by computational analysis. miRNAs as well as a large population of putative piRNAs (piwi-interacting RNAs) had dynamic accumulation profiles through early development. Defects in morphogenesis caused by loss of Drosha could be rescued with four miRNAs. Taken together our results indicate that post-transcriptional gene regulation directed by miRNAs is functionally important for early embryogenesis and is an integral part of the early embryonic gene regulatory network in *S. purpuratus*.

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## Introduction

Small RNAs are components of a conserved gene regulatory mechanism that includes microRNAs (miRNAs), short interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). miRNAs negatively regulate protein expression by binding to sequence-complementary target sites in messenger RNAs (mRNAs) which induces repression of mRNA translation or transcript destabilization and decay (Bartel, 2009; Brodersen and Voinnet, 2009; Ghildiyal and Zamore, 2009; Guo et al., 2010; Hendrickson et al., 2009; Rajewsky, 2006, 2011). In animals, miRNAs have thousands of targets and altogether regulate

a major portion of protein coding genes (Baek et al., 2008; Bartel, 2009; Friedman et al., 2009; Krek et al., 2005; Lewis et al., 2005; Selbach et al., 2008; Stark et al., 2005; Xie et al., 2005). The vast majority of miRNAs are initially processed by Drosha and its cofactor DGCR8 (Han et al., 2006; Lee et al., 2003) and the maturation of miRNAs and siRNAs requires Dicer. Dicer is a member of the RNase III ribonuclease family and is responsible for processing double-stranded RNA (dsRNA) to small interfering RNAs (siRNAs) during RNA interference (RNAi) (Zhang et al., 2002). It is also the key enzyme that mediates the final processing of most miRNAs from their precursors.

A number of fundamental steps in embryogenesis appear to be regulated by miRNAs and while the documentation of gene regulatory networks involved in cell fate specification and differentiation has revealed the importance of numerous signaling molecules and transcription factors, the diverse regulatory roles of miRNAs in early development are only now emerging (reviewed in Fabian et al., 2010; Ghildiyal and Zamore, 2009; Pauli et al., 2011). Recently a number of miRNAs were identified in the purple sea urchin, *Strongylocentrotus purpuratus* (Campo-Paysaa et al., 2011; Friedländer et al., 2011; Peterson et al., 2009; Wheeler et al., 2009), revealing many deeply conserved miRNAs also present in humans. Echinoderms are a sister group to the chordates and the function of miRNAs in these embryos may reflect transitions in deuterostome development. Armed with

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the in-depth knowledge of transcriptional gene regulatory networks in the sea urchin (see [www.spbase.org/endomes](http://www.spbase.org/endomes)), we set out to investigate the importance of miRNAs in early embryogenesis of this animal. We profiled and annotated small RNA expression from the ovary and several early embryonic stages by deep sequencing followed by computational analysis, including application of the miRNA identification tool "miRDeep" (Friedländer et al., 2008; Friedländer et al., 2011). Individual knockdowns of Dicer, Drosha and DGCR8 as well as miRNA rescue experiments suggest that the miRNA pathway plays an important functional role in early cell fate decisions of sea urchin embryogenesis and serves as a paradigm for an ancestral feature of the deuterostome lineage.

**Results**

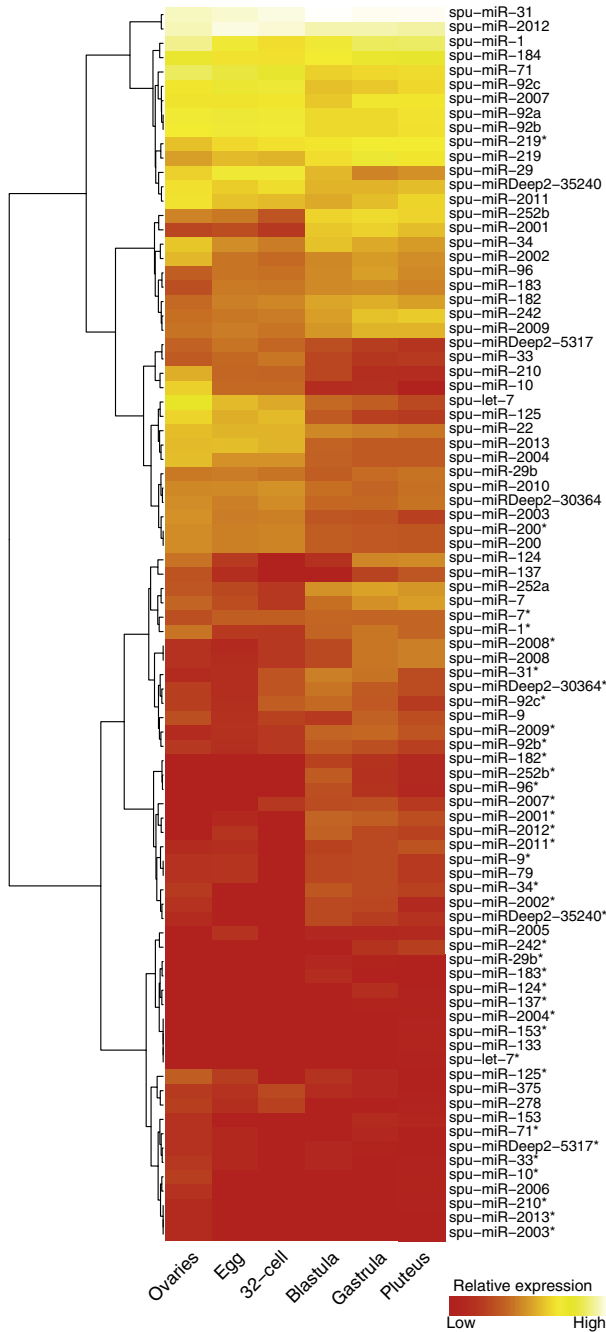
*Small RNA annotation and expression profiling*

We cloned and sequenced small RNA populations (18–40 nucleotides) from ovaries, eggs, 32-cell stage embryos (5 hours post fertilization [hpf]), blastulae (24 hpf), gastrulae (48 hpf), and early larvae (plutei; 72 hpf). Using miRDeep2 (Friedländer et al., 2008; Friedländer et al., 2011), a previously published algorithm that identifies miRNA genes based on sequenced Dicer hairpin products, we confidently identified 49 miRNAs in the ovary and five developmental stages of the sea urchin embryo (Fig. 1, Table S1), three of these identified miRNAs were novel miRNAs that were previously not annotated in miRBase version 16. Interestingly, one of these miRNAs is transcribed from the other genomic strand of a known miRNA locus, showing that bi-directional miRNA genes are deeply conserved (Stark et al., 2008; Tyler et al., 2008). Ten of the annotated miRNAs appear to be sea-urchin specific. Most of the miRNAs are present in the egg but have dynamic accumulation profiles with the majority of them upregulated by gastrulation (Fig. 1).

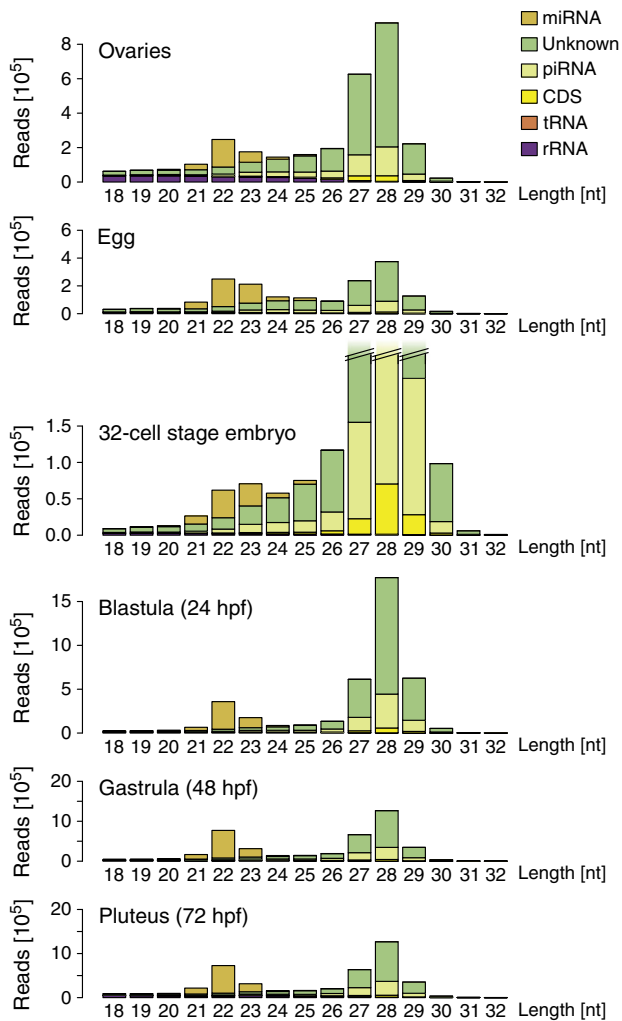
To investigate whether our deep sequencing data can accurately quantify differential miRNA expression, we tested the expression of 4 miRNAs (miR-31, -34, -252b, and -2009) using RT-qPCR Taqman assay. We observed an overall good correlation between deep sequencing and qPCR for all tested developmental stages (Fig. S1; square of the correlation coefficient lies between 0.78 and 0.98), except for the 48 h sample (square of the correlation coefficient is 0.49). The observed differences may be due to different sample preparations and/or efficiencies in reverse transcription, cDNA library construction, and the PCR amplification steps.

We next investigated the length distribution and annotation of all sequencing reads. Small RNAs showed a bimodal length distribution with 2 distinct peaks around 22 and 28 nucleotides (Fig. 2). All miRNAs identified by miRDeep account for a characteristic peak around 22 nucleotides. Interestingly, we found that most of the sequenced sea urchin RNAs that do not map to existing annotations have a distinct length profile peaking at 28 nucleotides, as has been observed for piwi-interacting RNAs (piRNAs) in other species. piRNAs are associated with silencing of transposable elements in the germline and have recently been shown to be involved in maternal mRNA deadenylation in the early embryo thus mediating the maternal-to-zygotic transition (Rouget et al., 2010). Further, we found that a large portion of these RNAs tend to overlap with each other by exactly ten nucleotides, with one read exhibiting a uridine bias at the 5' end and the other an adenine bias at the tenth nucleotide. These features are consistent with the conserved 'ping-pong' piRNA biogenesis pathway via mutual cleavage of the sense and antisense piRNA precursors by the Piwi proteins (Aravin et al., 2006; Brennecke et al., 2007; Girard et al., 2006; Grivna et al., 2006; Gunawardane et al., 2007; Houwing et al., 2007; Saito et al., 2006; Vagin et al., 2006; Watanabe et al., 2006). We therefore annotate the RNAs that overlap by exactly ten nucleotides as piRNAs and refer to the remaining small RNA species of around 28 nucleotides that do not overlap by ten nucleotides as 'unknown' sequences, although their length distribution suggests that they are likely highly enriched in piRNAs (Fig. 2).

We observed a significant decrease in total reads mapping to miRNAs at the 32-cell stage. This was correlated with an increase of reads mapping to putative piRNAs (Fig. 2). As distributions of sequenced reads do not reflect absolute abundance but rather relative frequencies, two possible interpretations to the 32-cell stage transition are that miRNAs are either cleared from the egg following fertilization, or that piRNAs strongly increase at the 32-cell stage. To distinguish these possibilities, we performed Northern blots for selected piRNA candidates. We observed a pronounced increase in 4 of the 5 detectable piRNAs in the 32-cell stage (Fig. S2). Moreover, RT-qPCR analysis did not illustrate a drastic decrease of the tested miRNAs in the 32-cell stage (Fig. S1). Taken together the results suggest that piRNAs



**Fig. 1.** Dynamic expressions of the sea urchin miRNAs. Hierarchical clustering of miRNAs based on gene expression patterns is plotted as a heat map. Most miRNAs are expressed maternally and are upregulated by 24 and 48 hpf. The "\*" corresponds to the minor miRNA precursor product found at lower concentration. Three novel miRNAs are *spu-miRDeep2-35240*, *spu-miRDeep2-5317*, and *spu-miRDeep2-30364*.



**Fig. 2.** Sea urchin small RNA composition and expression profile. Small RNAs from the ovary, egg, 32-cell stage, blastula (24 hpf), gastrula (48 hpf), and pluteus (72 hpf) were sequenced and annotated. miRNAs are 22 nts and putative piRNAs are between 27 and 29 nts. Putative piRNAs that overlap with each other in consistency with conserved 'ping-pong' mode of biogenesis (see Materials and methods) are labeled 'piRNA' while the remaining are labeled 'Unknown'. The figure is scaled to yield identical vertical extent of the 22 nucleotide miRNA fraction. Compare to Fig. S3 for an illustration with static scaling.

have a dynamic expression pattern in the early sea urchin embryo. This increase of piRNA expression may correspond with the specification of the *piwi*-positive small micromere lineage (Juliano et al., 2006; Rodriguez et al., 2005).

#### Key enzymes involved in miRNA biogenesis are required in early development

*Dicer*, the dsRNA processing enzyme that catalyzes the final cytoplasmic miRNA precursor cleavage reaction to generate mature miRNAs, is detected at relatively constant mRNA levels from the egg to the 64-cell stage, decreasing prior to gastrulation (Fig. S4A). In contrast, *Dicer* protein expression peaks in blastulae and decreases during gastrulation (Fig. S4B and S4C). To test the function of *Dicer* and its resulting miRNA products during early embryogenesis, eggs were microinjected with *Dicer* morpholino antisense oligonucleotides (MASO). Injected embryos showed an estimated 30% decrease in *Dicer* protein, as compared with the mock-injected embryos, as

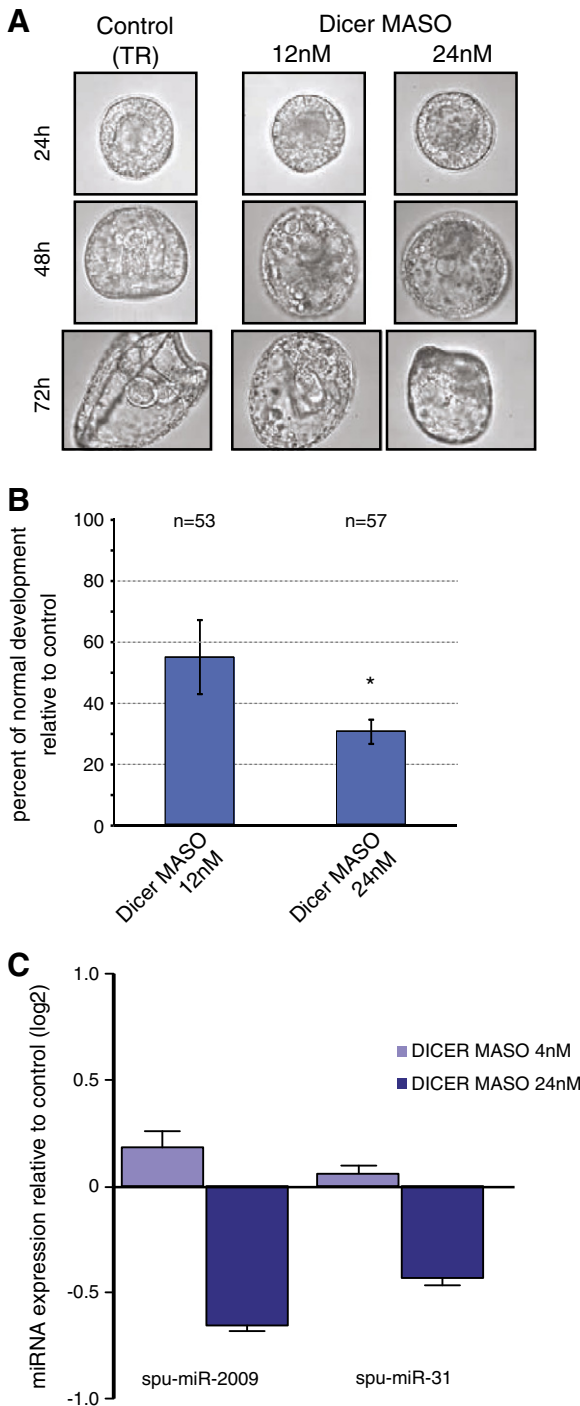
early as the 2-cell stage (Fig. S5). This suggests de novo *Dicer* translation during early development, and the fast decrease in *Dicer* protein after knockdown indicates a short half-life of the protein in the early embryo. We observed that most of the injected embryos developed normally into blastulae. However, by 48 hpf, *Dicer* MASO embryos failed to gastrulate, a phenotype dependent on the dose of MASO (Figs. 3A and B). Developmental defects in these embryos ranged from an overall delay in development, the failure to form a proper archenteron (the primitive gut), to embryonic lethality. These phenotypes were also observed with two different, non-overlapping *Dicer* MASOs (Fig. 3A and data not shown), but not with negative control MASOs (Fig. S6), nor when MASOs were used to knock down different gene functions irrelevant to the miRNA biogenesis pathway (Juliano et al., 2010, and data not shown).

To test if the knockdown of *Dicer* leads to decreased levels of mature miRNAs in the early embryo, we used RT-qPCR to quantify the sea urchin-specific miRNA, miR-2009, and a conserved vertebrate miRNA, miR-31, at the blastula stage (24 hpf). Compared to the mock-injected control, these miRNAs decreased up to 40% in *Dicer* MASO-injected embryos, suggesting that the decrease in *Dicer* protein resulted in a significant inhibition of miRNA biogenesis (Fig. 3C). The absence of complete miRNA knockdown may be explained by the stability of the assayed miRNAs or the lack of a complete *Dicer* gene knock-out using MASO, where residual *Dicer* still generated some functional miRNAs.

*Dicer* may have miRNA independent functions, such as centromeric silencing, the processing of endogenous siRNAs, and alternative deoxyribonuclease activities (Fukagawa et al., 2004; Kanellopoulou et al., 2005; Nakagawa et al., 2010). To test if the phenotypes we observed in *Dicer* knockdown embryos were specific to alterations in miRNA expression, we assayed knockdown phenotypes of *Drosha* and *DGCR8*, two other dsRNA processing enzymes catalyzing the nuclear primary miRNA cleavage which is critical for canonical miRNA biogenesis. Following MASO treatment for *Drosha* we observed earlier and more severe developmental defects compared to *Dicer*-deficient embryos (Figs. 4A and B). By 24 hpf, a delay in development was already observed in *Drosha* knockdown blastulae and by 72 hpf these embryos were either abnormal or were still in the gastrula stage, which is normally observed by 48 hpf of development. *DGCR8* MASO-injected embryos did not illustrate significant developmental effects (Fig. 4D), although the few aberrant embryos had similar morphological defects as the *Dicer* and *Drosha* knockdowns (Fig. 4C). This could be explained by a longer half life of *DGCR8* compared to the other proteins involved in miRNA biogenesis. However, the knockdown of a combination of *DGCR8* and *Dicer* or *DGCR8* and *Drosha* resulted in more pronounced developmental abnormalities as compared to *Dicer* or *Drosha* knockdown alone (Fig. 4E).

#### Alterations in miRNA biogenesis lead to misexpression of genes involved in early embryogenesis

*Dicer* knockdown embryos that failed to gastrulate exhibited a range of phenotypes largely between blastula (24 hpf) and gastrula (48 hpf) stages, when endodermal and mesodermal tissues are formed. Expression of the endodermal marker, *Endo1* (Wessel and McClay, 1985), is significantly decreased in *Dicer* knockdown embryos as compared to the mock-injected control embryos (Fig. 5A). We observed a similar decrease in expression level of a mesodermal marker, *Meso1* (Wessel and McClay, 1985), in *Dicer* knockdown embryos (Fig. 5B). These results indicate that the observed phenotypes caused by alterations in miRNA biogenesis most likely reflect a failure to properly specify various tissue types. We thus measured the transcript levels of a number of developmentally regulated molecules involved in cell signaling, transcriptional regulation, cell adhesion, cell movement, and cell proliferation (Fig. 5C) (Ben-Tabou de-Leon and Davidson, 2009; Byrum et al., 2009; Davidson et al., 2002; Duboc et al., 2004; Flowers et al., 2004; Logan et al., 1999; Peter and Davidson,



**Fig. 3.** Dicer is required for early sea urchin development. (A,B) *Dicer* morpholino anti-sense oligonucleotide (MASO)-injected embryos have dose-dependent developmental defects. (B) Barplots depicting the percentages of normal embryos in each experimental treatment relative to the percentage of normal embryos in the mock injected control at 48 hpf. *n* is the number of embryos analyzed. Unpaired Student *T*-test was used to determine the significance of the knockdown compared to the mock control. *p*-value was 0.025 for the Dicer MASO 24 nM sample. (C) Dicer knockdowns resulted in a decrease of miRNAs. A miRNA unique to the sea urchin *spu*-miR-2009 and miR-31, a conserved miRNA, were decreased in Dicer knockdown embryos compared to the control mock-injected embryos at 24 hpf. Standard deviation error bars are from 4 replicates.

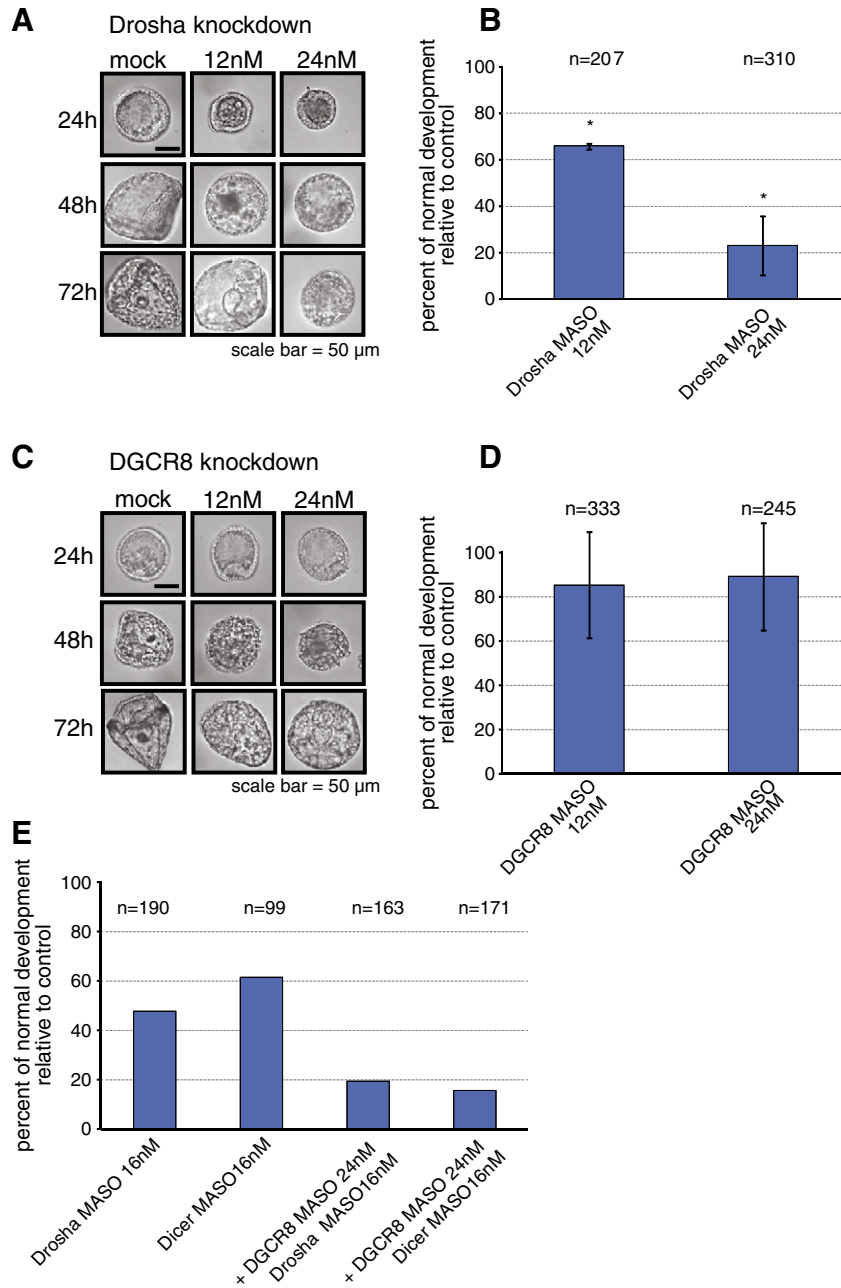
2011; Sherwood and McClay, 1999; Sweet et al., 1999; Sweet et al., 2002; Wikramanayake et al., 2004). The transcriptional repressor, Pmar1, is exclusively expressed in the skeletogenic micromere

lineage where it represses transcription of the ubiquitously present transcriptional repressor, HesC (Oliveri et al., 2002). This double-negative repression leads to the activation of the *delta* ligand and three regulatory genes, *alx1*, *ets1*, and *tbr* in the skeletogenic micromere lineage (Revilla-i-Domingo et al., 2007). The Delta/Notch pathway leads to the activation of *gcm*, a mesodermal gene regulator (Ransick and Davidson, 2006). At the tested time points, 17 and 24 hpf, we expect to capture critical gene expression changes that are essential for proper endomesodermal specification, which is likely affected by the global depletion of miRNAs (Fig. 5C). *β-catenin*, *wnt8*, *pmar1*, *hesC*, *delta*, and *nodal* mRNA levels are increased in *Droscha* knockdown embryos (Fig. 5C). Upregulation may suggest that these genes are directly regulated by miRNAs. Interestingly, the endodermal regulatory gene, *foxA*, and the mesodermal regulatory gene, *gcm*, are both decreased in mRNA levels by 24 h, instead suggesting an indirect miRNA regulatory pathway and consistent with our observation of decreased endo/mesodermal immunostaining in *Droscha* knockdown embryos (Figs. 5A,B). *β-catenin* is known to directly regulate the transcription of *pmar1* and *wnt8*; therefore, the increased mRNA levels of *wnt8* and *pmar1* may be a result of increased *β-catenin* and/or are directly regulated by miRNAs. Previous studies indicated that *pmar1* overexpression leads to increased *delta* and decreased *spec1* mRNA levels (Oliveri et al., 2002; Oliveri et al., 2003), which are consistent with what we observed with the QPCR data (Fig. 5C). However, results indicated that *gcm* and *foxA* mRNA levels are decreased when *delta* mRNAs are increased, indicating that *gcm* and *foxA* are likely to be regulated indirectly by miRNA-dependent mechanisms. Taken together, alterations in the miRNA biogenesis pathway lead to deregulation of most tested determinants of early sea urchin development. Whether these mRNA changes are directly or indirectly caused by alterations of miRNA expression remain to be determined.

*Rescue of the Droscha knockdown phenotype by miRNAs*

miR-1, miR-31, miR-2012, and miR-71 were sequenced at least 10 times more often than other miRNAs, likely indicating that these are among the highest expressed miRNAs in the sea urchin embryo (Fig. 1 and Table S2). We reasoned that their high levels could reflect broad and crucial functions in the early embryo. To test this assumption, we designed a rescue experiment in which we injected these four abundant miRNAs as double-stranded RNA (dsRNA) Dicer substrates into *Droscha* knockdown embryos. We found that the synthetic dsRNA duplexes significantly rescued *Droscha* knockdown embryos, including morphogenesis and function of each major cell type of the embryo (Fig. 6). While *Droscha* knockdown embryos have severe developmental defects, embryos rescued with dsRNA duplexes were able to develop into feeding larvae 5 days after fertilization (Fig. 6B). Complementation with miR-153 and miR-375, two of the least abundantly sequenced miRNAs, or a negative control dsRNA, however did not rescue the effect of *Droscha* knockdown, indicating sequence specificity of the miR-1, -31, -2012, and -71 rescue (Fig. 6).

To further dissect which miRNA is essential to rescue the *Droscha* knockdown phenotype, we set up experiments to test 16 combinations of dsRNA-1, -31, -71, and -2012 in the *Droscha* knockdown background (Fig. 7). We observed that single dsRNA-71 is able to rescue the *Droscha* knockdown phenotype from 9% of normal embryos in *Droscha* knockdown background to 50% normal embryos. The dsRNA-71 rescue effect of the *Droscha* knockdown-induced phenotype is not due to the amount of injected solutions (Fig. S7). Taken together, combinations of dsRNAs containing dsRNA-71 in the *Droscha* knockdown background rescued the *Droscha* knockdown phenotype most effectively, suggesting that miR-71 may be an essential component of the gene regulatory network in early developmental processes (Fig. 7).

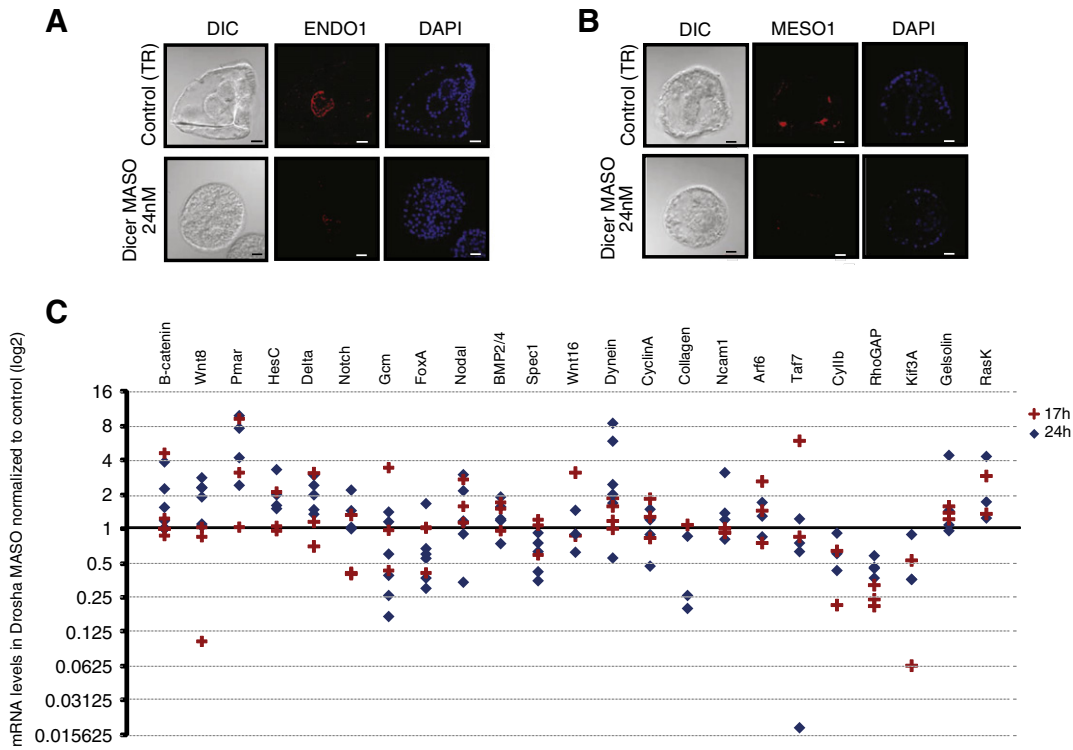


**Fig. 4.** *Drosha* knockdown displays similar developmental defects as the *dicer* knockdown. Morpholino antisense oligonucleotides of variable concentrations were injected into newly fertilized eggs. (A,C) DIC images of embryos at 24 hpf, 48 hpf, and 72 hpf. Scale bar is 50  $\mu$ m. (B,D,E) Barplots depicting the percentages of normal embryos in each experimental treatment relative to the percentage of normal embryos in the mock injected control at 48 hpf. *n* is the total number of injected embryos. Unpaired Student *T*-test was used to determine the significance level between the knockdown and the mock control. The *p*-values are 0.015 and 0.009 for *Drosha* MASO 12 nM and *Drosha* MASO 24 nM, respectively (A,B) *Drosha* MASO-injected embryos have dose-dependent developmental defects, whereas only very few (C,D) DGCR8 MASO-injected embryos show the same phenotype and most do not. (E) Injecting morpholinos against DGCR8 + *Drosha* or DGCR8 + *Dicer* leads to a decreased percentage of normal embryos as compared to each MASO injection alone. A lower concentration of *Drosha* and *Dicer* MASO at 16 nM instead of 24 nM (lanes 2 and 3) was used so that we can observe the further decrease in the percentage of normal embryos in MASO co-injections. An average of two biological replicates is presented.

## Discussion

Species-specific differences in the temporal expression and functional roles of miRNAs in development likely contribute to variations in the life histories of specific organisms. Given that posttranscriptional regulation throughout development has only been studied for a limited number of organisms, a more extensive investigation of the function of miRNAs is needed to achieve a fuller understanding of this aspect of evolution. We thus set out to investigate the role of miRNAs in early embryogenesis of the sea urchin *S. purpuratus*. We first confidently annotated a total of 49 miRNAs from the early stages of sea urchin

development and profiled their expression throughout early embryogenesis. The comparatively small number of identified miRNAs may correspond to morphological complexity (Campo-Paysaa et al., 2011; Peterson et al., 2009; Wheeler et al., 2009), or, alternatively, adult sea urchin tissues may harbor more miRNAs than we have detected in the embryonic stages (Campo-Paysaa et al., 2011; Christodoulou et al., 2010; Heimberg et al., 2008; Lee et al., 2007; Niwa and Slack, 2007; Sempere et al., 2006; Wheeler et al., 2009). Most of the annotated sea urchin miRNAs are maternally present and are dynamically expressed during the first 24 h (blastula) to 48 h (gastrula) of development (Fig. 1).



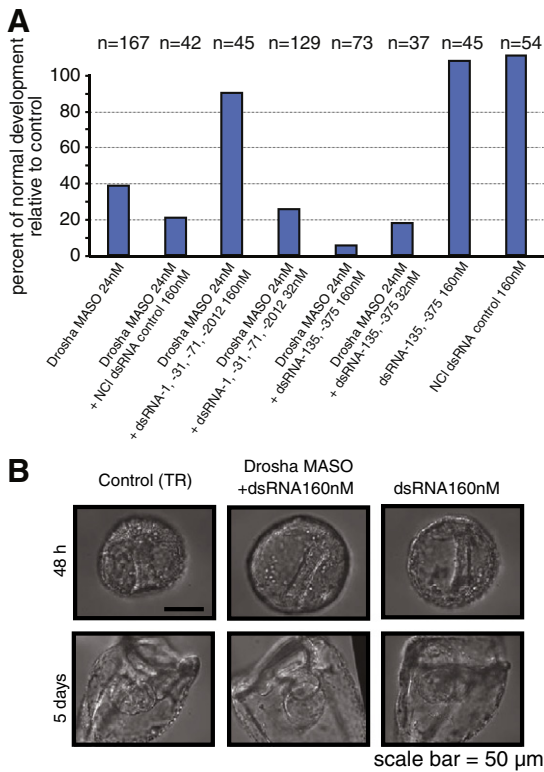
**Fig. 5.** miRNAs regulate broad gene sets. Embryos at 48 hpf were immunolabeled with Endo1 (A) and Meso1 (B) monoclonal antibodies to identify cell-type specific differentiation markers of endoderm and mesoderm, respectively. Scale bar is 20  $\mu$ m. (C) RT-QPCR measurement of mRNAs levels of molecules that mediate transcriptional regulation, cell signaling, cell adhesion, cell movement, and cell proliferation. QPCR results from 2 to 3 and from 3 to 6 independent biological experiments are measured in 17 h and 24 h embryos, respectively.

In *C. elegans*, about 60% of the total miRNAs are expressed in the zygote, which are presumably maternally deposited, and the greatest changes in miRNA dynamics occur around the time of gastrulation (Stoeckius et al., 2009). The majority of the zebrafish miRNAs are also expressed after gastrulation during the segmentation stage (10 to 24 hpf) in a tissue-specific manner (Wienholds et al., 2005). In Zebrafish miRNAs may not be essential for very early fate decisions but rather are thought to function in patterning and maintenance of tissue identity (Wienholds and Plasterk, 2005; Wienholds et al., 2005). Our data suggest that miRNAs play a central role in early cell fate decisions in the sea urchin embryo. This hypothesis is supported by our observed early embryonic lethal phenotypes upon inhibition of de novo synthesis of Droscha and Dicer in the sea urchin embryo. In zebrafish mutants where both maternal and zygotic Dicer is knocked out, the embryos displayed defects during gastrulation, resulting in embryos with defective brain morphogenesis and heart development (Giraldez et al., 2005). A partial rescue of the brain developmental defect is achieved with the addition of the most abundant zebrafish miRNAs, the miR-430 family (Giraldez et al., 2005). The miR-430 family is the earliest expressed miRNA, expressed from the onset of embryonic transcription (5 hpf), and has been shown to function in clearing maternal mRNA pools in the early embryo (Giraldez et al., 2005). Similar roles of specific miRNAs in the maternal-to-zygotic transition have been observed in *Drosophila melanogaster* (Bushati et al., 2008) and *Xenopus* (Lund et al., 2009). Our Droscha knockdown rescue experiments with combinations of 4 miRNAs suggest that the maternally expressed miR-71 may be particularly important for developmental processes prior to blastulation (Fig. 7 and Table S2). Moreover, the rescue with these miRNAs is more complete than shown in zebrafish with miR-430 (Giraldez et al., 2005). Previous studies demonstrate that mouse embryonic stem cells deficient in Dicer are viable, but fail to differentiate in vitro and in vivo, thus indicating that miRNAs are involved in the establishment of differentiated cell states. Therefore, establishment and

maintenance of a differentiated cell state may be a conserved function for miRNAs in the deuterostome lineage (Kanellopoulou et al., 2005).

The best computational tools for predicting miRNA target sequences scan mRNA 3'UTRs for short motifs complementary to nucleotides 2 to 7 (or 8) of the miRNA (Krek et al., 2005; Lewis et al., 2005). For confident target predictions, these tools incorporate sequence conservation of relatively closely related species in the search, which is unfortunately not available for the *S. purpuratus* genome. Nevertheless, we scanned the available 3'UTRs in the sea urchin transcriptome (Samanta et al., 2006) for sequences complementary to nucleotides 2 to 8 of the miRNA seed sequences within regulatory genes active during early embryogenesis (Fig. 5C and Table S5). Further experimental testing will be needed to test the direct regulation of specific miRNAs and their potential gene targets. It will be interesting to conduct the bioinformatic analysis once well-annotated 3'UTR sequences become available for a number of sea urchin sister species.

Given both the identification of dynamically expressed embryonic miRNAs and the functional requirement of proteins involved in miRNA biogenesis, our data indicate that post-transcriptional regulation by miRNAs is essential for developmental mechanisms early in the sea urchin embryo. Our results are consistent with a previous report in a different sea urchin species, *Hemicentrotus pulcherrimus*, which exhibited a dose-dependent severity of developmental defects with Dicer knockdown (Okamitsu et al., 2010). In silico and in vitro experiments have shown that a single miRNA can regulate many hundreds of targets at various levels of efficacy (Baek et al., 2008; Krek et al., 2005; Lewis et al., 2005; Selbach et al., 2008). Thus it is possible that certain miRNAs dominate the regulatory landscape in development. This conclusion is supported by the rescue experiments in which abnormal morphology associated with miRNA reduction can be obviated by supplying the four most abundant miRNAs (*spu*-miR-1, -31, -2012, and -71) to the early embryo and that miR-71 alone may be controlling key nodes in early developmental processes prior to the blastula stage. This result suggests that these miRNAs may



**Fig. 6.** Four miRNAs rescue developmental defects from Drosha loss of function. Embryos were injected with Drosha MASO (24 nM) with or without exogenous dsRNAs. Bar-plots depicting the percentages of normal embryos in the mock injected control at 48 hpf. (A) *Drosha* knockdown embryos (24 nM) were complemented with 160 nM or 32 nM of dsRNA duplexes, miR-1, -31, -2012, -71, -153, and -375, or a dsRNA negative control (NCI). Injections of dsRNA-1, -31, -2012, or -71 alone did not cause developmental defects (data not shown). *n* is the total number of injected embryos. (B) Embryos with Drosha knockdown (24 nM) complemented with dsRNA duplexes (160 nM) developed into normal larvae.

be important regulators within the gene regulatory network that directs early developmental signaling pathways that are essential for proper early embryogenesis.

## Conclusion

Our results support the hypothesis that miRNAs are essential for early embryogenesis. This study suggests for the first time that post-transcriptional regulation by miRNAs is an integral part of the gene

regulatory network that contributes to cell fate decisions and specification in the sea urchin embryo. A detailed examination of the functional roles of the 49 miRNAs in the sea urchin embryo will likely yield insight into the ancient and conserved function of miRNAs in early development throughout the deuterostome taxa. The comparably small total number of miRNAs and the lack of redundancy of multiple miRNA families in the sea urchin make it an attractive model to examine the functions of single miRNAs during embryogenesis (Fig. 1, Table S1 and Campo-Paysaa et al., 2011; Wheeler et al., 2009). In addition, the transcriptional network that directs sea urchin development is well understood (Davidson et al., 2002; Oliveri and Davidson, 2004) and thus will provide a strong framework for interpreting the functions of single miRNAs.

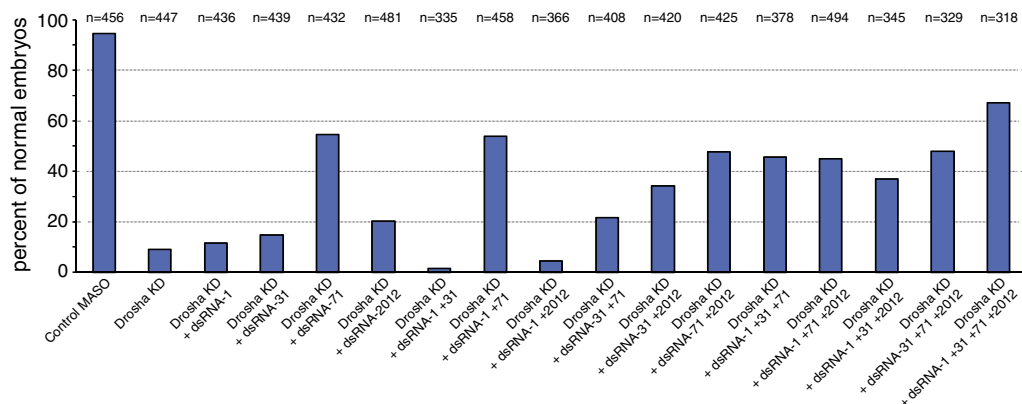
## Materials and methods

### Deep sequencing of small RNAs

Total RNA was extracted from the ovary, eggs, 32-cell stage (5 hpf), blastula (24 hpf), gastrula (48 hpf), and pluteus (72 hpf) stages with TriZol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with the following modifications: samples were initially extracted with chloroform several times until the aqueous phases were free of visible proteins and all RNAs were precipitated overnight at  $-20^{\circ}\text{C}$ . Small RNA were size selected (18–40 nt) on a 6% Urea-polyacrylamide gel. Sequencing libraries for each sample were prepared with a 5' monophosphate dependent cloning protocol from Illumina (DGE small RNA; Illumina Inc., CA) and sequenced on the Genome Analyzer 2 (Illumina). We obtained a total of 3.7 million sequence reads per sample. miRNAs are annotated by using miRDeep2 (Friedländer et al., 2008; Friedländer et al., 2011) against the miRBase version 17.

### Computational analysis of sequenced small RNA libraries

The six sequenced small RNA libraries were clipped and mapped in parallel using the Mapper module of miRDeep2 (Friedländer et al., 2011). Specifically, reads were parsed to fasta format and 3' adapters clipped by searching for perfect matches to the first six nucleotides of the adapter sequence, starting at position 18 in each read. If no matches to the first six nucleotides (nts) of the adaptor were found in a given read, then matches of the first five nts to the last five nts of the read were identified, then matches of the first four nts to the last four nts of the read and etc. We split the clipped reads by annotation by mapping them to reference databases, using an annotation hierarchy (Berninger et al., 2008): miRNA > mRNA > tRNA > rRNA > unknown. To identify miRNA reads, we mapped to



**Fig. 7.** miR-71 rescues significant Drosha knockdown phenotype. Single and combinations of dsRNAs corresponding to miR-1, -31, -71, and -2012 Dicer substrates were coinjected with the Drosha MASO into newly fertilized eggs. The percentages of normal and abnormal embryos (excluding dead embryos) are tabulated at 24 hpf. Two independent biological experiments were conducted.

the *S. purpuratus* miRNA precursors from miRBase version 16 and to three novel precursors (Friedländer et al., 2011). To identify mRNA reads, we mapped to the gene\_cds (coding) sequences from SpBase (<http://sugp.caltech.edu/SpBase/download/>). To identify tRNA reads we analyzed the *Spur\_v2.1* genome with tRNAscan-SE-1.23 using default eukaryotic parameters and mapped to the predicted tRNA sequences. To identify rRNA reads we mapped to the *S. purpuratus* 18S and 28S rRNA sequences obtained at GenBank (Benson et al., 2004). Last, all reads that mapped to the *Spur\_v2.1* genome but did not map to any of the above annotations were labeled 'unknown'. Reads were mapped with Bowtie (Langmead et al., 2009) with these options: -f -n 1 -e 80 -l 18 -a -best -strata.

Using this stringent procedure we successfully mapped between 43% and 55% of the clipped reads in each of the six datasets, corresponding to between 1.7 and 4.4 million reads. Even though some reads may not have been mapped because of the incomplete state of the genome assembly, these numbers were comparable to previous small RNA studies (e.g. Mayr and Bartel, 2009; Persson et al., 2009), showing the consistent high quality of the data. We annotated piRNAs in the following way: all reads that mapped to the genome but did not map to existing annotations ('unknown' reads) were pooled across samples. Using a custom ruby script we identified all instances where two of these reads overlap with each other such that they are on opposite genomic strands and their 5' ends overlap by exactly ten nucleotides. Since this overlap is in perfect accordance with the 'ping-pong' model of piRNA biogenesis, we annotated all such read pairs as piRNAs.

#### Microinjection approaches

Morpholino antisense oligonucleotides (MASO) against *dicer*, *droscha*, and *dgrc8* were ordered from GeneTools (Philomath, OR). The MASO sequences for *dgrc8* is 5' ACACGGTATGGCAGCCACTGGAACA 3'; for *droscha* is 5' TACCGGATCATTGCTACACGTACA 3', *dicer* 5' GGACTC-GATGGTGGCTCATCCATTC3' (used for the experiments presented here) and *Dicer*5UTR 5' GTACCAGAACTCTGAAAGATAGCAA3' (demonstrated the same phenotype as the first, data not shown). Standard control morpholino (5'CCTCTTACCTCAGTTACAATTATA3') was purchased from GeneTools (Philomath, OR). Microinjections were performed as previously described (Cheers and Etensohn, 2004) with modifications. MASO oligos were resuspended in sterile water and heated to 60 °C for 10 min prior to use. Injection solutions contain 20% sterile glycerol, 2 mg/ml 10,000 MW Texas Red lysine charged dextran (Molecular Probes, Carlsbad, CA) and varying concentrations of specific MASOs. Eggs from *S. purpuratus* were collected by injecting the animal with 0.5 M KCl to induce spawning. Eggs were dejellied in acidic sea water (pH 5.2) for 10 min on ice, followed by sea water washes. Dejellied eggs were rowed onto protamine sulfate-coated (4% w/v) 60 × 15 mm petri dishes. Eggs were fertilized with sperm in the presence of 1 mM 3-amino-triazol (Sigma, St. Louis, MO). Injections were performed using the Femto Jet injection system (Eppendorf; Hamberg, Germany). Injection needles 1x90mm glass capillaries with filaments (Narashige; Tokyo, Japan) were pulled on a vertical needle puller PL-10 (Narishige). The injection solution is calculated based on the injection bolus at about 1/5 of the egg diameter. The volume of the injection bolus was calculated and used to determine the μmoles of injected morpholino. The final concentration of the injected morpholino was determined by dividing μmol of injected morpholino with the volume of the egg calculated with a radius of 40 μm.

Droscha/DGCR8/Dicer knockdown abnormal phenotypes are defined either as embryos that were delayed in development, lacked gastrulation (limited or no gut formation), or had vacuolarization in the blastocoel. *Dicer*, *droscha*, and *dgrc8* MASO-injected embryos were imaged on a Zeiss AxioPlan microscope (Carl Zeiss Incorporation, Thorwood, NY) with an Orca-ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ).

#### Real time, quantitative PCR of miRNAs

Taqman miRNA primers against *spu*-miR-2009 and *spu*-miR-31 were used in real time, quantitative PCR. Total RNA was prepared from 120 *dicer* or *droscha* MASO-injected embryos 24 hpf using the miRvana miRNA isolation kit (Applied Biosystems, Foster City) to isolate small RNAs according to the manufacturer's instructions (Fig. 3C). Total RNA was resuspended in 50 μl of nuclease-free water. cDNA was prepared from 5 μl (20 ng) of the total RNA using the Taqman RT kit (Applied Biosystems, Foster City). Reverse transcription was conducted according to the TaqMan MicroRNA RT kit, with each reaction tube containing one single custom designed RT primer. QPCR was conducted according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster City). The Ct values of the *Dicer* MASO-injected embryos were normalized to the mock injected embryos as  $2^{-\Delta Ct}$  values. Two separate experiments were conducted with 4 replicates each.

A multiplex reverse transcription step was performed to detect *spu*-miR-2009, *spu*-miR-31, *spu*-miR34, *spu*-miR251, and ubiquitin expression levels in the egg, 32-cell stage, 24 h, 48 h, and 72 h embryos (Fig. S1). Total and small RNA populations were isolated from 300 eggs or embryos using the miRvana miRNA isolation kit (Applied Biosystems, Foster City). Each 20 μl reverse transcription reaction consists of 4 μl of the 5 × Taqman RT primer, 1 μl of oligo dT (500 μg/ml stock), 45 ng of small RNA and 45 ng of total RNA, 1 μl of dNTPs (100 mM each), 2 μl of MultiScribe Reverse Transcriptase (50 U/μl), 2 μl of 10 × RT Buffer, and 0.25 μl RNase Inhibitor. The QPCR reaction contains 1 μl of cDNA, 0.5 μl of 20 × TaqMan MicroRNA Assay, 5 μl of 2 × Universal Master Mix in a 10 μl total reaction. QPCR was conducted using the 7500 Real-Time FAST PCR cyclor system (Applied Biosystems, Foster City). Data are normalized to the internal control ubiquitin. Data are presented as fold changes of the egg sample.

#### Real time, quantitative PCR of mRNA

100 embryos at various time points were collected and total RNA were extracted using the Qiagen microRNeasy kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). cDNA was amplified using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City). QPCR was performed using the 7300 Real-Time PCR cyclor system (Applied Biosystems, Foster City). Two embryo equivalents were used for each QPCR reaction with the SYBER Green PCR Master Mix (Invitrogen). QPCR primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) (Table S3). Results were first normalized to ubiquitin levels and expressed as a fold difference compared to the uninjected embryos. 2–6 independent biological experiments with 3 replicates each were conducted.

#### Double stranded miRNA rescue duplexes

Synthetic double stranded RNA duplexes (Integrated DNA Technologies, Inc., Coralville, IA) were designed against the four most abundant miRNAs obtained from the deep sequencing data: *spu*-miR-1, *spu*-miR-71, *spu*-miR-31, and *spu*-miR-2012. *Dicer* substrate negative dsRNA control (DS NC1) was purchased from IDT (Integrated DNA Technologies, Inc., Coralville, IA). Double stranded RNA duplexes were designed based on the IDT protocol for *Dicer* substrates (idtdna.com). DsRNA duplexes were resuspended in sterile nuclease-free water to make 100 μM stock solutions and stored in aliquots at -20 °C.

#### Northern blotting

Testing of piRNA candidates was performed by Northern blot analysis as described previously (Lagos-Quintana et al., 2001). 70 μg of total RNA was used per sample. Equal loading and transfer were determined with methylene blue. Since selected piRNA candidates



were in low abundance, the imaging plates had to be exposed for up to one week. Pictures were obtained with an imaging plate reader and processed in Adobe Illustrator.

#### Immunological procedures

Dicer knockdown embryos were fixed in either 1% paraformaldehyde for 10 min at room temperature, followed by 1 min in 100% methanol (for Endo1 staining (Wessel and McClay, 1985) and Dicer) or 90% methanol for 1 h at  $-20^{\circ}\text{C}$  (for Meso1 staining; Wessel and McClay, 1985), followed by 5 PBST washes. Fixed embryos were blocked in 4% goat serum (Sigma, St. Louis, MO) in PBS-Tween for 1 h at room temperature then incubated in polyclonal Dicer or monoclonal Endo1 and Meso1 antibodies overnight at  $4^{\circ}\text{C}$ , washed 3 times in PBS containing 0.06% Tween (PBST), followed by incubation with goat anti-rabbit Alexa Fluor 488 conjugated antibody (Invitrogen) at 1:300 or goat anti mouse Cy3 conjugated antibody (Invitrogen) at 1:300 for 1 h at room temperature in blocking buffer. The embryos were washed 3 times with PBST and incubated with Hoechst (Molecular Probes; Carlsbad, CA) (10 mg/ml stock) at 1:1000 dilution for DNA labeling. Immunolabeled embryos were imaged on an LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY).

#### Dicer antibody generation and purification

Antiserum was raised in rabbits against the middle (amino acids GSQSQF to VIDTWD) and the carboxyl end (amino acid KQPAPA to KSQPKK) of the *S. purpuratus* Dicer fused to a 6XHIS tag using the pTAT vector (a generous gift of Steven F. Dowdy). Cell extracts were prepared as described previously with the following modifications (Leguia et al., 2006). The fusion constructs were transformed into BL21 cells for overexpression. Clones overexpressing the fusion proteins were identified by SDS-PAGE and immunoblot analysis of protein extracts using anti-HIS monoclonal antibodies diluted 1:3000. BL21 clones containing Dicer constructs were grown overnight in 5 ml cultures. Large scale cultures for protein purification was prepared with 5 ml of overnight culture diluted in 1 L of LB broth and 100  $\mu\text{g}/\text{ml}$  ampicillin at  $30^{\circ}\text{C}$  for 1 h, followed by addition of 1 mM IPTG for 3 h. Bacterial pellets were resuspended in Buffer Z (8 M urea, 100 mM NaCl, and 20 mM Hepes pH 8) containing 20 mM imidazole, lysed by sonication prior to purification on a ProBond Ni-NTA agarose column (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Lysates were dialyzed in water overnight and lyophilized. 0.5 mg of immunogen with Freund's adjuvant was injected in three booster shots into New Zealand white rabbits.

The Dicer antibody was affinity purified. Dicer protein eluted from Buffer Z containing 500 mM imidazole was concentrated with centricon column according to manufacturer's instructions (Millipore, Billerica, MA), followed by immobilization of Dicer protein to the Pierce Aminolink Plus Immobilization Kit (ThermoFisher Scientific; Rockford, IL). Rabbit serum containing Dicer antibodies was purified and eluted with Tris buffer, pH 9.5 and 1 ml of 100 mM Glycine, pH 2.5. 10  $\mu\text{g}$  of affinity purified antibody from the Dicer middle region recombinant protein was used for immunocytochemistry and western blotting.

#### Western blot

120 embryos were prepared by resuspending pelleted embryos in heated SDS sample loading buffer and boiled at  $100^{\circ}\text{C}$  with 1 mM DTT (Roche) for 10 min. Samples were stored at  $-80^{\circ}\text{C}$  if not run on the SDS-PAGE gel immediately. Samples were loaded onto Tris-Glycine 4–16% or 4–20% gradient gels (Invitrogen) and transferred to nitrocellulose (Pall Corporation, Pensacola, FL). Western blots were probed with 10  $\mu\text{g}$  of affinity purified Dicer antibody in blotto

(3% dry milk, 170 mM NaCl, 50 mM Tris, 0.05% Tween20) overnight at  $4^{\circ}\text{C}$ , followed by incubation with secondary antibody goat anti-rabbit HRP (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1:5000 in blotto for 1 h at room temperature. Signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific; Rockford, IL) according to the manufacturer's instructions. Quantitation of Dicer protein knockdown (Fig. S5) was performed by quantifying and normalizing the intensity of the bands of equal numbers of embryos in the Dicer MASO-injected embryos with the control. The pixel intensities were within the linear range of detection as determined by the Metamorph software v. 7.6 (Molecular Devices, Inc., Sunnyvale, CA).

#### Sequence raw data repository

Sequences are deposited in the GEO (gene expression omnibus) and SRA (short reads archive).

Supplementary materials related to this article can be found online at doi: [10.1016/j.ydbio.2011.11.015](https://doi.org/10.1016/j.ydbio.2011.11.015).

#### Competing interests statement

The authors declare no competing financial interests.

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