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miR-184 Has Multiple Roles in *Drosophila* Female Germline Development

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SUMMARY

Posttranscriptional regulation plays a crucial role in germline and early embryonic development, but the underlying mechanisms are only partially understood. Here we report the genetic and molecular analysis of the maternally and zygotically expressed microRNA *miR-184* in *Drosophila*. Loss of *miR-184* leads to multiple severe defects during oogenesis and early embryogenesis, culminating in the complete loss of egg production. Using both in vitro and in vivo assays, we characterize the relevant *miR-184* targets and target sites for three of the observed phenotypes. *miR-184* controls germline stem cell differentiation by tuning the DPP receptor Saxophone, dorsoventral patterning of the egg shell by regulating the *gurken* transport factor K10, and anteroposterior patterning of the blastoderm by tuning the transcriptional repressor Tramtrack69. Our study highlights the importance of microRNA-mediated regulation in the major developmental transitions of the female germline, and provides insights into several aspects of microRNA function.

INTRODUCTION

The regulation of gene expression during early development is very complex. In nonplacental organisms, the mother initiates and controls much of this process by placing mRNA transcripts in well-defined concentrations and locations within the developing egg. In many instances, these maternal "determinants" serve as morphogens—their absolute and relative concentrations are therefore crucial and under elaborate regulation, which includes mechanisms for transporting and localizing transcripts and tight control of their translation (St Johnston and Nusslein-Volhard, 1992; Ephrussi and St Johnston, 2004). During the midblastula transition, many of the maternal messages are destroyed, and zygotic expression takes over to mediate embryonic pattern formation and subsequent development (Bashirullah et al., 1999). Our mechanistic understanding of this early posttranscriptional regulation of maternally provided transcripts is still fragmentary, partly due to the difficulties in studying RNA-protein interactions and their lack of sequence specificity (Johnstone and Lasko, 2001).

Genomically encoded microRNAs (miRNAs) represent a new layer of posttranscriptional gene regulation that might play an important role in this context. miRNAs bind to specific sequences within the 3′UTRs of mRNAs, leading to degradation of the targeted mRNA or inhibition of protein synthesis (for a recent review, see Filipowicz et al., 2008). The nature and extent of their role in biological processes are still being debated, but both studies in which miRNA function is abolished wholesale by disrupting their biogenesis and analyses of individual miRNA genes reveal a strong requirement in the control of stem cell fate (Hatfield et al., 2005; Yi et al., 2008) and in early embryonic development, with higher fishes providing an apparent exception (Bernstein et al., 2003; Giraldez et al., 2005).

In *Drosophila*, the role of miRNAs in regulating stem cell behavior in the ovaries has been investigated by mosaic analysis of mutants that abrogate miRNA biogenesis. Presumably due to the perdurance of mature miRNAs, mutant clones show age-dependent phenotypes: after 12 days, the number of developing egg chambers is significantly depleted due to reduced division of germline stem cells (Hatfield et al., 2005); longer-term studies show a gradual loss of both germline and somatic stem cells; in both cases, the underlying causes are unclear (Jin and Xie, 2007). Forty-three miRNAs are expressed in the *Drosophila* germline (Neumüller et al., 2008), but none of their functions have been described.

Here we report the genomic knockout of the highly conserved miRNA *mir-184*, which is expressed in the female germline and has assumed control over multiple steps in oogenesis and early embryogenesis in *Drosophila*. We observe a range of phenotypes of varying penetrance, identify several of the responsible targets, and show that their protein levels are tuned by *miR-184* in vivo. Our results support the notion that an individual miRNA can exert phenotypically relevant control over multiple biological processes, and provide insight into the molecular mechanisms of miRNA-mediated regulation in female germline development.

RESULTS

Molecular and Genetic Characterization of the *mir-184* Locus

miR-184 was originally identified by expression cloning from the small RNA fraction of *Drosophila* embryos, but is conserved from insects to humans (Aravin et al., 2003). Northern analysis shows expression of *miR-184* throughout the life cycle, with a relatively

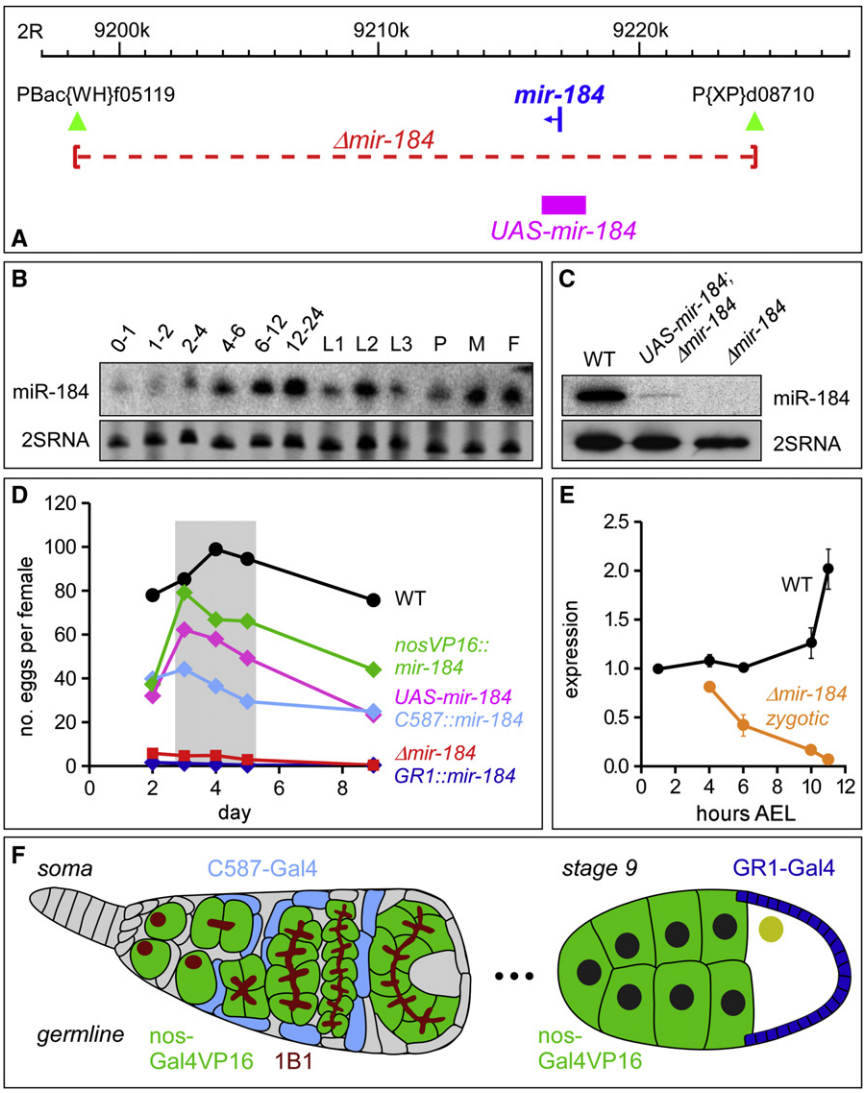

Figure 1. The *mir-184* Locus—Organization, Expression, and Rescue

(A) Organization of the *mir-184* locus, indicating the genomic coordinates of the mature miRNA, the *Δmir-184* deletion, and the *UAS-mir-184* construct.

(B and C) Northern analysis of *miR-184*, throughout the life cycle in wild-type (B) and in ovaries of wild-type and *Δmir-184* mutants with and without one copy of the *UAS-mir-184* transgene (C).

(D) Female egg laying in *Δmir-184* mutants and rescue achieved by expression of *UAS-mir-184* using different ovarian *Gal4* drivers, whose expression domains are depicted in F. Data represent averages from three to six independent experiments using four flies per chamber; with the exception of *Δmir-184* versus *GR1::mir-184*, all groups are significantly different from all others ($p < 0.01$), based on one-way ANOVA of average egg production from days 3–5 (gray box) with Student-Newman-Keuls post hoc test. For control experiments using the *Gal4* lines alone in wild-type and in the *Δmir-184* mutant background, see Figure S2.

(E) Expression level of mature *miR-184* in wild-type and in *Δmir-184* zygotic mutant embryos, as determined by qPCR; data represent averages ± SEM from two to four independent replicates. Note the persistence and slow decay of miRNA levels into midembryogenesis.

(F) Schematic depiction of the germarium and early oogenesis, with expression patterns of the different *Gal4* drivers used for the rescue of the *Δmir-184* egg production phenotype in D.

weak maternal contribution but strong subsequent zygotic expression (Figure 1B) (Aravin et al., 2003; Leaman et al., 2005); notably, we find strong expression in ovaries (Figure 1C). RNA in situ hybridization using the primary transcript as probe shows strong expression in a highly dynamic pattern throughout embryogenesis (see Figure S1 available online). *miR-184* is also one of the few miRNAs that are expressed in Schneider (S2) cells in significant copy number (Leaman et al., 2005; Kertesz et al., 2007).

mir-184 is a single copy gene and lies isolated within a 50 kb region on the right arm of the second chromosome (50A; 9217K) (Figure 1A). The genomic region is rich in extant P element insertions, including several FRT site-containing elements (PBac{WH}, P-element{XP}; Exelixis Collection), which we used to generate an FLP-induced deletion of 22 kb between the elements PBac{WH}f05119 and P{XP}d08710, following established procedures (Parks et al., 2004). Multiple recombination/deletion events were collected and confirmed by genomic PCR and sequence analysis. In order to be able to carry out rescue and misexpression experiments, we generated a *UAS-mir-184* strain, which contains 1.5 kb of genomic sequence surrounding the *mir-184* gene (1 kb upstream, 0.5 kb downstream) (Figure 1A).

Δmir-184 zygotic mutant flies eclose at a normal Mendelian ratio and appear morphologically normal, indicating that loss of zygotic expression has no detectable effect on adult viability and no obvious effect on development and overall morphology, which is surprising given the strong and complex expression of the *mir-184* transcript throughout embryogenesis. Among adults homozygous for *Δmir-184*, male fertility is normal; however, females lay far fewer eggs than in wild-type, and the eggs and embryos that are produced show severe abnormalities. Strikingly, the defects become progressively worse over time: young (2- to 3-day-old) *Δmir-184* females lay 5–10 eggs per day, which represents <10% of wild-type production (Figure 1D). Approximately 70% of the eggs have normal (external) morphology and are fertilized; however, most of these embryos (85%) show severe defects in anteroposterior patterning, and many also show severe defects during cellularization; only about 1% of all progeny develop to adulthood. As the females age, egg production declines further and the number of eggs with an abnormal external morphology increases. Eggs from 3- to 4-day-old females are typically smaller than wild-type, and many show defects in dorsoventral patterning of the egg shell, as judged by the position and length of the dorsal

appendages. *Δmir-184* females that are 5 days or older lay almost no eggs (Figure 1D). Thus, progressive failure of egg production is the prevalent phenotype in the *Δmir-184* mutant and supersedes all others within a week. However, its incomplete or delayed penetrance makes it possible to observe a range of distinct other defects as well, indicating that *miR-184* function is required for multiple successive steps of oogenesis and early embryogenesis.

The observed phenotypes point to a requirement for *miR-184* in either the female germline itself or in the somatic cells of the ovary. RNA in situ hybridization in ovaries is often difficult, and we were not able to obtain consistent interpretable results when attempting to detect the *miR-184* primary transcript. To determine where the requirement lies, we therefore decided to express the *UAS-mir-184* transgene in different cell populations of the ovary using established *Gal4* drivers and examine under which conditions the sterility phenotype can be rescued, mindful of the possibility that ectopic or even overexpression might lead to phenotypic defects by itself. *nos-Gal4VP16* (Van Doren et al., 1998) drives expression in the germline cells, *C587-Gal4* (Zhu and Xie, 2003) in most somatic cells of the ovary excluding the cap cells, and *GR1-Gal4* (gift from T. Schupbach) drives expression in the follicle cells that envelope the oocyte and produce the egg shell (Figure 1F); these drivers provide no rescue ability on their own (Figure S2). We find that expression of *mir-184* in the germline (*nos-Gal4VP16*) strongly rescues the sterility of *Δmir-184* females: egg production approaches wild-type levels, and almost all eggs and embryos appear morphologically normal (Figures 1D and S3). This indicates that *miR-184* is required in the germline, which is consistent with the fact that expression of mature *miR-184* is detected in northerns of freshly laid eggs/embryos, that is, prior to the onset of zygotic transcription (Figure 1B). Notably, we also observe substantial rescue of egg production, although not egg morphology, by simply introducing *UAS-mir-184* into the *Δmir-184* background. This suggests that, due to the inclusion of 1 kb upstream sequence, the *UAS-mir-184* transgene on its own drives moderate expression in the germline. Northern analysis of ovaries from *Δmir-184* females that carry the *UAS-mir-184* transgene indeed reveals weak expression of mature *miR-184*, at about 10% of the level observed in wild-type (Figure 1C), indicating that the 1 kb upstream sequence included in the UAS construct contains at least part of a germline promoter. Expression of *mir-184* in the somatic cell populations of the ovary leads to different results: driving expression using *C587-Gal4* has no effect beyond that of *UAS-mir-184* alone, whereas driving expression in the follicle cells (*GR1-Gal4*) leads to severe sterility, suggesting that ectopic or overexpression of *mir-184* in follicle cells is in itself detrimental to oogenesis (Figures 1D and S2).

Given the lack of obvious developmental defects as a result of the removal of zygotic *miR-184* alone, we sought to examine the perdurance of maternally provided *miR-184*. We quantified the amount of mature *miR-184* at different time points of development (see Experimental Procedures) and found that in the *Δmir-184* zygotic mutant the mature miRNA is still present at close to the initial (1 hr) level after 4 hr and then declines, reaching about 20% of initial levels at 10 hr (Figure 1E). This suggests stage-dependent turnover of *miR-184* and a half-life of ~3 hr in midembryogenesis. Given the capacity of low amounts of *miR-184* to provide biological function (see above), it is thus very possible that the loss of the zygotic transcript is (partially) rescued by the maternal component, as is the case for many other genes with a maternal contribution.

To gain more specific insight into the biological role of *miR-184*, we investigated three of the observed phenotypes in greater detail: the defect in anteroposterior patterning in early embryos, the defect in dorsal-ventral patterning of the egg shell, and the loss of egg production itself. For all analyses, we crossed zygotic *Δmir-184* females and males inter se; thus, the resulting offspring are both maternal and zygotic nulls. Because sterility worsens with increasing age, we carefully staged females and examined their ovaries and progeny with different sets of markers appropriate for the developmental stage under observation. We then searched the computational miRNA target predictions, both our own (PITA; Kertesz et al., 2007) and those of others (Pictar; Grun et al., 2005; Stark et al., 2005) for candidates that might be responsible for the observed defects. Our target prediction algorithm PITA (Kertesz et al., 2007) takes into consideration mRNA secondary structure and accessibility of the target sites, without employing evolutionary conservation filters; potential targets are scored by computing the difference ($\Delta\Delta G$) between the free energy gained by formation of the miRNA-mRNA duplex (ΔG_{duplex}) and the energetic cost of unpairing the target site to make it accessible to the miRNA (ΔG_{open}). Twenty-four candidate sites were first tested in vitro using an assay for translation efficacy that we recently developed; the assay uses transfection of a dual luciferase reporter into S2 cells, which naturally express *mir-184* at significant levels, and is highly sensitive, quantitative, linear, and reproducible (Kertesz et al., 2007) (Figures 2B and S4). We tested ~200 bp 3′UTR fragments centered around the putative site; corresponding 3′UTR sequences in which the site was deleted served as control (see Experimental Procedures). To examine whether the candidates function as targets of *miR-184* in vivo, we then measured the respective protein levels either in situ or using quantitative western analysis, and tested for genetic interaction with *mir-184*.

mir-184 Is Required for Normal Anteroposterior Patterning and Cellularization of the Embryo

Approximately 85% of embryos from morphologically normal eggs laid by 2- to 3-day-old *Δmir-184* females show severe defects in anteroposterior patterning. When examining the timing and pattern of expression of the early zygotic segmentation genes (St Johnston and Nusslein-Volhard, 1992; Rivera-Pomar and Jackle, 1996) in *Δmir-184* embryos, we find that the gap genes (*hunchback*, *giant*, *Kruppel*, *knirps*, and *tailless*) appear normal, but the expression of pair rule genes is severely affected: the onset and development of the pattern is delayed (*fushi tarazu* [*ftz*] and *odd-skipped odd*]) (Figures 3A–3F and 3J–3O), or some pattern elements are missing (*runt*) (Figure S5). A similar phenotype has been described for ubiquitous overexpression of the transcriptional repressor Tramtrack69 (TTK69) under heat shock control (Brown and Wu, 1993), suggesting that TTK69 might be the responsible *miR-184* target in this context. TTK69 is required for the proper timing and patterning of pair rule gene expression; it has been shown to bind to the *ftz* promoter (Brown et al., 1991), but whether it also binds to

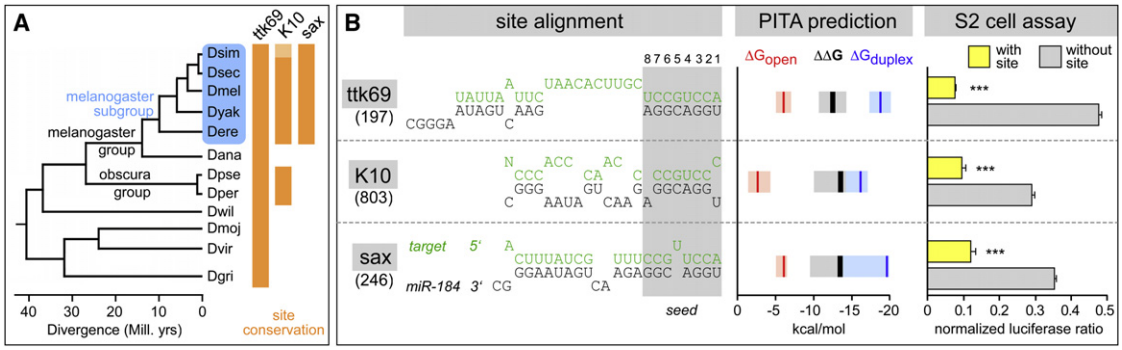

Figure 2. *miR-184* Target Sites in the 3′UTRs of *ttk69*, *K10*, and *sax*

(A) Phylogenetic tree of Drosophilids, with conservation of target site 5′ seeds indicated by orange bars.

(B) Quantitative features of the target sites. Left: alignment of *miR-184* (black) with target mRNA sequences (green); 5′ seed region is highlighted in gray, and parentheses indicate position within the 3′UTR. Middle: PITA predictions for the free energy of duplex formation (ΔG_{duplex}), the energy required to open the target site (ΔG_{open}), and the difference between the two ($\Delta\Delta G$); vertical lines indicate values for *Drosophila melanogaster*, and surrounding light-colored boxes represent average ± 1 standard deviation of values for the other species in which the site is conserved. Right: results for S2 cell dual luciferase assay; shown are average values ± SEM of normalized *Renilla*/firefly ratios obtained from 6–12 replicates for *ttk69*, *K10*, and *sax* 3′UTR sequences centered around the *miR-184* target sites (yellow bars) and for mutated 3′UTR sequences in which the sites are deleted (gray bars). Asterisks indicate the statistical significance of the difference between the wild-type and the mutated 3′UTR sequence, as determined by t test, n = 6–12, ***$p < 0.001$.

other pair rule gene promoters is unknown—for example, the effects on *odd* expression could be indirect, because FTZ itself acts as the key activator of *odd* expression. The 3′UTR of the *ttk69* mRNA contains a good *miR-184* target site at position 197 (Figure 2B); the site is conserved across all Drosophilids (Figure 2A) and is predicted by PITA as well as by Pictar and Stark et al. (2005). In our S2 cell dual luciferase reporter assay, this site confers strong translational repression, in contrast to the control sequence in which the site is deleted (Figure 2B).

To examine whether this repression also occurs in vivo, we compared the TTK69 protein levels of wild-type and *Δmir-184* embryos using quantitative western analysis (see Experimental Procedures). For each genotype, we individually tested ~25 carefully staged embryos (0–1 hr) and found a 2.5-fold average increase of TTK69 protein in *Δmir-184*, supporting the idea that *miR-184* downregulates TTK69 protein levels in vivo (Figures 3S and 3T). We reasoned that if *miR-184* represses TTK69, partial removal of *ttk69* should lead to a suppression of the *Δmir-184* phenotype. To test this, we removed one maternal copy of the *ttk* gene (ttk^{e11}) and examined whether the effects on *ftz* and *odd* expression are ameliorated compared to the *Δmir-184* background. We find that in embryos derived from *Δmir-184; ttk^{e11}/+* females, the expression of both *ftz* and *odd* is indeed less delayed and, at the end of the blastoderm, the patterns show markedly improved resolution of stripes (Figures 3G–3I and 3P–3R). Taken together, our results indicate that maternally provided *miR-184* controls the proper timing of pair rule gene expression in the blastoderm by tuning the expression level of the transcriptional repressor TTK69.

Aside from the defect in anteroposterior patterning, many embryos from young *Δmir-184* females show a very peculiar defect in cellularization that has not been reported in any of the maternal and zygotic loss-of-function screens (Nusslein-Volhard and Wieschaus, 1980; Schupbach and Wieschaus, 1991). In wild-type embryos, zygotic nuclei occupy an ellipsoid field from 80% to 20% egg length by the fifth cleavage, and move to the periphery by the eighth cleavage (Campos-Ortega and Hartenstein, 1985). In *Δmir-184* maternal and zygotic mutants, anterior nuclei fail to move to the anterior tip of the embryo and instead remain at ~80% egg length; although cellularization then occurs, the anterior tip remains devoid of nuclei and merely fills with yolk (Figures 3U–3W). These embryos develop further and undergo gastrulation and germband extension/retraction, but die in midembryogenesis (~stage 13). The fact that this defect occurs only in the anterior—the movement of nuclei to the posterior pole is entirely normal—reveals an underlying asymmetry in the mechanism by which nuclei reach the periphery. One validated *miR-184* target that might be responsible for this effect is the kinesin motor KIF3C (Figure S4).

mir-184 Is Required for Axis Formation of the Egg Chamber

As described above, eggs produced by 3- to 4-day-old *Δmir-184* females are often smaller than wild-type, a phenotype that is indicative of defects in vitellogenesis (oogenesis stage 11) and results from a failure of nurse cells to fully deliver their content to the oocyte (Spradling, 1993). In addition, most eggs (80%) show defects in dorsoventral patterning of the egg shell: nearly all of these are dorsalized, with the dorsal appendages more widely spaced and shortened compared to wild-type (Figures 4A–4C) (Schupbach and Wieschaus, 1991); very occasionally, we find ventralized eggs. To separate the polarity from the size phenotype, we decided to investigate the polarity defects with molecular markers during the previtellogenic stages of oogenesis (stage 9).

The key component in regulating dorsoventral patterning of the egg chamber is the TGF-α homolog Gurken (GRK), which is secreted by the oocyte and activates the EGF receptor in the overlying somatic follicle cells (Nilson and Schupbach, 1999). During mid-to-late oogenesis, after the oocyte nucleus has moved to an anterior-dorsal position, *grk* mRNA and protein strongly accumulate at the anterior-dorsal corner, in a tight cap directly overlying the oocyte nucleus. The precise localization

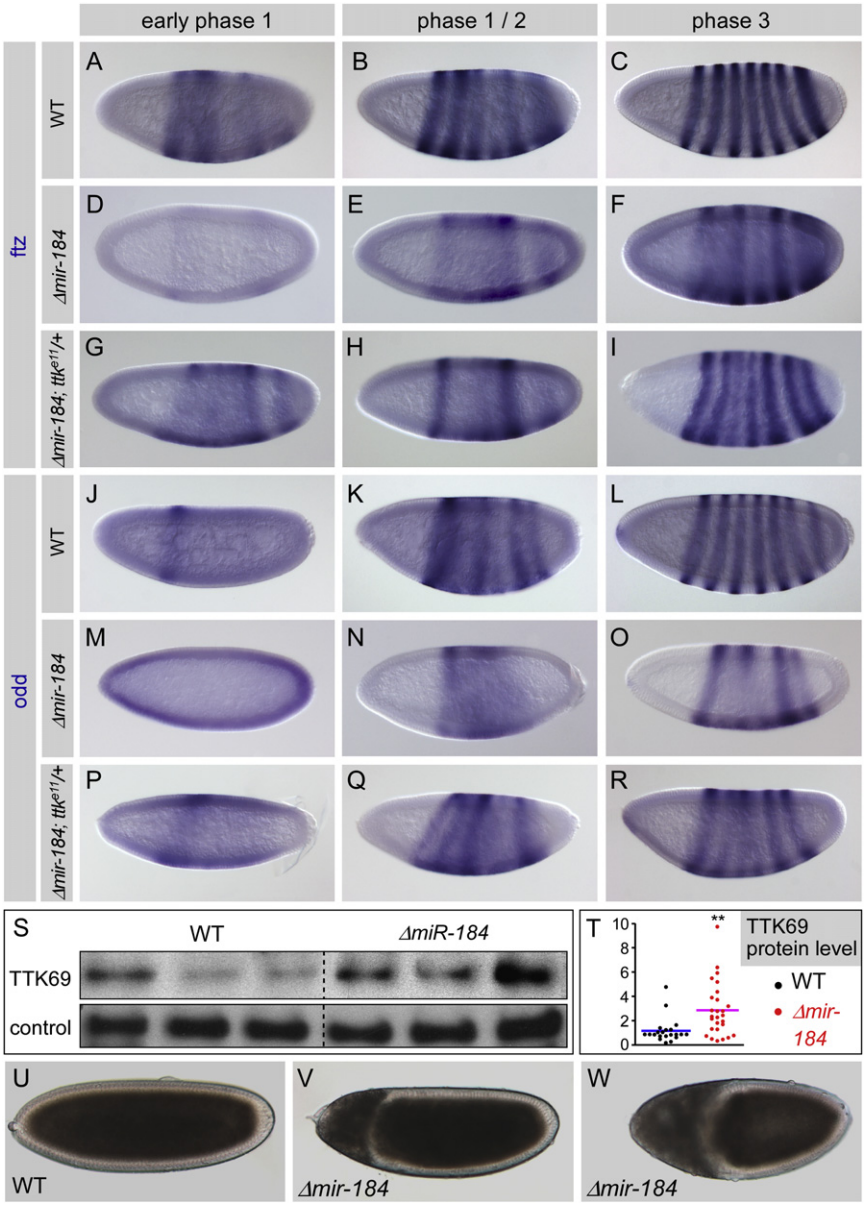

Figure 3. *mir-184* Is Required for Normal Anterior-Posterior Patterning and Cellularization of the Blastoderm

(A–R) Lateral views of carefully staged blastoderm embryos, probed with *ftz* and *odd* antisense RNA; phases 1–3 are defined by progress of cellularization following Lecuit and Wieschaus (2000). Expression and pattern evolution of *ftz* and *odd* are markedly delayed in *Δmir-184* embryos (D–F and M–O) compared to wild-type (A–C and J–K); removal of one copy of *ttk* partially rescues the *Δmir-184* phenotype (G–I and P–R).

(S) Western analysis of individual 0–1 hr embryos probed with TTK69 antibodies. In the *Δmir-184* mutant, the protein level of TTK69 is significantly increased compared to wild-type; a TTK-unrelated band recognized by the antiserum is used as loading control.

(T) Quantitation of western analysis. Dots represent TTK69 protein levels of individual embryos, with values normalized to the wild-type average (y axis). Averages are indicated by horizontal lines; asterisks indicate statistical significance of the difference between wild-type and mutant as determined by t test, n = 22–27, $^{**}p < 0.01$. Note that the *Δmir-184* mutant embryos are taken from the pool of all eggs that are morphologically normal; because the phenotype is not fully penetrant, ~15% these embryos are expected to develop normal anteroposterior patterning.

(U–W) Lateral views of live blastoderm-stage embryos, showing that in *Δmir-184* mutants the anterior portion of the embryo often fails to cellularize.

of *grk* mRNA is a complex process that requires several factors, including Squid, K10, Transportin, Bruno, Cornichon, Capuccino, and Spire, which all act as positive regulators.

In *Δmir-184* mutants, we find the oocyte nucleus in its normal position; GRK protein is present at normal levels, but more spread out along the oocyte cortex compared to wild-type, consistent with the observed (moderate) dorsalization of the egg shell (Figures 4D–4F). *grk* mRNA does not contain any *miR-184* binding sites, and among the known *grk* regulators only *K10* contains an *miR-184* site in its 3′UTR (position 802). This site has a short (6-mer) 5′ seed, but lies in a very accessible region of the 3′UTR (ΔG_{open} −2); notably, the site is conserved only in the *melanogaster* and *pseudoobscura* subgroups of Drosophilids and is not predicted by Pictar and Stark et al. (2005) (Figure 2A). When tested in our S2 cell assay, the *K10* site confers almost as strong repression as the 8-mer seed *ttk69* site, consistent with the fact that the two sites have nearly the same predicted ΔΔG (~12) (Figure 2B).

K10 encodes a nuclear protein (Serano and Cohen, 1995) that, together with Squid (Norvell et al., 1999), is required for the directed nuclear export of *grk* mRNA from the oocyte nucleus to the dorso-anterior corner of the oocyte. In *K10* null mutants, *grk* mRNA and, consequently, GRK protein are spread out over the entire anterior cortex of the oocyte, leading to severe dorsalization of the egg chamber (Nilson and Schupbach, 1999); however, the effects of *partial* loss or *gain* of *K10* function have not been described. Interestingly, *K10* is not transcribed in the oocyte nucleus itself but rather in the nurse cells and is rapidly transported into the oocyte at the beginning of oogenesis (stage 2), where the mRNA eventually localizes to the anterior cortex. K10 protein is then strongly synthesized during stages 8–10 and transported into the oocyte nucleus (Serano and Cohen, 1995). There is thus a substantial time lag of 4 days between the onset of *K10* transcription and the onset of its translation.

In *Δmir-184* mutant ovaries, we find K10 protein expressed early (stage 2) and at much higher levels than in wild-type (Figures 4J–4L), suggesting that *miR-184* is indeed required for the repression of K10 translation in vivo. Strikingly, later on

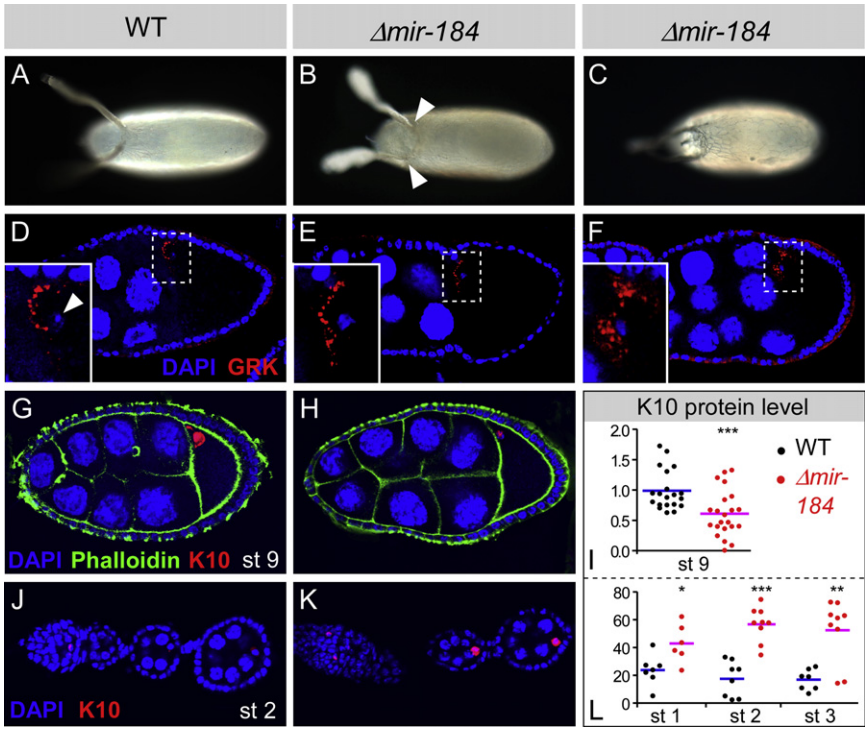

Figure 4. *mir-184* Is Required for Normal Dorsoventral Patterning of the Egg Chamber and Vitellogenesis

(A–C) Dorsal view of live eggs. Most *Δmir-184* eggs show more lateral positioning of the dorsal appendages (B, arrowheads) compared to wild-type (A), but often eggs are also smaller than wild-type (compare C and A).

(D–F) Single confocal sections of stage 9 oocytes in lateral view, labeled with DAPI (blue) and GRK antibodies (red). In wild-type, GRK protein is tightly concentrated in a dorso-anterior cap around the oocyte nucleus (arrowhead in D); in *Δmir-184* mutants, GRK protein is often smeared out along the anterior cortex of the oocyte; see enlarged view of boxed areas at left.

(G–L) K10 immunohistochemistry (G, H, J, and K) and quantitation (I and L). Single confocal sections of stage 9 (G and H) and stage 2 (J and K) oocytes in lateral view, labeled with DAPI (blue), the F-actin marker phalloidin (green), and K10 (red). In the early stages of oogenesis (J–L), K10 is more strongly expressed in *Δmir-184* mutants than in wild-type, but at later stages (G–I) its levels are reduced. (I and L) Quantitation of K10 protein levels (see Experimental Procedures). Dots represent values for individual oocytes; averages are indicated by horizontal lines; asterisks indicate the statistical significance of the difference between wild-type and mutant as determined by t test, n = 6–22, ***p < 0.001, **p < 0.01, *p < 0.05.

(stage 9), the K10 protein level is *reduced* by about 50% on average compared to wild-type (Figures 4G–4I). Thus, the loss of *mir-184*-mediated repression leads to a precocious initiation of K10 translation, followed by a reduction of its protein level in the oocyte nucleus at the time when it is required for *grk* mRNA transport. This partial loss of K10 protein at the critical stage is consistent with the observed mislocalization of GRK protein and thus explains the moderate dorsalization defect in the egg shells of *Δmir-184* mutants (Figures 4A–4C). It is possible that the precocious K10 translation is itself responsible for the later reduction in K10 protein level, but the involvement of additional *miR-184* targets cannot be excluded (see Discussion).

miR-184 Is Required for Stem Cell Differentiation

Egg production in *Δmir-184* mutant females ceases almost completely after 5 days. To gain insight into the causes of this defect, we used a panel of markers to examine the ovaries, in particular the germarium, in which the earliest stages of oogenesis take place (for a review, see Gilboa and Lehmann, 2004; Morrison and Spradling, 2008) (schematic in Figure 5P). In wild-type, two germline stem cells (GSCs) are embedded within a somatic cell niche, which consists of a stack of terminal filament cells and six cap cells. The GSCs undergo asymmetric divisions, where one of the daughters loses contact with the niche, becomes a cystoblast, and undergoes four mitotic divisions with incomplete cytokinesis, resulting in the formation of a cyst of 16 cells that are connected by cytoplasmic bridges (fusome). The 16-cell cyst becomes enveloped by somatic follicle cells that will produce the egg chamber. All germline cells are VASA positive, the GSCs are recognizable by their spectrosome (spherical fusome), cystoblasts by their differentiation marker Bag-of-marbles (BAM) (McKearin and Spradling, 1990), and the cysts by their branching fusome (Hay et al., 1988; Lin et al., 1994) (Figures 5A and 5D). The spectrosome/fusome can be visualized by 1B1/HTS antibodies, the nuclei by DAPI, and the somatic cells based on their strong actin cytoskeleton (phalloidin).

Previous studies have shown that the differentiation of cystoblasts is driven by BAM. In the GSCs, BAM expression is suppressed (and the stem cell character maintained) by DPP signaling that responds to ligand secreted from the somatic niche cells (Xie and Spradling, 1998). As the cystoblasts move away from the niche, they receive less DPP signal, which leads to de-repression of *bam* transcription, thereby initiating their differentiation (Casanueva and Ferguson, 2004). In the absence of *bam*, no cystoblast differentiation takes place and the germarium becomes filled with undifferentiated GSC-like cells ("bag of marbles" phenotype) (McKearin and Spradling, 1990). This phenotype can be mimicked by overexpression of DPP in the somatic cells or of the activated type I Dpp receptor Thickveins (TKV) in the germline: both lead to ectopic DPP signaling activity and repression of *bam* in the cystoblasts (Xie and Spradling, 1998; Casanueva and Ferguson, 2004). An overactive but ligand-dependent mutant allele of the second type I DPP receptor, Saxophone (SAX), leads to a milder increase in signaling that does not cause a *bam*-like phenotype by itself, but does so in combination with an additional mild boost in signal, such as adding a third genomic copy of *dpp* (Casanueva and Ferguson, 2004).

In the germaria of 5-day-old *Δmir-184* mutant females, we observe a large number of GSC-like cells (VASA-positive, spherical fusome), accompanied by an absence of BAM-positive cystoblasts and of multicellular cysts and egg chamber formation

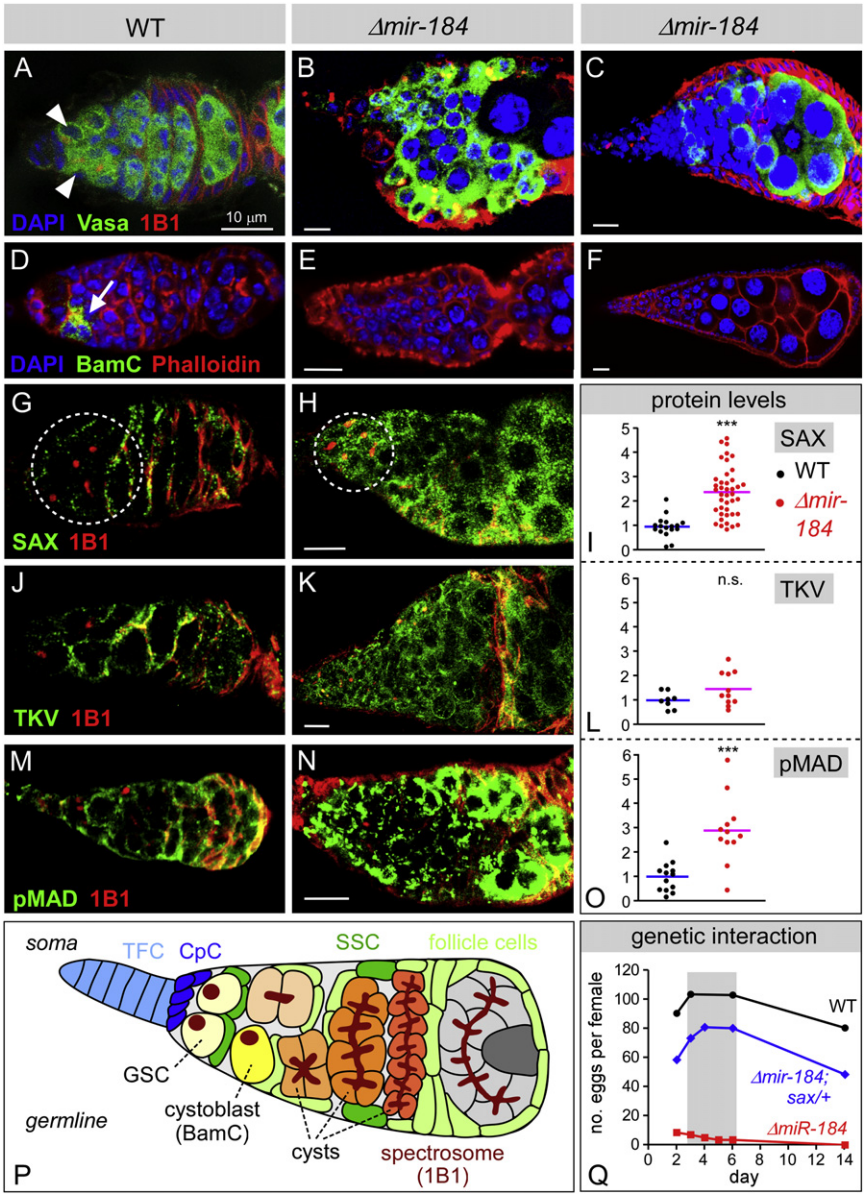

Figure 5. *mir-184* Is Required for Normal Cystoblast Differentiation in the Germarium

(A–C) Lateral views of germaria stained with DAPI (blue), the germline marker VASA (green), and the spectrosome marker 1B1 (red); the wild-type position of the two GSCs is indicated by arrowheads in (A). The ovarioles of 5-day-old *Δmir-184* females are filled with GSC-like cells, but no cysts.

(D–F) The cystoblast differentiation marker BAM-C (green; arrow in D) is not expressed in the *Δmir-184* mutant ovarioles.

(G–I) In the mutant (H), SAX protein (green) levels are highly increased compared to wild-type. Approximate regions of interest used to quantitate protein levels (I) are indicated by dashed circles.

(J–L) TKV (green) is mislocalized similarly to SAX (compare K and H), but its protein levels are not significantly increased (L).

(M–O) pMAD protein (green) levels and thus DPP signaling is highly increased in the mutant compared to wild-type. All images show single confocal sections. To capture the overgrowth phenotype in the mutant, *Δmir-184* embryos are imaged at lower magnification, as indicated by scale bars (which represent 10 μm in all panels).

(I, L, and O) Quantitation of protein levels as determined by measuring average pixel intensity in equivalent regions of interest from confocal micrographs (see Experimental Procedures). Dots represent values for individual germaria, normalized to the wild-type average; averages are indicated by horizontal lines; asterisks indicate the statistical significance of the difference between wild-type and mutant as determined by t test, n = 8–41, ***p < 0.001; n.s., not significant.

(P) Schema depicting early oogenesis in the germarium. TFC, terminal filament cells; CpC, cap cells; SSC, somatic stem cells; GSC, germline stem cells.

(Q) Removal of one genomic copy of *sax* in *Δmir-184* mutant females substantially rescues their infertility. Data represent egg-laying averages from two to five independent experiments; all groups are significantly different from all others (p < 0.001), based on one-way ANOVA of average egg production from days 3–6 (gray box) with Student-Newman-Keuls post hoc test.

(Figures 5B and 5C). This phenotype is very similar to that of *bam* itself and indicates an inability to differentiate cystoblasts (Figures 5E and 5F). Of the genes that need to be *downregulated* for cystoblast differentiation to occur (Gilboa and Lehmann, 2004; Morrison and Spradling, 2008), only the *sax* mRNA contains an *miR-184* target site. The site contains a mismatch in the 5′ seed but shows strong pairing in the 3′ portion and lies in a reasonably accessible region of the 3′UTR (ΔG_{open} −6), resulting in a very good ΔΔG score (−14) (Figure 2B). As in the case of *K10*, the site is not conserved across all Drosophilids (Figure 2A) and is not predicted by Pictar and Stark et al. (2005). In our S2 luciferase reporter assay, the site confers strong translational repression similar in strength to that of the *ttk69* and *K10 miR-184* sites (Figure 2B). This effect is also found in vivo: in wild-type germaria, SAX protein shows moderate levels of expression and is concentrated in patches at the plasma membrane (Figure 5G). In the *Δmir-184* mutant, we observe SAX protein present at much higher levels (Figure 5I) and mislocalized within the cells, with distribution along the entire plasma membrane and also at high concentrations within the cytoplasm (Figure 5H). We further examined whether this strong increase and intracellular mislocalization of the SAX receptor is accompanied by increased downstream signaling activity, such as increased phosphorylation of MAD (Tanimoto et al., 2000). Indeed, we find a strong increase in pMAD levels in the *Δmir-184* mutant compared to wild-type (Figures 5M–5O), which is consistent with the observed reduction in BAM expression and the *bam*-like phenotype (McKearin and Spradling, 1990). To determine whether the observed increase in SAX protein levels is responsible for the *bam*-like phenotype, we tested for genetic interaction between *mir-184* and *sax*: we removed one maternal copy of the *sax* gene (sax^4) (Singer et al., 1997) and asked whether fertility and ovarian phenotypes are ameliorated compared to the *Δmir-184* background. We find that *Δmir-184*;

sax^4/+ females produce many more eggs and do not become sterile over time (Figure 5Q); consistent with the maintenance of fertility, the ovaries of 7-day-old females show no *bam*-like phenotype. Taken together, our results indicate that *mir-184* controls germline cell differentiation by tuning the levels of the SAX receptor, thereby modulating the amount of DPP signal the GSCs receive.

Previous work had shown that wild-type levels of an overly active SAX receptor had to be combined with a mild increase in DPP ligand expression to produce a *bam*-like phenotype; we therefore wondered whether in our case with strongly increased levels of *wild-type* SAX receptor additional synergistic input might also be contributing to the phenotype. Because TKV is not a target of *miR-184* but is thought to form heterodimers with SAX (Haerry et al., 1998), we examined whether TKV protein levels and/or distribution are (indirectly) affected in *Δmir-184*. In wild-type, TKV protein appears more broadly expressed than SAX, but also localized to patches at the plasma membranes of germ cells, similar to SAX (Figure 5J). Interestingly, in the *Δmir-184* mutant, we find no significant increase in TKV protein levels but mislocalization both at the plasma membrane and within the cytoplasm, that is, a distribution resembling that of SAX (Figures 5K and 5L). This suggests that the *Δmir-184*-induced overexpression and mislocalization of SAX also result in a redistribution of TKV, which may contribute to the observed increase in DPP signaling activity/pMAD and thus to the *bam*-like phenotype in *Δmir-184* mutants.

In addition to the overgrowth phenotype, the ovaries of 5-day-old *Δmir-184* females contain many empty ovarioles, and those of older females are completely devoid of any germ cells, indicating the gradual loss of GSC function over time. This phenomenon might be a secondary consequence of the differentiation defect, as it is also observed under *bam* loss-of-function conditions: in older *bam* as well as in *Δmir-184* females, the overgrowth subsides and the GSCs begin to lose expression of markers such as VASA (Figure 5C, data not shown). Because the stem cell niche itself appears intact, the reasons for this regression are unclear.

DISCUSSION

Our study demonstrates the important role of miRNA-mediated regulation in the development of the female germline in *Drosophila*. We show that *miR-184*, strongly expressed in the germline and deposited in the egg, regulates several distinct steps during oogenesis and early embryogenesis, including stem cell differentiation and axis formation of both egg chamber and embryo, and we characterize the underlying molecular mechanism by identifying three relevant *miR-184* targets. Female germline development has long been known to be a carefully regulated process in which the spatiotemporal pattern and activity level of key factors is kept in check by multiple levels of control (Johnstone and Lasko, 2001; Gilboa and Lehmann, 2004). Our results now show that *miR-184* provides a crucial additional layer of regulation. Interestingly, *miR-184* does not target the key developmental regulators and morphogens themselves but components involved in their regulation, namely a signal transduction receptor, a transport factor, and a general transcription factor.

Developmentally, the first process *miR-184* regulates is the interaction between somatic niche and germline stem cells. Previous genetic analysis of this process has focused on the role of TKV in mediating the DPP signal in stem cell maintenance and cystoblast differentiation (Xie and Spradling, 1998; Casanueva and Ferguson, 2004). We have now demonstrated that *miR-184*-mediated translational repression of SAX protein levels, potentially combined with indirect effects on TKV protein distribution, are a crucial mechanism in dampening DPP signal reception and thus promoting cystoblast differentiation. The substantial rescue of egg production that we observe when halving the gene dose of *sax* suggests that the lack of cystoblast differentiation (and the subsequent loss of germline stem cells) is responsible for the reduction and ultimate loss of fertility in *Δmir-184* mutants.

miR-184's role in establishing egg chamber polarity is more complex. miRNAs have frequently been viewed as performing a clean-up task—suppressing translation of residual transcript after developmental decisions have been made (Giraldez et al., 2006; Bushati et al., 2008). The misregulation of K10 in *Δmir-184* mutants argues that precocious translation, even within the proper cell (oocyte), may also be deleterious. However, we currently do not understand the mechanistic connection between the early overproduction and the later depletion of K10 protein. Because actively translated transcripts are generally considered to be more protected against degradation (Johnstone and Lasko, 2001), a partial loss of *K10* transcript seems unlikely. Given that *K10* mRNA is bound by translational regulators (Bicaudal D and Egalitarian) and K10 protein interacts with other proteins (Squid) (Roth et al., 1995; Norvell et al., 1999), it is possible that these factors themselves are limiting and titrated away by the precocious translation and strong accumulation of K10 protein, but we cannot exclude the possibility that other *miR-184* targets not yet implicated in dorsoventral patterning of the egg are also involved.

Finally, in early embryonic development, *miR-184* tunes the potent transcriptional repressor TTK69, thereby ensuring the proper timing of pair rule gene expression and anterior-posterior patterning. Several additional phenotypes are readily visible in the mutant that indicate *miR-184*'s involvement in processes known to be tightly regulated, such as the transition into the vitellogenic state, which is stringently controlled by several hormone systems, but also in processes where this is unexpected, such as cortical nuclear migration in the syncytial blastoderm. Detection of the entire range of distinct phenotypes in the *Δmir-184* mutant was only possible due to their partial penetrance; however, eventually the requirement for GSC differentiation becomes absolute and, thus, within a week, the loss of egg production supersedes all other phenotypes.

The phenotypes we observe in the *Δmir-184* mutant partially overlap with those seen in mutants in which miRNA biogenesis is disrupted (Hatfield et al., 2005; Jin and Xie, 2007). However, these experiments are difficult to compare: biogenesis mutants presumably affect all 43 miRNAs normally expressed in the germline (Neumüller et al., 2008), causing additional phenotypes that are likely to epistatically mask effects visible in *Δmir-184*; in addition, these studies have to be conducted under mosaic conditions, where perdurance of mature miRNAs may add another layer of complication. The polarity and vitellogenesis defects but not the germarium overgrowth we find in the *Δmir-184* mutant have been reported for *dcr-1* germline clones

(Hatfield et al., 2005; Jin and Xie, 2007). Conversely, *dcr-1* germline clones show cell-autonomous cell-cycle defects that we do not observe (N.I. and U.G., unpublished data) and GSC maintenance defects (Hatfield et al., 2005) that simply cannot be observed in the *Δmir-184* mutant, due to its rapid tumorous growth and subsequent regression phenotype.

Our study also sheds light on important mechanistic aspects of miRNA function. Most of the defects in the *Δmir-184* mutant can be rescued by germline-specific expression of *mir-184*, indicating that the miRNA is coexpressed with its targets in the same cell and tunes their expression. Loss of *mir-184* function leads to increases in protein level in the 2- to 5-fold range, with the mutant showing increased variability in protein level compared to wild-type, concordant with the observed incomplete penetrance and variability in phenotype. Our findings support the idea that miRNAs regulate a large number of different targets in vivo (Selbach et al., 2008). Depending on the stoichiometry and affinity between miRNA and mRNA as well as the critical level of the cognate protein, some of this regulation, although quantifiable at the expression level, may be phenotypically silent. However, the fact that several distinct and molecularly attributable defects are observed in the *Δmir-184* mutant clearly indicates that the loss of proper tuning of protein levels frequently becomes phenotypically visible. This is consistent with the longstanding knowledge that many biological processes are sensitive to changes in the activity level of their key components.

Both our genetic and our molecular analyses demonstrate the key role of the maternal component of *miR-184*. *miR-184* is strongly expressed in the ovaries and later in a highly dynamic pattern throughout embryogenesis, but we observe a pronounced difference in phenotypic impact: loss of the zygotic component has no discernable effect on adult morphology and viability, yet loss from the female germline results in severe morphologic defects in oogenesis and embryonic development. Notably, much of this germline requirement can be rescued by much lower levels of *miR-184* than are expressed in wild-type. Moreover, the maternal contribution of *miR-184* persists stably through the first 3 hr of development and is then slowly degraded with a half-life of ~3 hr. This long perdurance is common to many maternally provided transcripts and typically results in rescue into larval stages and beyond. Thus, it is quite possible that also in the case of *miR-184*, the persisting maternal contribution rescues whatever zygotic function the miRNA may have, implying that the high level and complex pattern of its embryonic expression might be (partially) redundant.

The remarkable functionality carried by low concentrations of the miRNA highlights the need for complete removal of the maternal contributions of miRNAs when undertaking functional studies. Surprisingly, this consideration has frequently been neglected in current genetic analyses of *Drosophila* miRNAs, despite the fact that many of those under investigation have weak (similar to *miR-184*) or even strong maternal contributions (e.g., *miR-6* and *miR-286*) (Leaman et al., 2005; Neumüller et al., 2008). This disregard of maternal contribution and of functional redundancy between family members may be partially responsible for the unusual situation that for *Drosophila* miRNAs, primarily postembryonic and more subtle phenotypes have been reported (e.g., Li et al., 2006; Bushati et al., 2008), whereas for most vertebrate miRNAs, severe, even embryonic, phenotypes are observed (e.g., Martello et al., 2007; Zhao et al., 2007).

Another intriguing finding of our study is that while *miR-184* itself is highly conserved, two of the three *miR-184* target sites we identified are only partially conserved across the Drosophilids, suggesting that the acquisition of molecular targets and thus of regulatory function is in evolutionary flux (Lu et al., 2008). The fact that poorly conserved sites and even sites with mismatch in the 5′ seed region can confer significant and phenotypically relevant repression, as we show here and others have demonstrated previously (Vella et al., 2004; Didiano and Hobert, 2008), draws into question, from a developmental biologist's perspective, the rationale for filtering computational target site predictions based on evolutionary conservation and of applying overly stringent seed matching rules. Our results suggest that considering other features of target candidates, such as site accessibility, can provide an important complement to purely sequence-based approaches.

EXPERIMENTAL PROCEDURES

Genetics, Transgenes, and Fly Strains

The *Δmir-184* deletion was created by FLP-mediated recombination of FRT-bearing P elements (PBac{WH}f05119 and P {XP}d08710) (Parks et al., 2004), which was detected by loss of w^+ and confirmed molecularly by genomic PCR and sequence analysis. The *UAS$_t$-mir-184* construct was created using a 1.5 kb genomic fragment containing the miRNA (see Table S1 available online). The following Gal4 drivers and mutant strains were used: *nos-Gal4VP16* and *c587-Gal4* (R. Lehmann), *GR1-Gal4* (T. Schupbach), *UAS-sax-wt* (K. Wharton, M. O'Connor), *ttk^{e11}* (A. Travers), *sax^4* (Bloomington Stock Center 5404), *K10^{130}* (Bloomington Stock Center 7385), and *bam^{Δ86}* (R. Lehmann).

Molecular Biology

Our dual luciferase assay was conducted as described in Kertesz et al. (2007). The assay makes use of endogenously expressed *miR-184*, which is present in substantial copy number in S2 cells (Kertesz et al., 2007) and employs a modified version of the psiCHECK-2 dual luciferase vector (Promega). 3′UTR (~200 bp) fragments centered around the putative target sites were amplified by PCR from genomic or plasmid DNA; mutated versions of the 3′UTR fragment without the target site were generated by oligo annealing. All tested sequences are listed in Table S1. One million S2 cells were transfected with reporter plasmid (1 μg) using Cellfectin (Invitrogen) and, after 20 hr, lysed and tested for luciferase activity. The *Renilla*/firefly luciferase ratios were normalized against the empty vector and averaged over 3–12 replicates.

miRNA northerns were carried out as described in Kertesz et al. (2007), except that 15 μg of total RNA was loaded per lane. qPCR for miRNA quantification was done on single eggs using a Taqman miRNA assay kit from Applied Biosystems, following the manufacturer's instructions. Eggs were genotyped by RT-PCR based on the presence/absence of the *mir-184* precursor. Subsequently, for each genotyped RNA extract, the amount of mature *miR-184* was measured by qPCR and internally normalized against the levels of ribosomal RNA. For quantitative westerns of TTK69, single staged eggs were collected, ground up in Laemmli buffer, and run on a 10% polyacrylamide gel, blotted onto nylon membrane, and incubated with rb-anti-TTK69 at 1:1000 (F. Azorin, A. Travers) and HRP-anti-rabbit at 1:200 (Pierce); signal was quantified using a Fuji BAS100 bioimager and ImageGauge.

Histochemistry and Imaging

RNA in situ hybridization procedures and probes are described in Schroeder et al. (2004); for *miR-184* the primary transcript was detected (for genomic fragment, see Table S1). Immunohistochemistry was performed as described in Pane et al. (2007), using the following antibodies: rat-anti-K10 1:2000 (R. Cohen), mouse-anti-GRK 1:20 and goat-anti-VASA 1:1000 (T. Schupbach), rb-anti-SAX 1:200 (Abcam), rb-anti-TKV 1:100 (M. O'Connor,

M. Gonzalez-Gaitan), rb-anti-pMAD 1:100 (P. ten Dijke), mouse-anti-BAM 1:5 (D. McKearin), mouse-anti-HTS 1:50 (1B1; Developmental Studies Hybridoma Bank), and Texas-red- or Alexa-488-coupled phalloidin (Invitrogen, Molecular Probes); secondary antibodies were at 1:1000 (Molecular Probes).

Stainings were imaged on an inverted Zeiss LSM510 fitted with a UV laser. To quantify protein levels, stacks of ~30 0.48 μm confocal sections of staged germaria and oocytes were projected, and average pixel intensities were measured for equivalent regions of interest using ImageJ and Metamorph. Statistical significance of differences was assessed using the t test. All data to be compared were collected with identical microscope and software parameter settings.

SUPPLEMENTAL DATA

Supplemental Data include five figures and one table and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00249-4.

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