A polycistronic locus encodes both a tumor-suppressive microRNA and a long non-coding RNA regulating testis morphology in Drosophila

Travis D. Carney[1,2] and Halyna R. Shcherbata[1,2]

[1]Institute of Cell Biochemistry, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625, Hannover, Germany

[2]Mount Desert Island Biological Laboratory, Bar Harbor, ME 04609, USA

*Corresponding author: Shcherbata.Halyna@mh-hannover.de

Abstract

The impact of non-coding RNAs on stem cell biology and differentiation processes is an important and incompletely understood area of research. Using the testes of *Drosophila melanogaster* as a valuable system for investigating these processes, we identified a polycistronic locus from which two non-coding transcripts, *miR-317* and the long non-coding RNA (lncRNA) *Peony*, are produced, with alternative polyadenylation implicated in regulation of their differential expression levels. We report here that each transcript has a distinct role in Drosophila testes; the increased expression of *Peony* results in the disruption of the muscle sheath covering the testis, and the absence of *miR-317* leads to the emergence of germ-cell tumors in developing flies. The deficiency of *miR-317* increases Notch signaling activity in somatic cyst cells, upregulates multiple predicted targets of *miR-317*, and drives germline tumorigenesis. This work implicates *miR-317* as a tumor suppressor that modulates Notch signaling strength and suggests alternative polyadenylation as a novel mechanism for regulation of miRNA expression.

Keywords: miRNA, lncRNA, gene expression, germ-cell tumor, Notch signaling, tumor suppressor, Drosophila testes, alternative polyadenylation

Running Title: Tumor suppressor *miR-317* and lncRNA *Peony* are expressed from a polycistronic non-coding RNA locus

Introduction

Over three decades of research have revealed the involvement of microRNAs (miRNAs) in many aspects of stem cell biology and in processes of differentiation. MiRNAs have been shown to modulate cell division in human embryonic stem cells by targeting key cell cycle checkpoint components (Qi et al. 2009). In Drosophila, miRNAs are required for cell cycle progression as well as maintenance of ovarian germline stem cells (GSCs) (Hatfield et al. 2005; Shcherbata et al. 2007). In addition to roles in stem cell function and homeostasis, we have previously shown that miRNAs affect aspects of cell fate determination in multiple cell types in Drosophila (Kucherenko et al. 2012; Yatsenko and Shcherbata 2014). These studies demonstrate the importance of miRNAs in the crucial processes of stem cell function and cellular specification and differentiation.

The Drosophila testis has proven to be an invaluable model for the study of stem cell biology, intercellular signaling, and differentiation. The testis is a coiled tube, closed at the apical (anterior) end, in which the processes of spermatogenesis take place (Figure 1A). At the testis apex, a

cluster of specialized somatic cells called the hub forms a stem cell niche capable of maintaining two populations of stem cells: GSCs and somatic cyst stem cells (CySCs) (de Cuevas and Matunis 2011) (Figure 1B). Both are maintained through direct contact with the hub and through multiple cell-signaling pathways. GSCs and CySCs divide asymmetrically such that one daughter cell retains both niche contact and stemness, while the other daughter, no longer proximal to the self-renewal-promoting signals from the niche, begins a differentiation program. The differentiating daughter of the GSC, a gonialblast, becomes encapsulated by two somatic daughter cells, termed cyst cells, which themselves differentiate in concert with the germ cells and maintain this association throughout spermatogenesis. Cyst cells serve as a differentiation niche, supporting spermatogenesis processes and protecting the germ cells from exogenous signals (Decotto and Spradling 2005; Zoller and Schulz 2012; Papagiannouli 2014). The gonialblast undergoes four rounds of transit-amplifying mitotic divisions to form a 16-cell cluster of cells termed spermatogonia (Greenspan et al. 2015). This cell cluster and the two encapsulating cyst cells comprise a single cyst. Spermatogonial cells mature into primary spermatocytes, substantially increase their volume, and undergo meiosis to form spermatids, each of which elongates, matures, and is released as a mature sperm to be stored in the seminal vesicle until copulation. Therefore, a single GSC division has the potential to result in the formation of 64 haploid gametes (Greenspan et al. 2015).

The processes of proliferation and differentiation must be carefully balanced to ensure that each cyst produces the proper number of gametes while avoiding tumor-like overgrowth. In particular, precisely four spermatogonial mitotic divisions must occur to form a normal, 16-cell spermatocyte cyst. Numerous instances have been reported in which these processes fail and result in overproliferation. The genes *bag of marbles* (*bam*), *benign gonial cell neoplasm* (*bgcn*), and *Rbfox1* act cell-autonomously in the germline, and mutation of these genes results in overproliferation of early, undifferentiated germ cells (McKearin and Spradling 1990; Gonczy et al. 1997; Tastan et al. 2010). In addition, genes affecting signaling, transport, and numerous other processes in the somatic cyst cells have been shown to affect the germline non-autonomously, interfering with the differentiation program and causing dramatic overproliferation of undifferentiated germ cells (Matunis et al. 1997; Kiger et al. 2000; Tran et al. 2000; Joti et al. 2011; Fairchild et al. 2017; Tang et al. 2017). From these studies, a clear picture has emerged in which the close association and signaling relationship between germline and somatic cells is crucial for proper spermatogenesis.

Recent studies have shown that long non-coding RNA transcripts (lncRNAs) are expressed in a tissue-specific manner and are particularly enriched in the testes of Drosophila and other animals, including mammals (Cabili et al. 2011; Young et al. 2012; Brown et al. 2014; Necsulea et al. 2014). Many testis-expressed lncRNAs are enriched in late developmental stages including meiotic and elongated cysts (Wen et al. 2016; Vedelek et al. 2018). Some of them have roles in the late stages of spermatogenesis, as mutations cause spermatid defects including defective chromatin compaction and sperm individualization (Wen et al. 2016).

We are interested in elucidating the effects that non-coding RNAs (ncRNAs), in particular miRNAs, have on processes related to stem cell biology and differentiation and how these processes can be perturbed when miRNAs are dysregulated. It is also important to determine which miRNA target genes are in turn affected in these mutants. To this end, we screened a number of miRNAs reported to exhibit expression in testes and/or ovaries. We closely examined

gonads of flies mutant for these miRNAs for phenotypes consistent with stem cell division/self-renewal problems or differentiation defects.

Here we report that deficiency of the miRNA *miR-317* causes the formation of germ-cell tumors in developing fly testes. Previous reports have implicated *miR-317* in several processes and behaviors, including negative regulation of the Toll immune response (Li et al. 2017; Li et al. 2019); larval ovary morphogenesis and development (Yang et al. 2016); startle-induced locomotion (Yamamoto et al. 2008); aggressive behavioral response (Edwards et al. 2009); female response to mating (Fricke et al. 2014); and regulation of Cyclin B expression during embryonic development (Pushpavalli et al. 2014). Furthermore, overexpression (OE) of *miR-317* is sufficient to prevent the formation of imaginal disc tumors in the potently oncogenic background of simultaneous *RasV12* OE and *discs large* mutation (Wang et al. 2022)**.** However, the present study is the first to demonstrate an endogenous role of *miR-317* as a tumor suppressor.

The *miR-317* mutant allele *miR-317[ko]* results in the concomitant upregulation of a co-transcribed lncRNA *CR45054* (FBgn0266414), which causes a disruption of the muscle sheath surrounding the testis and a pronounced enlargement of the testis apex. This distinct phenotype causes the testis to resemble the appearance of a peony flower. Consequently, we have named the lncRNA *CR45054* as "*Peony.*" Our data indicate that *miR-317* and *Peony* are mutually expressed from a single polycistronic non-coding RNA locus, and their expression depends on differential polyadenylation.

Furthermore, we find that OE of either of two putative *miR-317* targets*,* Rotund (Rn) or CG18265, or the Notch signaling cofactor, Mastermind (Mam), is sufficient to cause the formation of testis tumors. All three encode transcriptional regulators. Deficiency of *miR-317* results in increased Notch pathway signaling activity in somatic cyst cells, and the gene dose reduction of *mam* results in a partial abrogation of tumor formation. Our results show that *miR-317* is a novel tumor suppressor, and this important function requires the repression of multiple targets in the somatic cells of the Drosophila testis.

Results

miR-317[ko] males have multiple testis phenotypes

The Drosophila testis serves as a convenient system for detecting defects in division and maintenance of stem cells as well as the differentiation of their progeny. At the apex of control testes, the region containing transit-amplifying spermatogonia is characterized by bright DAPI staining, owing to small cell size and tightly condensed chromatin. As germ cells mature into spermatocytes, the cells grow substantially and their chromatin decondenses, leading to much more diffuse DAPI staining (Figures 1C & 1E). In searching for miRNA genes involved in processes of stem cell function and differentiation, we examined the gonads of a loss-of-function (LOF) mutant, *miR-317[ko]*, in which the pre-miRNA has been precisely replaced with a *mini-white* cassette by ends-out homologous recombination (Chen et al. 2014) (Figures 1D, 1F, & 1G). In some *miR-317[ko]* testes, we observed a dramatic enlargement at the testis apex, a phenotype referred to here as apical enlargement (AE; Figure 1D). We also observed an incidence of tumors in *miR-317[ko]* testes, identifiable in adults by very bright DAPI staining apart from the apical, spermatogonial region as well as the strong expression of Vasa, an RNA-binding helicase commonly used as a germ-cell marker (Figure 1F).

To reduce the likelihood of genetic background-induced phenotypic artifacts, we wished to analyze the *miR-317* LOF phenotypes using trans-heterozygous allelic combinations. However, aside from *miR-317[ko]* described above, the only *miR-317* mutant stocks available possess large deficiencies that uncover not only the locus of interest, but also many other genes. This is particularly disadvantageous for the analysis of miRNA phenotypes, which tend to be subtle and easily modified by environmental conditions as well as genetic backgrounds (Vidigal and Ventura 2015). Indeed, miRNA mutants often fail to exhibit a phenotype except in a sensitized genetic background (Brenner et al. 2010). Therefore, we generated a new deficiency allele using FLP-mediated recombination between FRT sites present in transposon insertions *PBac{WH}f04665* and *P{XP}d02752* (Parks et al. 2004). This recombination resulted in the deletion of 5.5 kb that removes only *miR-317* as well as the large second exon of the nearby long non-coding RNA (lncRNA) gene *Peony* (Figure 1G). We predict that this deletion, which we call *Df-miR-317*, represents a LOF allele for both *miR-317* and *Peony*.

We compared testes from *miR-317[ko]* homozygotes and trans-heterozygous (*miR-317[ko]/Df*) adult males and found that testis tumors exist in flies of both genotypes with similar frequency, strongly suggesting that this phenotype is attributable to the loss of the miRNA (Figure 1H). Interestingly, we noted that while *miR-317[ko]* and *miR-317[ko]/Df* testes exhibit an apical enlargement (AE) phenotype, we very rarely observe this phenotype in *Df-miR-317* testes (Figure 1I). As the primary difference between *miR-317[ko]* and *Df-miR-317* is the deletion of *Peony* only in the latter, this discrepancy raises the possibility that the lncRNA is involved in the development of the AE phenotype. Below, we look first into the roles of the *Peony* lncRNA in testes phenotypes and then at the role of *miR-317* as a tumor suppressor. In all subsequent experiments requiring *miR-317* LOF, we use either *miR-317[ko]* homozygotes or *miR-317[ko]/Df* flies.

RNA-seq in *miR-317* mutant testes and ovaries reveals differential expression of genes important for cell signaling and differentiation

To attain a global view of the genes perturbed in *miR-317* mutants, we performed RNA-seq analyses comparing a control genotype, *w[1118]*, to the LOF mutant, *miR-317[ko]*. As the *miR-317[ko]* phenotype indicated a potential issue in germline differentiation, we performed this comparison not only on testes but also on ovaries from adult animals. This allowed us to explore the differences in transcriptional changes between the gonads of both sexes (see Materials and Methods). We detected more total genes in testes than in ovaries (13232 vs. 8894), but we found that more genes are dysregulated in *miR-317* mutant ovaries than in testes, both in number of dysregulated genes and as a percentage of total genes detected: 2944 genes (33.1% of all genes detected) are dysregulated in ovaries, while 1844 genes (13.9% of all genes detected) are dysregulated in testes. In both organs, more genes are downregulated than upregulated in *miR-317* mutants (59.1% in testes, 55.7% in ovaries; Supplemental Tables 1 & 2; Supplemental Figure 1A).

We found that the transcriptional effects of *miR-317* deficiency have both similarities and interesting differences in testes and ovaries. We performed a Gene Ontology (GO)-term enrichment analysis to investigate the biological processes that are overrepresented among the genes dysregulated in both organs. Ovaries and testes share enriched terms pertaining to signal transduction and cell differentiation, as well as cell communication, cell migration, and morphogenesis, all of which terms could be relevant to the tumor phenotype that we discovered in testes. Interestingly, GO terms related to gene expression regulation and cell cycle are highly

enriched in mutant ovaries but not in testes, while the terms cell junction organization and myofibril assembly are enriched only in testes and not in ovaries (Supplemental Figure 1B).

Consistent with previous reports (Djebali et al. 2012; Brown et al. 2014; Wen et al. 2016; Joshi and Rajender 2020; Hong et al. 2021), we detected a substantially higher proportion of lncRNA genes in testes than in ovaries (Supplemental Tables 1 & 2; Supplemental Figure 1A). In contrast to protein-coding genes, lncRNAs in both organs are more likely to be up- than downregulated. This is particularly striking in the testes, in which the five most-upregulated genes, and more than a third of the top 100 most-upregulated genes, are ncRNAs (Supplemental Table 1). Among those lncRNA genes strongly upregulated in *miR-317[ko]* testes and ovaries is *Peony,* the gene just upstream of *miR-317* (Supplemental Figure 1C; Supplemental Tables 1 & 2).

In spite of the large number of genes and important processes that are disrupted in *miR-317* mutant ovaries, we did not observe any incidence of tumors or other remarkable phenotypes similar to those found in testes. Therefore, we opted to focus the rest of our study on testes.

Upregulation of *Peony* causes disruption of the testis muscle sheath

Analyses of mutant testes revealed that AE is concomitant with a disruption of the muscle sheath that surrounds the testis (Figures 2A-C). While nearly 80% of *miR-317[ko]* testes exhibit some degree of muscle disruption, *Df-miR-317* testes muscles appear intact as in controls (Figure 2D). Since *miR-317* mutants have significantly upregulated *Peony* (Supplemental Tables 1 & 2; Supplemental Figure 1C), we generated a *UAS-Peony* transgene to allow conditional OE of a full-length cDNA. To determine whether *Peony* plays a role in the AE or muscle disruption phenotype, we overexpressed *Peony* using Gal4 lines that express in various cell types in testes (Supplemental Figure 2). Indeed, we found that muscle disruption is evident upon *Peony* OE in some cell types. Expression of *Peony* in somatic cells or germ cells – using *tj-Gal4* or *vasa-Gal4+nos-Gal4*, respectively – causes muscle disruption in 13-34% of testes (Figure 2D; Supplemental Figures 2A & 2B). OE using *bab1-Gal4*, expressed in somatic cyst cells, muscle cells, and pigment cells (Supplemental Figures 2C & 2D), causes muscle sheath disruption in 42% of testes. OE with *Mef2-Gal4*, expressed in muscle cells, causes muscle sheath disruption in 33% of testes, while OE with *MJ12a-Gal4*, which drives expression only in the pigment cell layer that also surrounds the testis (Hrdlicka et al. 2002) (Supplemental Figures 2E & 2F), does not cause muscle disruption (Figure 2D).

Since none of the conditions described above result in a muscle disruption phenotype as severe as *miR-317[ko]*, we turned to ubiquitous OE using *Tub-Gal4*. This condition causes a high degree of lethality, so we used a *Tub-Gal4; Tub-Gal80[ts]* combination (*Tub-Gal4[ts]*) to block expression until late larval stages. This results in a strong incidence of muscle disruption very similar to that seen in *miR-317[ko]* testes (Figure 2D). Moreover, while OE using the other cell-type-specific drivers fails to result in AE, this phenotype does manifest upon ubiquitous OE (Supplemental Figures 3A & 3B). Increased *Peony* levels do not, however, result in the formation of tumors with any driver. We conclude that while tumor formation is due to a loss of *miR-317* function, the AE phenotype is a result of the upregulation of *Peony* that occurs in *miR-317[ko]* testes or upon exogenous OE.

Differential levels of *Peony* and *miR-317* rely on alternative polyadenylation

The strong upregulation of *Peony* in *miR-317[ko]* mutant gonads led us to investigate how their expression is interrelated. We first hypothesized that this upregulation may be due to direct

targeting of the lncRNA by *miR-317.* We examined all 192 lncRNAs dysregulated in mutant testes for the presence of *miR-317* binding sites. We found that 18 (35%) of the 52 downregulated lncRNAs and 38 (27%) of the 140 upregulated lncRNAs – including *Peony* – have predicted target sites (Supplemental Table 1). Thus, the presence of a *miR-317* binding site is not predictive of a lncRNA's upregulation in the absence of *miR-317*. Furthermore, the strong OE of *miR-317* results in only a small reduction in *Peony* expression (Supplemental Figure 3C). We conclude that the regulation of *Peony* is likely not due to direct targeting by *miR-317*.

It has been proposed that *Peony* serves as the primary miRNA transcript from which *miR-317* is processed (Kadener et al. 2009). Consistent with this, we observe that while a transcription start site (TSS) is predicted near the 5′ end of *Peony*, there is no independent TSS predicted upstream of the miRNA (Figure 2E). We confirmed co-expression by performing reverse transcription (RT) using either oligo(dT) or a reverse primer downstream of *miR-317*, Peony_R6, followed by qPCR using primers within *Peony's* second exon (Figure 2E). The successful amplification using these approaches confirms not only that *Peony* and *miR-317* are expressed in a common primary transcript but also that at least a portion of the transcript is polyadenylated (Supplemental Figure 3D). Notably, this result does not exclude the presence of another polyadenylation (polyA) site between *Peony* and *miR-317*. We propose that *Peony* is upregulated in *miR-317[ko]* tissues due to stabilization of the transcript, either because of the strong polyA site in the *mini-white* present in *miR-317[ko]* or due to the lack of the miRNA hairpin.

We next wished to determine whether *Peony* and *miR-317* co-expression is constitutive or if it is separable. We used pan-neuronal *elav-Gal4 Gal80[ts]* (*elav[ts]*) to drive expression from a UAS insertion in the intron of *Peony*, *P{GSV6}GS11364* (Figure 2E). This is sufficient to cause upregulation not only of *Peony* sequence downstream of the insertion site, but also of *miR-317* (Supplemental Figure 3C). Further, to test the necessity of *Peony* expression for production of *miR-317*, we assayed *miR-317* levels in flies homozygous for *MI02131,* a MiMIC gene trap containing a transcriptional stop signal (Figure 2E). Similar to *miR317[ko]* and *Df-miR-317* flies, in which *miR-317* is undetectable as expected, *MI02131* causes a strong loss of *miR-317* expression (Supplemental Figure 3E). We conclude that *Peony* expression is required and sufficient for production of *miR-317*.

Three polyA sites are predicted between *Peony* and *miR-317* (RegRNA 2.0) (Chang et al. 2013), indicating that in some circumstances, transcription may terminate before the expression of the miRNA (Figure 2E). This raises the interesting possibility that the differential usage of polyA sites may play a role in regulating the expression of *miR-317*. To investigate this possibility, we decided to compare adult testes to heads because numerous studies have demonstrated that polyadenylation is regulated differently in brains than in other somatic tissues (Beaudoing and Gautheret 2001; Smibert et al. 2012; Yatsenko et al. 2014; MacDonald 2019). Reasoning that using heads allows the strong enrichment of brain-expressed RNAs, we extracted RNA from testes and heads of male *w[1118]* flies, completed RT using an oligo-dT reverse primer, and used the resulting cDNA to perform qPCR using two sets of primers: one targeting the pre-miRNA region and another in the second exon of *Peony* (Figure 2E). In this way, we were able to quantify both regions of the parent transcript and calculate a *Peony:miR-317* ratio for different tissues. Comparing RNA extracted from testes and male heads of *w[1118]* flies, we found a striking difference in the ratio between these tissues (52:1 ratio in heads vs 9:1 in testes; Figure 2F). This result shows that *Peony* abundance is much greater than that of *miR-317* and that their relative expression levels vary widely between different tissues. One explanation is that alternative polyA

sites exist: a proximal site between the two genes and a distal site downstream of the miRNA (Figure 2E). The differential expression of the RNAs may result either from a propensity for the transcription machinery to utilize the proximal site, the rapid processing of the miRNA hairpin and subsequent degradation of the miRNA-containing transcript, or both.

To further test whether differential polyadenylation plays a substantial role in the observed prevalence of the *Peony* transcript, we made use of *RpII215[c4],* an RNA polymerase II subunit mutation that results in reduced transcription elongation speed. This slowed elongation imparts to the transcription apparatus the tendency to utilize a proximal polyA site (Liu et al. 2017). In the context of coding genes, the choice of a proximal or a distal polyA site usually results in a transcript with a shorter or a longer 3′UTR, respectively. We sought to test whether forcing a proximal polyA site bias results in an increased expression of *Peony* at the expense of *miR-317*. We compared *Peony:miR-317* ratios in male heads and testes between *w[1118]* control flies and *RpII215[c4]* mutants. In male heads, we found that this ratio does not change significantly in the slow elongation mutant (Figure 2F). In contrast, the ratio of *Peony:miR-317* increases nearly 2-fold in *RpII215[c4]* testes, from 8.5 to 15.3 (p=.025, Figure 2F). Consistent with our observations, while the *RpII215[c4]* mutant was shown to enhance the usage of proximal polyA sites in Drosophila bodies, this phenomenon was not observed in heads (Liu et al. 2017), possibly due to additional stringent controls regulating 3′UTR length in the brain.

These results led us to attempt to determine the presence of a proximal polyA site using a modified 3′ rapid amplification of cDNA ends (3′ RACE) approach (see Materials and Methods). Using RNA extracted from *RpII215[c4]* male flies, which we expect to have a higher incidence of proximal polyA site usage, we confirmed the presence of a cleavage and polyA site only 22 bp downstream of the *Peony* transcript's annotated 3′ end (Figures 2E & 2G). These results strongly suggest that, in testes, the relative levels of *Peony* and *miR-317* are affected by the differential choice of either a proximal or a distal polyA site.

Recent reports have demonstrated that primary miRNA transcripts can have roles in addition to the action of the miRNA, particularly affecting expression levels of neighboring genes (He et al. 2021). Moreover, it has been shown in plants (Lauressergues et al. 2015; Sharma et al. 2020) and in humans (Kang et al. 2020) that primary miRNA transcripts can encode small regulatory peptides that act in a feedback mechanism to upregulate their parent transcript, thus promoting the expression of the miRNAs. While *Peony* serves as the primary transcript for *miR-317* and is predicted to be non-coding, its sequence includes an open reading frame that would encode an 83-amino-acid peptide. To determine whether this putative peptide, or the *Peony* transcript itself, is involved in regulation of its own expression, we overexpressed *Peony* in larval brains using our UAS transgene (*elav>Peony*). Since the *UAS-Peony* transgene was created using a full-length cDNA (see Materials and Methods), qPCR primers within the transcript sequence could not be used to assay expression levels of the endogenous gene; therefore, we made use of the gene trap *MI02131*, which is inserted in exon 2 of *Peony* (Figure 2E) and is thus included in a truncated *Peony* transcript. Using primers against the *GFP* coding sequence within the MiMIC transposon, we performed RT-qPCR on larval brains with or without the OE of *Peony.* We found that, as expected, *GFP* expression is absent from brains lacking the MiMIC insertion and present in those containing MiMIC. Importantly, *GFP* expression does not change upon OE of *Peony* (Supplemental Figure 3F). This indicates that the increased expression of *Peony* does not affect transcription at its endogenous locus and that no feedback mechanism is involved in its regulation.

miR-317 LOF tumors consist of GSC-like and early spermatogonial cells

Next, we turned our attention from the lncRNA, *Peony*, to examine the tumor suppressive functions of *miR-317*. To more fully characterize the *miR-317[ko]* testis tumor phenotype, we stained control and mutant testes with antibodies that mark different cell types and indicate different stages of differentiation. The strong expression of Vasa and Adducin (Add) suggests that the tumors are largely composed of germ cells, not somatic cells (Figures 3A & 3A′). We used several additional means to verify this. Add interacts with the actin cytoskeleton and labels distinct germline-specific structures in early germ cells: spectrosomes and fusomes. The morphology of these structures can indicate the identity (i.e., differentiation state) of these cells: spherical spectrosomes are indicative of a GSC or gonialblast (the immediate daughter of a GSC) identity, while branched fusomes indicate a more mature spermatogonial identity. We found that the tumors in *miR-317* mutants possess mostly spectrosomes but can also contain a smaller amount of branched fusomes, sometimes within a single tumor (Figure 3A″). These results demonstrate that the majority of the cells within the tumors have an early germ-cell identity.

The pro-differentiation protein Bam is an indicator of the differentiation state of germ cells. In control testes, Bam is expressed in germ cells of 4- to 8-cell cysts and is absent from GSCs, gonialblasts, and 2-cell cysts. *bam-GFP* is a reporter construct consisting of *bam* regulatory elements driving the expression of GFP (Chen and McKearin 2003). As a result, GFP is expressed in the endogenous *bam* pattern: excluded from the most apical germ cells but expressed in 4- and 8-cell cysts. Unlike Bam protein, GFP perdures into later spermatogonial and spermatocyte stages (Figures 3B-3B′). Deficiency of *miR-317* does not change this pattern of GFP expression (Figures 3C-3C′). We found that *bam-GFP* is not expressed in *miR-317[ko]* tumors (Figures 3D-3D′), indicating that in terms of differentiation state, the germ cells comprising the tumor are earlier than the spermatogonia of 4-cell cysts. Taken together, our data suggest that *miR-317* testis tumors consist of early, undifferentiated germ cells similar to GSCs, gonialblasts, or 2-cell cysts. Next, we wanted to determine whether somatic cells also contribute to the cellular composition of the tumors.

The soma-expressed transcription factor, Traffic jam (Tj), is expressed in CySCs and early cyst cells near the testis apex in control and *miR-317* mutants but is not found in cells in or around the tumors in *miR-317* LOF testes (Figures 3E′-3G′). However, we observed that while Tj^+ nuclei cluster close to the apex in controls, in *miR-317* LOF testes, they are found significantly further removed from the apex (Supplemental Figures 4A-C). This suggests that early cyst cells may exhibit either a delay in differentiation or increased mobility in *miR-317* mutants. The testis hub comprises another population of somatic cells at the apex. We stained these cells with an antibody against Fasciclin III, which allowed us to quantify the number of hub cells. We found that *miR-317[ko/Df]* hubs contain significantly more cells than controls (Supplemental Figure 4D). This further suggests that there may be a signaling defect in the somatic cells of the mutant testis apex.

To investigate the role of *mir-317* in the somatic cells, we attempted a rescue of the tumor phenotype using an early cyst-cell driver, *zfh1::T2A::Gal4*, in which the self-cleaving peptide T2A and Gal4 sequences have been fused to the C-terminus of Zfh1 by CRISPR-Cas9-mediated genome editing (Albert et al. 2018). We found that *miR-317[ko/Df]* combined with a heterozygous *zfh1::T2A::Gal4* allele results in an incidence of tumors of about 11%, and addition of *UAS-miR-317* elicits a significant rescue, reducing the incidence of tumors to about 3% (Supplemental

Figure 4E). This result strongly suggests that *miR-317*'s tumor suppressive activity takes place in the early somatic cells.

Next, to investigate whether tumor cells are proliferative in adult testes, we stained for the mitotic marker, phosphorylated histone H3 (PH3). The spermatogonial cells within each normal cyst divide synchronously in clusters of 2, 4, or 8 cells. We found that while some cells in each tumor are $PH3^+$, demonstrating that they are mitotically active, most of the dividing cells are dispersed, not clustered (Figure 3F′-3G′). This indicates that tumors consist of cells that divide similarly to GSCs or early spermatogonia.

We also stained testes with multiple other somatic cell markers. Eyes absent (Eya) is a transcription factor expressed in mature somatic cyst cells, while β-3-Tubulin marks the cortexes of somatic cells and thus nicely outlines differentiating cysts (Figures 3H-H′). We found that Eya^+ somatic cells can be found adjacent to tumors, but not within them; similarly, tumors express β-3-Tubulin at their periphery, but this somatic marker does not extend within the tumor (Figures 3I-J′). Our results lead us to conclude that *miR-317* mutant testis tumors consist not of soma but of germ cells, probably a mix of cells similar in appearance to GSCs and their spermatogonial progeny. Hereafter we refer to these tumors as germ-cell tumors (GCTs).

GCTs arise developmentally and disintegrate in aging testes

As our investigations of the GCT phenotypes described above were limited to young, 0-3-day-old males, we next sought to determine whether the GCT phenotype persists in aging flies. We dissected 1-3-day-old, 2-week-old, and 4-week-old males. Control males do not exhibit GCTs (Figures 4A, 4C, & 4E). We found that approximately the same proportion of mutant testes exhibit GCTs at the 1-3-day-old and 2-week-old time points; however, in older, 4-week-old testes, we found a pronounced decrease in the incidence of GCTs (Figures 4F & 4G). These results indicate that new tumors do not form in aging, adult flies.

In young *miR-317[ko]/Df* males, as described above, we found GCTs mostly near the apexes of testes (Figure 4B), while in 2-week-old testes, GCTs are located more basally (Figure 4D). We measured the location of GCTs at these time points as a percent displacement between the apex (0%) and the base of the testis, before the seminal vesicle (100%) and found that GCT distance from the apex increases markedly from 17.7% in 1-3-day-old to 70.2% in 2-week-old mutants (Figure 4H). In addition, although very few GCTs exist in 4-week-old mutants, we found evidence of the fragmented remains of tumors near the testis base, in the terminal epithelial region just apical to the seminal vesicles, evidenced by clusters of small cells stained very strongly with Vasa (Figure 4F-4F′). 29% of 4-week-old mutant testes exhibit this phenotype, which is never seen in control testes (Figure 4E). We conclude that GCTs in *miR-317* LOF testes originate from germ cells near the hub and then migrate posteriorly through the testis, finally disintegrating in the terminal epithelial region near the testis base. Furthermore, we find that the formation of these GCTs is a phenomenon that occurs during development and in very young flies but does not persist in aging, adult males.

Next, we wished to determine whether *miR-317* deficiency affects male fertility. We performed fertility assays on young, 2-week-old, and 4-week-old males as previously described (Herrera et al. 2021). A subset of both control and mutant males exhibit sterility at all ages. This number is not significantly different in 1-3-day old controls and mutants (5% and 14%, respectively; $p=0.14$, chi-square analysis). However, the incidence of sterility increases with age more in mutants than

in controls, and by 4 weeks of age, significantly more mutant males (48%) fail to produce progeny than controls (27%; p=0.03). Overall, we found that no difference in fertility exists in 1-3-day- or 2-week-old flies, but that 4-week-old mutants are significantly less fertile than controls (Figure 4I), largely due to the pronounced increase in sterility in the population. This result confirms that *miR-317* is necessary for proper fertility in aging male flies.

Relevant *miR-317* targets involved in mutant phenotypes

To investigate the molecular mechanism of *miR-317* involvement in GCTs, we used multiple online databases to compile a list of over 1000 predicted *miR-317* target genes (see Materials and Methods). 919 of these predicted targets were detected in our testis RNA-seq data, of which 142 are significantly dysregulated in *miR-317[ko]* testes ($p<.05$; 88 downregulated and 54 upregulated; Supplemental Table 3). Although mostly highly significant, the levels of dysregulation of these genes are quite modest, with Log_2 fold changes (Log_2FC) ranging from 0.18-1.29 upregulated and -0.19 to -2.48 downregulated. This is consistent with previous results demonstrating that while each miRNA affects numerous targets, the change in expression of each mRNA target may be minor (Baek et al. 2008). Moreover, translational repression of a transcript by miRNAs can affect protein output without causing degradation of the mRNA; in this case, even effective miRNA targeting can yield little to no transcriptional changes.

Genes dysregulated in *miR-317[ko]* are expected to include both direct *miR-317* targets and indirectly affected, downstream genes. For this reason, we placed the highest priority on upregulated genes because we expect transcripts that are directly targeted by *miR-317* to be more highly expressed in the mutant. Applying a stringent requirement that a gene should be upregulated with at least a Log_2FC of 0.5 reduces the list of upregulated genes from 54 to 15 genes; we propose that these 15 genes are strong candidates to be direct targets of *miR-317* in testes (Table 1; Supplemental Figure 1A).

We next sought to recapitulate *miR-317[ko]* mutant phenotypes via the upregulation of putative *miR-317* targets. For seven of the candidates in Table 1, we were able to acquire Drosophila stocks predicted to cause Gal4-induced OE (Table 2). In addition, Table 2 includes information regarding OE of *UAS-Peony* as well as stocks utilized for the OE of Notch pathway-related genes (*Notch, Delta,* and *mastermind*), for reasons outlined below. Each of these stocks is either an in vitro-cloned UAS line, a UAS-containing transposon insertion, or a CRISPR-OE line. The latter is a stock that ubiquitously expresses a guide RNA (gRNA) specific for a gene of interest; when crossed to a stock containing both an appropriate Gal4 driver and *UAS-dCas9.VPR*, a construct expressing a nuclease-dead Cas9 protein fused with a transcriptional activation domain, this can cause the OE of the gene of interest from its endogenous locus (Lin et al. 2015).

For germline OE, we used *nanos-Gal4* (*nos-Gal4*), which is expressed in GSCs and early germ cells, and for somatic OE, we used *C587-Gal4*, which is expressed in cyst stem cells and cyst cells (Kawase et al. 2004) (Supplemental Figures 2G & 2H). First, we overexpressed in germ cells those genes with UAS lines available and were unable to detect the AE phenotype or the formation of GCTs (Table 2). Next, we overexpressed in somatic cells using either *C587-Gal4* or *C587-Gal4; UAS-dCas9.VPR*, as appropriate (Table 2). The AE phenotype was not observed in any of the OE experiments. Notably, however, somatic expression of either *rotund* (*rn*) or *CG18265* is sufficient to cause GCTs (Table 2; Figures 5A-C). Both *rn* and *CG18265* encode transcriptional regulators with no previously known roles in tumorigenesis. We conclude that OE of multiple putative *miR-317* target genes can contribute to the formation of GCTs in the testis

and propose that in wild-type testes, the miRNA *miR-317* is active in repression of multiple targets in its role as a tumor suppressor.

The Notch cofactor Mastermind is implicated in the formation of GCTs

Among the genes predicted to be targeted by *miR-317* are many interactors and regulators of Notch signaling (Supplemental Table 4). To address the involvement of Notch in GCT formation, we attempted to augment Notch signaling strength by overexpressing Notch pathway components. We performed germline OE of the Notch ligand Delta or somatic OE of multiple forms of activated Notch. We also overexpressed Notch clonally using the Flp-out method (Struhl and Basler 1993). While Notch OE consistently causes a high degree of lethality, none of these conditions is sufficient to cause the formation of GCTs. We noted that *mastermind* (*mam*), which encodes a transcriptional cofactor in canonical Notch signaling, is among the transcripts with predicted *miR-317* binding sites (Supplemental Table 4), although it is not upregulated in our RNA-seq dataset (Supplemental Tables 1 & 2). In another attempt to increase Notch signaling strength, we overexpressed *mam* in somatic cells using *C587-Gal4* and found that this condition results in formation of GCTs but not AE (Figure 5D). Furthermore, OE of *mam* in Flp-out clones results in tumors, similar to its somatic OE (Table 2).

To further investigate a potential involvement of the Notch pathway in *miR-317* LOF phenotypes, we tested Notch signaling strength using a reporter in which GFP is under the transcriptional control of a Notch responsive element (*NRE-GFP*) (Saj et al. 2010). We found that the GFP signal is nearly undetectable in most control testes from young, 1-day-old flies. In those that express detectable GFP, the pattern is consistent with weak Notch signaling activity in cyst cells (Figures 5E-E′). Upon *miR-317* LOF, GFP expression is strongly upregulated in mature cyst cells while remaining undetectable in the cells closest to the apex (Figures 5F-F′). We conclude that *miR-317* normally acts to repress Notch signaling in cyst cells.

Next, we tested whether Mam is necessary for either the GCT or the AE phenotype by introducing a strong *mam* LOF allele, *mam[8],* into the *miR-317[ko]* background. We found that the loss of one copy of *mam* fails to modify the AE phenotype (p=0.34; Figure 5G). In contrast, the incidence of GCTs is marginally suppressed (p=0.08) by *mam* heterozygosity (Figure 5H). Taken together, our results strongly suggest that Mam is both necessary and sufficient for testis GCT formation. Furthermore, as Mam is a crucial Notch cofactor, these results also raise the possibility that increased Notch signaling strength could serve to promote GCT formation in *miR-317* mutant testes.

Finally, to check the levels of *CG18265* and *rn*, the identified *miR-317* targets, during development, we isolated RNA from L3 larval males and subjected it to qRT-PCR. We also assayed the levels of *mam* in these animals. Consistent with our RNA-seq results (Supplemental Tables 1&2), we found that *CG18265* and *rn* are significantly upregulated in *miR-317* mutant L3 larvae (Figure 5I). Also consistent with RNA-seq, we found that *mam* is not upregulated in mutant larvae, implying that in spite of its *miR-317* binding site, *mam* is likely not a direct target of the miRNA (Figure 5I).

Our data demonstrate that *Peony* and *miR-317* have separable effects: upregulation of the *Peony* transcript causes lesions to appear in the testis muscle sheath leading to AE, and loss of *miR-317* causes upregulation of *CG18265* and *rn*, increased Notch signaling strength, and formation of GCTs (Figure 5J). Consistent with these conclusions, OE of *CG18265*, *rn*, or *mam* causes the

formation of GCTs but does not lead to muscle disruption or AE. Ubiquitous OE of *Peony* from our inducible transgene does not cause GCT formation but results in a testis muscle breakage phenotype as well as a concomitant apical enlargement similar to that seen in *miR-317[ko]* testes (Figure 5J).

Discussion

Sufficiency of Mam and Notch in driving GCT formation

We show in the present study that OE of Mam in cyst cells is sufficient to cause GCT formation, and reduction of Mam partially abrogates the incidence of GCTs. As Mam has been well-studied as an important transcriptional cofactor in canonical Notch signaling, these results suggest the intriguing possibility that increased Notch signaling strength is causative in GCT formation. However, it has also been reported that in various contexts, Mam functions in a Notch-independent manner (Kankel et al. 2007; Vied and Kalderon 2009; Zhang et al. 2011; Lobo-Pecellin et al. 2019). Our results do not allow us to distinguish between a canonical or Notch-independent function for Mam in tumor formation; therefore, a Notch-independent Mam function remains a possibility in the context of testis GCT formation. However, in light of our finding that *miR-317[ko]* causes increased Notch activity in cyst cells, the same cell type in which Mam OE results in GCTs, we favor a model in which cyst cell-expressed Mam participates in this Notch signaling.

While we were unable to show that overexpressed Notch drives GCT formation, the possibility remains that increased Notch signaling may drive tumorigenesis in conditions that we did not test. Further manipulation of the timing and strength of the Notch pathway would be required to truly exclude this possibility; additionally, a more detailed exploration of the genetic interaction between *Notch* and *miR-317* should prove informative. Alternatively, Notch OE may not induce GCTs if Mam is a limiting factor in determining the strength of Notch signaling. In this instance, reducing the gene copy number of *mam* decreases the incidence of tumors as we demonstrate here, but increasing Notch without simultaneously increasing Mam is insufficient to induce tumor formation. Consistent with this possibility, in Drosophila ovaries, somatic OE of activated Notch and Mam simultaneously results in tumor-like overgrowth, while expression of Notch alone does not (Defreitas et al. 2020).

Intriguingly, the consequences of Notch involvement in various processes differ in a cell- and context-dependent manner. For instance, Notch can behave either as an oncogene or as a tumor suppressor, depending on the cell type; similarly, either proliferation or terminal differentiation can be promoted by increased Notch signaling (Koch et al. 2013; Yatsenko and Shcherbata 2018; Yatsenko and Shcherbata 2021; Zamfirescu et al. 2022). In Drosophila, OE of Notch in neural stem cells induces tumorigenesis, while in intestinal stem cell lineages, increased Notch is required for differentiation (Ohlstein and Spradling 2006; Wang et al. 2006; Ohlstein and Spradling 2007; Bowman et al. 2008).

In the Drosophila testis, Notch signaling was shown to be required for germ cell survival: Delta produced in the germline and Notch in the somatic cyst cells are both required to prevent cell death of the germ cells (Ng et al. 2019). In light of our results showing that increased Notch signaling in *miR-317* mutants coincides with tumor formation, it is possible that the levels of Notch components or the strength of Notch signaling must be maintained within a particular range to

prevent either cell death or overproliferation. Another possibility is that cysts at different developmental stages have variable competence to undergo Notch-mediated germline overproliferation. It has been reported that Notch signaling is not active in the youngest cyst cells near the apex, rather only in more differentiated, slightly more basal cells (Ng et al. 2019). Thus, increased Notch signaling strength in earlier cyst cells in the spermatogonial region may have the propensity non-autonomously to cause overproliferation of their associated germ cells. Future studies will be required to assess the competence of different stages of cysts to undergo tumorigenesis.

MiR-317 **regulates multiple genes to repress tumor formation in developing testes**

MiRNAs or miRNA clusters can target multiple genes in the same pathway to ensure a speedy and robust response (Mestdagh et al. 2010; Uhlmann et al. 2012; Cicek et al. 2016; Hart et al. 2019). We report here that repression of at least two putative *miR-317* targets, *rn* and *CG18265*, is necessary to prevent the onset of tumors in Drosophila testes. The upregulation of both of these genes in *miR-317* mutants is modest (1.26 to 1.61-fold) as measured both by RNA-seq (Supplemental Table 1) and by qRT-PCR (Figure 5I). This is illustrative of the miRNA's typical regulation of multiple targets, in which the effect is not a binary on/off result but rather a fine-tuning of gene expression (Baek et al. 2008), by which the miRNA can serve to reinforce cell fates, regulate responses to environmental cues, or buffer stochastic fluctuations in gene expression or signaling. We also note that the penetrance of the GCT formation phenotype is low and variable, in that most germline cysts escape tumorigenesis and apparently undergo spermatogenesis properly. In fact, in most experiments, the majority of males examined had no tumors. We propose that this reflects the effect of *miR-317*'s expression tuning on the remarkably robust processes of spermatogenesis, and that *miR-317* deficiency sensitizes the system such that these processes are marginally more prone to failure, leading to an infrequent tumorigenic event.

Both *rn* and *CG18265* encode C2H2 zinc-finger transcription factors. Interestingly, Rn is reported to affect Notch signaling in multiple contexts: *rn* mutation dominantly enhances the *split* phenotype caused by a recessive *Notch* allele (Brand and Campos-Ortega 1990); and the expression patterns of the Notch ligands Delta and Serrate are perturbed by *rn* mutation in imaginal discs (St Pierre et al. 2002). To our knowledge, no link between *CG18265* and Notch signaling has yet been demonstrated.

Rn has important roles in olfactory neuron specification and imaginal disc development. While not an essential gene, both male and female *rn* mutants are sterile. This sterility is not germline-dependent (Kerridge and Thomas-Cavallin 1988), suggesting that Rn has a role in the somatic gonadal cells. While it is not known why *rn* mutants are sterile, our findings suggest that *miR-317* acts in somatic cells, at least in the testes, to modulate the levels or expression pattern of *rn* and prevent germline overproliferation. Notably, *rn* is not dysregulated in ovaries per our RNA-seq data (Supplemental Table 2), implying that it is not similarly affected by *miR-317* in both organs.

Our data show that GCTs in *miR-317* LOF testes arise during development, such that they already exist in newly eclosed males, and our evidence suggests that new tumors do not form during adulthood. This is consistent with our finding that the *miR-317* targets *CG18265* and *rn* are upregulated in *miR-317* LOF L3 larvae and young adult testes (Figure 5I; Supplemental Table 1), suggesting that the crucial time for *miR-317*'s repressive action in terms of tumor formation is during development. As metamorphosis is analogous to human puberty (Guirado et al. 2023), the

timing of GCTs in *miR-317* LOF animals is reminiscent of the incidence of human seminoma, which is a common type of GCT in males that frequently manifests beginning in adolescence (Frazier et al. 2012). Seminomas are tumors composed of germ cells that remain in their undifferentiated state (Penn et al. 2018), contrasting with other testicular neoplasms such as teratomas, in which pluripotent cells are capable of giving rise to progeny of all three germ layers (Nettersheim et al. 2016). The *miR-317* mutant GCTs, similar to seminomas, appear to contain only undifferentiated germ cells.

Regulation of miRNA expression by differential polyadenylation

We demonstrate here that *Peony* and *miR-317* comprise a polycistronic unit and mutually affect one another's expression levels. We identified a proximal polyA site near the annotated end of *Peony* and show evidence for another site, not yet molecularly defined, downstream of *miR-317*. We propose that transcriptional read-through of the proximal polyA site is required for expression of the miRNA, and the choice of polyA site determines whether *Peony* alone is expressed, or *miR-317* as well. Indeed, in the event that the polyA site is bypassed and *miR-317* is transcribed, pre-miRNA processing and cleavage would be predicted to de-stabilize the 5′ portion of the transcript that includes *Peony*. Therefore, expression of the two genes could in principle be mutually exclusive: proximal polyadenylation stabilizes the *Peony* transcript but prevents expression of *miR-317*, while transcriptional read-through allows miRNA processing but causes degradation of *Peony*.

It has been suggested that *miR-317* is expressed as a cluster with two downstream miRNA genes, *miR-277* and *miR-34*, both of which are processed from the lncRNA gene *CR43459* (Kadener et al. 2009; Zhou et al. 2018). Our data suggest that this is not the case, at least in testes and ovaries. First, although our RNA-seq data demonstrate that *Peony* is strongly upregulated in *miR-317[ko]* testes and ovaries, expression levels of *CR43459* do not change in either organ (Supplemental Tables 1&2). Additionally, a TSS has been identified at the beginning of each of these ncRNA genes, *Peony* and *CR43459*, consistent with their independent transcription in spite of their close proximity (Zhou et al. 2018). Moreover, a genome-wide analysis of primary miRNAs in humans and mice suggests that a widespread mechanism leading to differential transcription of clustered miRNAs is through the use of alternative promoters (Chang et al. 2015).

An important and enticing question is whether other miRNA genes are regulated in a way similar to the *Peony-miR-317* locus. We looked carefully at the genomic loci of all known Drosophila miRNA genes, of which there are over 260 (Supplemental Table 5; Supplemental Figure 5A). The most common genomic context for miRNA genes is presence in the intron of a coding gene (118/264 miRNA genes, 44.7%). Many others are located in exons or in intergenic space, while an appreciable minority (46/264 genes, 17.4%) reside closely upstream or downstream of adjacent genes in a way similar to the close proximity of *Peony* and *miR-317* (Supplemental Figure 5B). This 17.4% of miRNA genes, which we term "putatively co-transcribed," represents an intriguing avenue for further investigation to determine if they are indeed co-expressed with adjacent genes and whether alternative polyadenylation may play a role in their differential expression.

Possible implication of *Peony* in phenotypes attributed to *miR-317*

In light of our finding that the functional lncRNA *Peony* is adjacent to and co-transcribed with *miR-317*, phenotypes that would ordinarily have been ascribed to the miRNA should be scrutinized

carefully for involvement of *Peony*. For instance, several studies reported phenotypes that they attributed to loss of *miR-317*, when the allele used was a P{GT1} transposon (BG01900) inserted in *Peony*, 1730 bp 5′ of *miR-317* (Yamamoto et al. 2008; Edwards et al. 2009; Fricke et al. 2014). Moreover, care should be given in interpretation of phenotypes caused by the *miR-317[ko]* allele since, as demonstrated here, this allele results in a strong upregulation of *Peony*, which could produce confounding results. Potentially helpful in this respect is the new *Df-miR-317* allele generated for this work, which removes both *miR-317* and *Peony*, facilitating the unambiguous attribution of phenotypes to the miRNA.

The cryptic yet crucial functions of lncRNAs

It has recently become appreciated that eukaryotic genomes undergo pervasive transcription, in stark contrast to the historical view that the transcriptome consists mainly of protein-coding transcripts and other RNAs involved primarily in translation: tRNAs, rRNAs, and a few others. This widespread transcription results in a vast number of non-coding transcripts, the functions of most of which are still unknown (Jensen et al. 2013). MiRNAs comprise one class of non-coding RNAs (ncRNAs), but while the canonical function of miRNAs is well-known, the same cannot be said for the lncRNAs. Indeed, although many thousands of these transcripts exist in animal cells, their functions remain mostly obscure. It is a subject of much debate whether many lncRNAs are the result of transcriptional noise with no bona fide functions within the cell. Counter to this proposition has been the elucidation of important functions for an ever-increasing number of lncRNA genes as well as clear instances of evolutionary conservation, which would be unexpected for transcripts with no importance (Guttman et al. 2009; Chen 2016; Li and Liu 2019). Indeed, the study of lncRNAs' molecular and cellular functions and the determination of their roles in living cells remains an important and tantalizing problem in modern biology. Our finding that *Peony* is co-transcribed with an important tumor-suppressive miRNA and that the lncRNA itself also affects muscle integrity and testis morphology adds an important element to the growing body of evidence demonstrating the important roles of lncRNAs in numerous contexts.

Materials and methods

Fly Stocks and Genetics

Flies were maintained at 25°C at constant humidity and a 12h-12h light-dark cycle. They were kept on standard cornmeal-yeast-agar fly food. All genotypes used are listed in Key Resources Table. For experiments utilizing *Tub-Gal80[ts],* crosses were performed and progeny were allowed to develop for 3 days at 25°C, at which temperature Gal4 activity is low in the presence of Gal80[ts]. After 3 days, larvae were shifted to 29°C to allow Gal4-induced expression until dissection (L3 or adult).

The following stocks were provided by the Bloomington Drosophila Stock Center (BDSC): *w[1118]* (BDSC 6326); *OR-R* (BDSC 5); *C587-Gal4* (BDSC 67747); *P{UAS-3xFLAG.dCas9.VPR}attP40* (BDSC 66561); *P{w[+mC]=EP} EP3412* (BDSC 17125); *P{w[+mC]=EP}Ada2b[G3857]* (BDSC 31778); *w[*]; P{w[+mW.hs]=GawB}bab1[Pgal4-2]/TM6B, Tb[1]* (BDSC 6803); *P{w[+mC] EPgy2}CG18265[EY11347]* (BDSC 20298); *P{y[+t7.7] v[+t1.8]=TOE.GS02678}* (BDSC 79975); *P{y[+t7.7] v[+t1.8]=TOE.GS03029}* (BDSC 80311); *P{w[+mGS]=GSV1}EP-820[EP-820]* (BDSC 43450); *P{y[+t7.7] v[+t1.8]=TOE.GS03028}* (BDSC 80310); *Mi{MIC}MI02131* (BDSC 37146); *P{w[+mW.hs]=GawB}elav[C155]* (BDSC 458); *P{y[+mDint2] w[+mC]=EPgy2}EY14693* (BDSC

21439); *w*; P{UAS-mam.A}2* (BDSC 27743); *mam[8]/CyO* (BDSC 1596); *miR-317[ko]* (BDSC 58926); *P{w[+mW.hs]=GawB}MJ12a* (BDSC 6991); *w[1118]; P{w[+m*]=NRE-EGFP.S}5A* (BDSC 30727); *P{y[+t7.7] v[+t1.8]=TOE.GS01580}* (BDSC 85759); *P{w[+mC]=UAS-rn.SP}1* (BDSC 7403); *RpII215[c4]* (BDSC 3663); *y1 w*; P{tubP-GAL4}LL7/TM3, Sb1 Ser1* (BDSC 5138); *w[*]; P{w[+mC]=tubP-GAL80[ts]}10* (BDSC 7108); and *w[*]; P{w[+mC]=tubP-GAL80[ts]}2* (BDSC 7017). The following stocks were provided by the Kyoto Drosophila Stock Center at the Department of Drosophila Genomics and Genetic Resources (DGGR), Kyoto Institute of Technology: *P{GSV6}GS11364* (DGGR 203275); *y* w*; P{w+mW.hs=GawB}NP1624* (DGGR 104055); and *P{w+mC=vas-GAL4.2.6}2* (DGGR 109996). The following stocks were procured from the Exelixis collection at Harvard Medical School: *PBac{WH}f04665* and *P{XP}d02752*.

Other stocks used were: *UAS-Peony* (this study); *Df-miR-317* (this study); *P{w+mC=bam-GFP.-799/+133}3LR* (Chen and McKearin 2003); *P[UASp-Delta-2]* (Ward et al. 2006); *w*; P{GAL4-nos.NGT}40; P{GAL4::VP16-nos.UTR}CG6325MVD1* (a gift from Hannele Ruohola-Baker); *Mef2-Gal4* (a gift from Gerd Vorbrüggen); *Tub-Gal4* (chromosome 2; a gift from Stefan Jakobs); and *zfh1::T2A::Gal4* (a gift from Christian Bökel). Alleles used to express activated forms of Notch were *UAS-N[cdc10]*, which expresses an intracellular fragment of Notch (Brennan et al. 1999) and *UAS-N[B33c-3]*, which results in the expression of a constitutively active form of Notch lacking most of the extracellular domain (Larkin et al. 1996).

Fertility assay

Fertility assay was performed as previously reported (Herrera et al. 2021). Individual control (*w[1118]/OR-R*) or mutant (*miR-317[ko/Df]*) males of either 0-3 days, 2 weeks, or 4 weeks of age were crossed to two 1-3-day-old control virgin females. For each time point, 50 males were initially crossed. Flies were allowed to mate for 48 hours, then they were removed from the vials. Any crosses in which the male or either female died within the 48-hour period were not included in the analysis. After 10-12 days, the progeny from each cross were counted as a measure of each male's fertility.

Immunofluorescence and confocal microscopy

Adult flies were dissected with Dumont #5 forceps (Fine Science Tools) in cold phosphate-buffered saline (PBS). Testes were fixed in 4% formaldehyde (Polysciences 18814-20) in PBT (PBS + 0.2% Triton-X 100 (Sigma 108603)) for 20 minutes, then rinsed in PBT multiple times for a total of at least 30 minutes. Then, testes were blocked in PBTB solution (PBT + 5% normal goat serum (Abcam ab7481) and 0.1% bovine serum albumin (Applichem A1391)) for at least 30 minutes. Next, testes were incubated in a solution of primary antibodies diluted in PBTB overnight at 4°C. After primary antibody incubation, testes were rinsed multiple times for at least 30 minutes, blocked in PBTB for at least 30 minutes, and incubated in a solution of secondary antibodies diluted in PBTB, either at room temperature for 2.5 hours or at 4°C overnight. Then, testes were rinsed multiple times in PBT, incubated in a 1 μg/mL solution of DAPI in PBT for 10 minutes, and rinsed three more times with PBT. Finally, the buffer was removed and testes were stored in Vectashield (Vector Labs) until being mounted on slides and imaged.

The following mouse monoclonal antibodies were procured from the Developmental Studies Hybridoma Bank and used at the dilutions shown: anti-Adducin (1:20), anti-LaminC (1:20), anti-Dlg (1:20), anti-Eyes absent (1:100), anti-Fas3 (1:20), and anti-β-Galactosidase (1:200). Other antibodies used were: chicken anti-GFP (1:5000, Abcam); rabbit anti-mCherry (1:200, Abcam);

rabbit anti-Phosphohistone H3 (1:10000, Upstate Biotechnology); guinea pig anti-Traffic jam (1:5000, a gift from Dorothea Godt); guinea pig anti-β3-Tubulin (1:1500, a gift from Renate Renkawitz-Pohl); and rabbit anti-Vasa (1:5000, a gift from Herbert Jäckle). Tissues were also counterstained using the DNA stain DAPI (VWR) to mark nuclei and Cy3-labeled phalloidin (ThermoFisher) to mark actin filaments. Fluor-conjugated secondary antibodies were obtained from Jackson Immunoresearch or ThermoFisher and used at a 1:500 dilution.

Imaging at Hannover Medical School was performed using a Zeiss LSM 700 confocal microscope equipped with dual photomultiplier tubes (PMTs); four laser lines at 405, 488, 555, and 647 nm; and the following objectives: 10x air (numerical aperture (NA) 0.45), 25x oil (NA 0.8), and 40x oil (NA 1.3). Imaging at the Mount Desert Island Biological Laboratory was performed using a spinning-disk confocal (CSU-W1, Yokogawa, Japan) on a Nikon inverted Ti-Eclipse microscope stand (Nikon Instruments Inc., Japan), using a Nikon 10x air objective (NA 0.45; ref: MRD70170). DAPI and AF488 fluorescence were excited using a 405 nm and 488 nm lasers and collected using bandpass filters (Chroma ref: ET436/20x and Semrock ref: FF01-525/50). Images were acquired in 1024x1024 pixels with a Scientific CMOS Zyla 4.2 (Andor Technology, United Kingdom) controlled with NIS AR 5.41.02 software (build 1711, Nikon Instruments Inc., Japan) and saved in nd2 file format. Z-stack images were taken of all samples. Either single focal plane or z-projections of multiple focal planes were used in figures, as appropriate to best depict each phenotype. Images were processed using Fiji software (Schindelin et al. 2012) and assembled in Adobe Illustrator (Adobe, Inc.).

miRNA target prediction

The following databases were used to search for predicted targets of *miR-317*: TargetScanFly (http://www.targetscan.org), MinoTar (https://www.flyrnai.org/cgi-bin/DRSC_MinoTar.pl), and microRNA.org (http://www.microrna.org). 975 targets were predicted by microRNA.org, 31 by MinoTar, and 102 by TargetScanFly; 76 targets were predicted by multiple databases for a total of 1030 predicted targets. These databases only consider transcripts of coding genes as potential miRNA targets, so to identify *miR-317* binding sites in lncRNAs, we performed a manual curation. The predominant *miR-317* species is *miR-317-3p*: UGAACACAGUGCUAAAUGAAAGA. We used the seed sequence (positions 2-7, GAACAC) as well as one base on either side of the seed to define potential targets. According to the TargetScanFly custom, transcripts were considered potential targets if they possessed the following: 8mer site UGUGUUCA; 7mer-m8 site UGUGUUC; 7mer-A1 site GUGUUCA; offset 6mer site UGUGUU; or 6mer site GUGUUC. We downloaded the sequences of all lncRNAs that were dysregulated in testis RNA-seq and searched for these sequences. Of the 192 lncRNAs dysregulated in testes, we found that 56 possess potential *miR-317* binding sites (Supplemental Table 1).

qRT-PCR

Drosophila tissues were dissected in cold PBS and transferred to microcentrifuge tubes. Total RNA was prepared using a standard Trizol-chloroform extraction followed by an isopropanol precipitation. RNA was quantified using a Nano-drop spectrophotometer (ThermoFisher) and treated with DNaseI. For expression levels of mRNA, lncRNA, or pre-miRNA, SYBR Green-based qPCR was performed. For measuring mature miRNA levels, a TaqMan-based assay was performed. Reactions were run in triplicate. Either α-Tubulin 84B (SYBR Green qPCR) or 2S rRNA (TaqMan qPCR) was used as an endogenous control. Expression fold change (FC) was determined using the expression $FC = 2^{-\Delta\Delta Ct}$.

3′ RACE PCR

To identify the site of polyadenylation between *Peony* and *miR-317*, a 3′ RACE procedure was performed. Total RNA was extracted from five adult male flies of the genotype *RpII215[c4]* using TRIzol-chloroform extraction and isopropanol precipitation. Briefly, the flies were homogenized in 400 µL of TRIzol, 80 µL of chloroform was added, and the sample was shaken vigorously by hand. The sample was centrifuged for 15 minutes at 4°C and 12,000 x g. The upper, aqueous phase was moved to a new tube, and isopropanol was used to precipitate RNA at room temperature for 10 minutes. The sample was centrifuged at 4°C and 12,000 x g for 10 minutes to pellet RNA. The pellet was washed with 75% ethanol, dried, and suspended in RNAse-free water. 4 µg of RNA was used for each reverse transcription reaction.

Reverse transcription was performed using SuperScript II (Invitrogen) according to the manufacturer's instructions. A control reaction lacking reverse transcriptase (-RT) was performed in parallel with the experimental reaction. An anchored oligo(dT) primer appended with a 5′ adapter sequence (5′-GGCCACGCGTCGACTAGTAC(T_{17})V-3′; Integrated DNA Technologies, IDT) was used for the RT reaction. The RT reaction progressed for two hours at 42°C, followed by 15 minutes at 95°C to inactivate the polymerase.

Identification of the polyA site was performed using a semi-nested PCR protocol. First, an "outer" nested reaction was performed using a gene-specific primer (*RACE_F1*, 5′-TTCGTTCGCTTGTGAGTGTCC-3′) within the second exon of *Peony*, and as reverse primer, the adapter portion of the anchored oligo(dT) was used: *3′ RACE PCR* (5′-GGCCACGCGTCGACTAGTAC-3′; IDT). This reaction proceeded for 25 cycles of PCR. Next, this PCR product was diluted 1:100 in water and used as template for the "inner" nested PCR. For this reaction, a forward primer (*RACE_F2*, 5′-ACACATACACGCACACAAACGG-3′) was used that is downstream of *RACE_F1*, still within the second exon of *Peony*. The same reverse primer, *3′ RACE PCR* (IDT) was used. PCR was performed for 35 cycles in three 50 µL reactions. The PCR product was run on a 1% agarose gel, and a prominent band at 400 bp was excised from the gel and the DNA isolated using a gel-extraction kit (Zymo). This DNA was Sanger-sequenced using the *RACE_F2* primer.

Generation of *UAS-Peony*

The pUASt-attB vector was linearized with XhoI. The *Peony* cDNA was generated from RNA taken from *w[1118]* flies and PCR amplified using the following primers (lowercase bases indicate overlap to the vector; uppercase bases correspond to genomic sequence): *UAS-Peony_F* aacagatctgcggccgcggcGAATTCTGAACACAGCAAAAAGTACTGCATATAATAC; *UAS-Peony_R2* aagatcctctagaggtacccGCTCAGCAGAGACACAAACCG. Gibson assembly was used to assemble insert and vector. Transgenesis was performed by GenetiVision (Houston, Texas); the transgene was site-specifically integrated at the VK1 attP site on chromosome 2R.

RNA-sequencing

RNA was extracted from ovaries and testes from young, 0-1-day-old *w[1118]* and *miR-317[ko]* flies. Accessory glands and other components of the male reproductive system were separated from testes, and mature eggs were removed from ovaries prior to RNA extraction. All samples were in triplicate. Total RNA was treated with DNaseI and submitted to the Transcriptome and Genome Analysis Laboratory (TAL) at the Georg-August-University, Göttingen, Germany. RNA

quality was assessed by TAL prior to library preparation and sequencing. Sequencing was performed on an Illumina HiSeq4000 system, generating 50-bp reads and approximately 50 million reads per sample. Alignment and differential expression analysis was performed by TAL, resulting in the identification of 2944 genes in ovaries and 1844 genes in testes that were dysregulated in *miR-317[ko]* (Supplemental Tables 1 and 2).

Declaration of interests

The authors declare that they have no competing interests.

This study includes no data deposited in external repositories.

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Author contributions

Conceptualization, TDC, HRS; Methodology, TDC, HRS; Investigation, TDC; Writing, TDC, HRS; Visualization, TDC, HRS; Funding Acquisition, HRS.

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Key Resources Table

Reagent type	Source	Identifier	Gene
Drosophila stock	BDSC #5	*OR-R*	-
Drosophila stock	BDSC #67747	*C587-Gal4*	-
Drosophila stock	BDSC #66561	*P{UAS-3xFLAG.dCas9.VPR}attP40*	-
Drosophila stock	BDSC #17125	*P{w[+mC]=EP}EP3412*	*Ada2b*
Drosophila stock	BDSC #31778	*P{w[+mC]=EP}Ada2b[G3857]*	*Ada2b*
Drosophila stock	BDSC #79975	*P{y[+t7.7] v[+t1.8]=TOE.GS02678}*	*Ada2b*
Drosophila stock	BDSC #6803	*w[*]; P{w[+mW.hs]=GawB}bab1[Pgal4-2]/TM6B, Tb[1]*	*bab1-Gal4*
Drosophila stock	BDSC #20298	*P{w[+mC] EPgy2}CG18265[EY11347]*	*CG18265*
Drosophila stock	BDSC #80311	*P{y[+t7.7] v[+t1.8]=TOE.GS03029}*	*CG18265*
Drosophila stock	BDSC #43450	*P{w[+mGS]=GSV1}EP-820[EP-820]*	*CG4467*
Drosophila stock	BDSC #21439	*P{y[+mDint2] w[+mC]=EPgy2}EY14693*	*CG6356*
Drosophila stock	BDSC #80310	*P{y[+t7.7] v[+t1.8]=TOE.GS03028}*	*CG9705*
Drosophila stock	BDSC #37146	*Mi{MIC}MI02131*	*Peony*
Drosophila stock	BDSC #458	*P{w[+mW.hs]=GawB}elav[C155]*	*elav-Gal4*
Drosophila stock	BDSC #27743	*w*; P{UAS-mam.A}2*	*mastermind*
Drosophila stock	BDSC #1596	*mam[8]/CyO*	*mastermind*
Drosophila stock	BDSC #58926	*miR-317[ko]*	*miR-317*
Drosophila stock	BDSC #6991	*P{w[+mW.hs]=GawB}MJ12a*	*MJ12a-Gal4*
Drosophila stock	BDSC #30727	*w[1118]; P{w[+m*]=NRE-EGFP.S}5A*	*NRE-GFP*
Drosophila stock	BDSC #85759	*P{y[+t7.7] v[+t1.8]=TOE.GS01580}*	*PIP5K59B*
Drosophila stock	BDSC #7403	*P{w[+mC]=UAS-rn.SP}1*	*rotund*
Drosophila stock	BDSC #3663	*RpII215[c4]*	*RpII215*
Drosophila stock	BDSC #5138	*y1 w*; P{tubP-GAL4}LL7/TM3, Sb1 Ser1*	*Tub-Gal4 (III)*
Drosophila stock	BDSC #7108	*w[*]; P{w[+mC]=tubP-GAL80[TS]}10*	*Tub-Gal80[ts] (II)*
Drosophila stock	BDSC #7017	*w[*]; P{w[+mC]=tubP-GAL80[TS]}2*	*Tub-Gal80[ts] (III)*
Drosophila stock	BDSC #6326	*w[1118]*	*white*
Drosophila stock	DGGR #203275	*P{GSV6}GS11364*	*Peony*
Drosophila stock	DGGR #104055	*y* w*; P{w+mW.hs=GawB}NP1624*	*tj-Gal4*
Drosophila stock	DGGR #109996	*P{w^{+mC}=vas-GAL4.2.6}2*	*vasa-Gal4*
Drosophila stock	Exelixis #f04665	*PBac{WH}f04665*	*Peony*
Drosophila stock	Exelixis #d02752	*P{XP}d02752*	*Peony*
Drosophila stock	this study	*UAS-Peony*	*Peony*
Drosophila stock	this study	*Df-miR-317*	*miR-317*
Drosophila stock	(Brennan et al, 1999)	*UAS-N[cdc10]*	*Notch*

Drosophila stock	(Chen & McKearin, 2003)	*P{w+mC=bam-GFP.-799/+133}3LR*	*bam*
Drosophila stock	(Larkin et al, 1996)	*UAS-N[B33c-3]*	*Notch*
Drosophila stock	(Ward, et al. 2006)	*P[UASp-Delta-2]*	*Delta*
Drosophila stock	Gift from Hannele Ruohola-Baker	*w*; P{GAL4-nos.NGT}40; P{GAL4::VP16-nos.UTR}CG6325MVD1*	*nos-Gal4*
Drosophila stock	Gift from Gerd Vorbrüggen	*Mef2-Gal4*	*Mef2-Gal4*
Drosophila stock	Gift from Stefan Jakobs	*Tub-Gal4*	*Tub-Gal4 (II)*
Drosophila stock	Gift from Christian Bökel	*zfh1:T2A:Gal4*	*zfh1*
Antibody	DSHB	Mouse anti-Adducin 1:20	NA
Antibody	DSHB	Mouse anti-Eyes absent 1:100	NA
Antibody	DSHB	Mouse anti-β-Galactosidase 1:200	NA
Antibody	DSHB	Mouse anti-LaminC 1:20	NA
Antibody	Abcam	Chicken anti-GFP 1:5000	NA
Antibody	Abcam	Rabbit anti-mCherry 1:200	NA
Antibody	Upstate Biotechnology	Rabbit anti-Phosphohistone H3 1:10000	NA
Antibody	Gift from Dorothea Godt	Guinea pig anti-Traffic jam 1:5000	NA
Antibody	Gift from Renate Renkawitz-Pohl	Guinea pig anti-β3-Tubulin 1:1500	NA
Antibody	Gift from Herbert Jäckle	Rabbit anti-Vasa 1:5000	NA
Stain	VWR	DAPI	NA
Stain	Thermofisher	Cy3-labeled Phalloidin	NA

DGGR = Department of Drosophila Genomics and Genetic Resources, Kyoto Institute of Technology

BDSC = Bloomington Drosophila Stock Center

DSHB = Developmental Studies Hybridoma Bank

Table 1: Predicted *miR-317* targets upregulated in testes in *miR-317[ko]* vs control; p<0.05 and $Log_2FC>0.5$

Gene ID	Name	Protein Description	Log_2 Fold Change	Adjusted p-value
FBgn0037427	*Osi17*	Osiris 17 ; integral component of plasma membrane	1.29	4.12E-09
FBgn0037419	*Osi12*	Osiris 12 ; integral component of plasma membrane	1.22	2.53E-08
FBgn0039178	*CG6356*	CG6356 ; transmembrane transport	1.05	2.63E-11
FBgn0066114	*GlcAT-I*	GlcAT-I ; Golgi membrane	1.02	9.05E-11
FBgn0036661	*CG9705*	Cold shock domain-containing protein CG9705 ; regulation of transcription, DNA-templated	0.81	1.03E-21
FBgn0037845	*CG14694*	IP11787p ; transmembrane transporter activity	0.79	1.62E-04
FBgn0267337	*rn*	Rotund ; transcription factor activity	0.69	3.58E-11
FBgn0031001	*CG7884*	CG7884, isoform B ; no biological data available	0.68	1.44E-03
FBgn0034789	*PIP5K59B*	PIP5K59B ; Phosphatidylinositol phosphate kinase activity	0.67	3.30E-03
FBgn0036725	*CG18265*	GH10077p ; nucleic acid binding	0.67	2.46E-03
FBgn0037555	*Ada2b*	Transcriptional Adaptor 2b ; regulation of transcription, DNA-templated	0.61	6.22E-07
FBgn0039064	*CG4467*	CG4467, isoform B ; proteolysis	0.58	2.93E-02
FBgn0037837	*CG14693*	Uncharacterized protein, isoform E	0.56	3.38E-08
FBgn0031275	*GABA-B-R3*	Metabotropic GABA-B receptor subtype 3 ; G-protein coupled receptor signaling pathway	0.54	1.98E-08
FBgn0033476	*oys*	Oysgedart ; lysophospholipid acyltransferase activity	0.53	1.46E-09

Table 2: Testing for GCT formation caused by overexpression (OE) of candidate genes

Gene	OE line	BDSC#	Type of OE line	Notes on OE line	Germline OE	Somatic OE	FLP-out OE
Predicted *miR-317* target genes upregulated in testes							
rn	*P{w[+mC]=UAS-rn.SP}1*	7403	in vitro-constructed UAS-gene fusion	*UAS-rn* (ii)	no tumors	**some tumors**	no tumors
CG18265	*P{w[+mC] EPgy2}CG18265 [EY11347]*	20298	UAS-containing transposon insertion	inserted in 5'UTR intron	no tumors	**some tumors**	n.d.*
CG18265	*P{y[+t7.7] v[+t1.8]=TOE.GS03029}*	80311	CRISPR-OE line	CG18265 OE gRNA	n.d.	**some tumors**	n.d.
Ada2b	*P{w[+mC]=EP}EP3412*	17125	UAS-containing transposon insertion	upstream of *Ada2b*	no tumors	no tumors	n.d.
Ada2b	*P{w[+mC]=EP}Ada2b[G3857]*	31778	UAS-containing transposon insertion	inserted in 5'UTR	no tumors	no tumors	n.d.
Ada2b	*P{y[+t7.7] v[+t1.8]=TOE.GS02678}*	79975	CRISPR-OE line	*Ada2b* OE gRNA	n.d.	no tumors	n.d.
CG4467	*P{w[+mGS]=GSV1}EP-820[EP-820]*	43450	UAS-containing transposon insertion	inserted upstream of *CG4467*	no tumors	no tumors	n.d.
CG6356	*P{y[+mDint2] w[+mC]=EPgy2}EY14693*	21439	UAS-containing transposon insertion	*CG6356* OE	no tumors	no tumors	n.d.
CG9705	*P{y[+t7.7] v[+t1.8]=TOE.GS03028}*	80310	CRISPR-OE line	*CG9705* OE gRNA	n.d.	no tumors	n.d.
PIP5K59B	*P{y[+t7.7] v[+t1.8]=TOE.GS01580}*	85759	CRISPR-OE line	*PIP5K59B* OE gRNA	n.d.	no tumors	n.d.
Notch-related genes							
Delta	*UASp-Delta*	NA	in vitro-constructed UAS-gene fusion	OE full-length Dl	no tumors	n.d.	n.d.
Notch	*UAS-N[cdc10]*	NA	in vitro-constructed UAS-gene fusion	OE of NICD	lethal	mostly lethal; no tumors	no tumors
Notch	*UAS-N[B33c-3]*	NA	in vitro-constructed UAS-gene fusion	OE constitutively active N	lethal	mostly lethal; no tumors	mostly lethal; no tumors
mam	*w*; P{UAS-mam.A}2*	27743	in vitro-constructed UAS-gene fusion	n.d.	n.d.	**some tumors**	**some tumors**
***Peony* OE**							
Peony	*UAS-Peony*	NA	in vitro-constructed UAS-gene fusion	OE full-length *Peony* cDNA	no tumors	no tumors	no tumors

* n.d., experiment not done

Carney_Figure_1

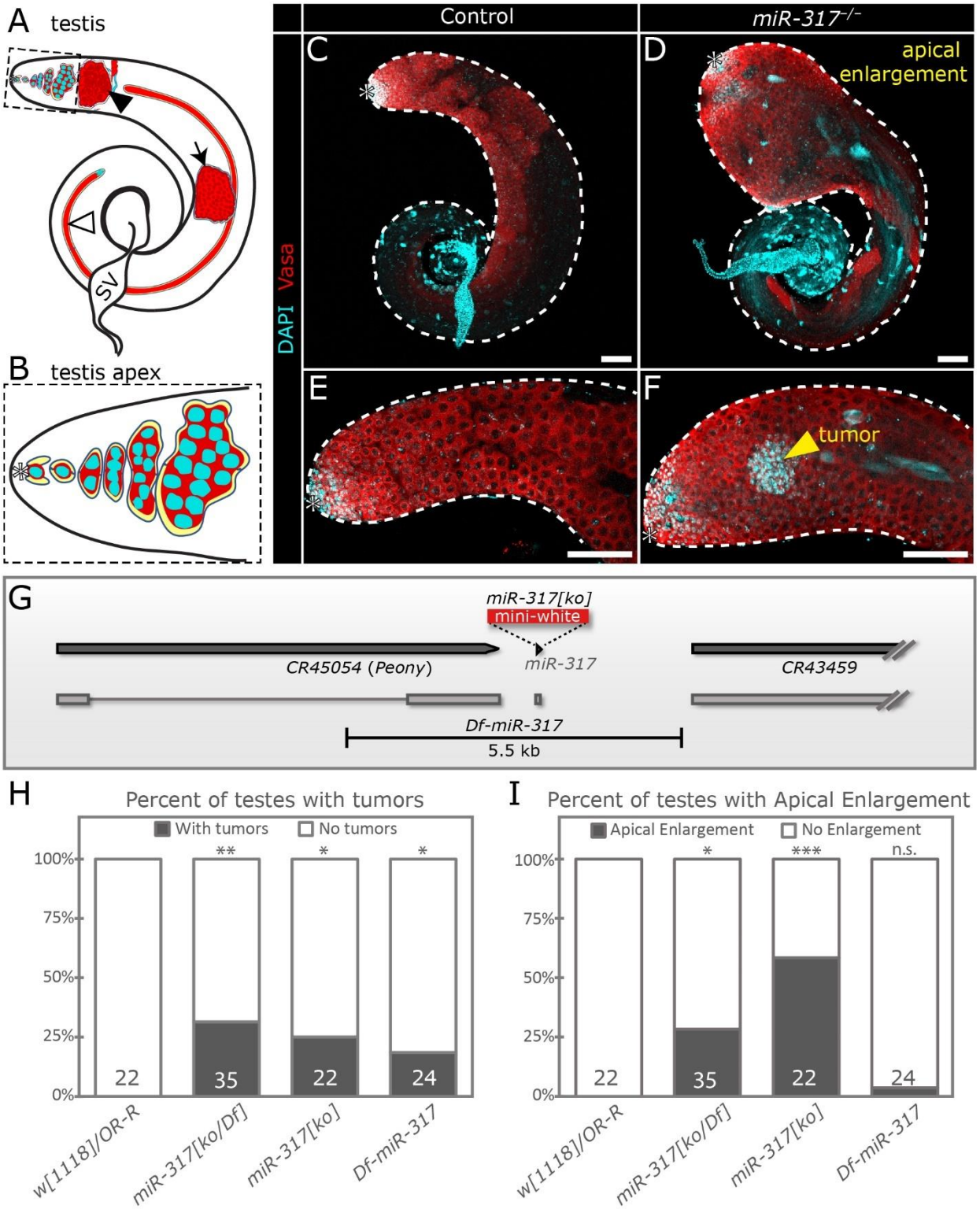


Figure 1. *miR-317* mutation causes testis phenotypes in adult males

A Schematic of one adult testis. Shown are stem cell niche (hub; denoted by asterisk); somatic cyst cells (yellow) surrounding germline cysts; 16-cell spermatocyte cyst (solid arrowhead); 64-cell post-meiotic cyst (black arrow); elongated spermatid-stage cyst (open arrowhead); and seminal vesicle (SV). Dashed box outlines the apex, enlarged in B. **B** Testis apex. Hub (asterisk); germline cells (red) surrounded by somatic cyst cells (yellow). Germline cells divide to form 16-cell cysts. **C** Control testis is a coiled, closed-ended tube (outlined with white dashed line). **D** *miR-317*$^{-/-}$ testis exhibiting apical enlargement phenotype. **E** Control testis apex. **F** *miR-317*$^{-/-}$ apex containing a tumor (yellow arrowhead), identified by strong expression of Vasa (red) as well as strong DAPI staining (cyan) distal from the apex. **G** Genomic context of *miR-317*, which is closely apposed to the lncRNA gene *CR45054* (*Peony*). *miR-317[ko]* is a knock-in mutant in which the pre-miRNA is replaced by a *mini-white* cassette. *Df-miR-317* is a 5.5-kb deletion of *miR-317* and the large second exon of *Peony*. **H-I** Quantification of the percent of testes exhibiting tumors (H) or apical enlargement (I). Scale bars in C-F are 100 µm. *, $p<.05$, **, $p<.01$; ***, $p<.001$ as determined by chi-square analysis; n=22-35 testes per genotype.

Carney_Figure_2

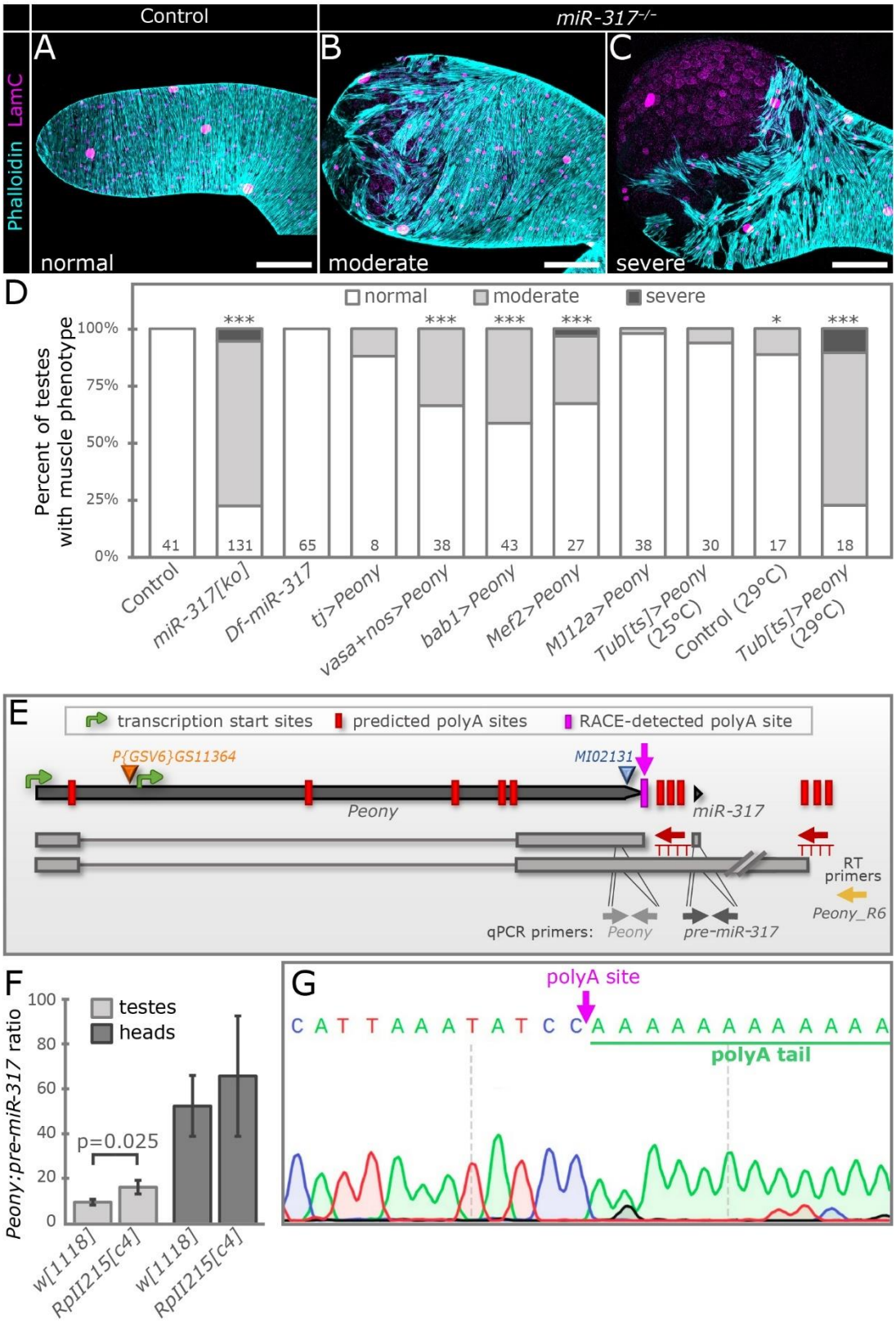


Figure 2. *Peony* levels are regulated by alternative polyadenylation and affect testis morphology

A Control testis apex stained with Phalloidin (cyan) to mark the muscle sheath and anti-LamC (magenta) to mark nuclear envelopes. Multinucleated muscle cells have many small nuclei; pigment cells have large, sparsely spaced nuclei. **B-C** *miR-317*$^{-/-}$ testis apexes with moderate (B) and severe (C) muscle sheath disruption. In C, the muscle sheath is severely disrupted, and the underlying germline is revealed by LamC staining. **D** Quantification of muscle disruption phenotypes in testes of various genotypes: *miR-317* mutants and *Peony* overexpression using cell- and tissue-specific drivers. **E** Schematic of the *Peony-miR-317* locus showing predicted transcription start sites and polyA sites; polyA site detected using 3′RACE; transgenic insertions used in this study; and locations of primers used for reverse transcription and qPCR. The most distal polyadenylation sites are not annotated and are predicted based on this work. Transcripts (light gray rectangles) depict putative alternative isoforms that differ based on alternative polyadenylation. **F** *Peony:*pre-*miR-317* ratios resulting from qRT-PCR using the qPCR primers shown in panel E in two tissues (testes and heads) from two genotypes: *w[1118]* and *RpII215[c4].* Shown are mean ± standard deviation from three biological replicates; statistical significance determined using a Student's t-test. **G** Sequencing result of 3′RACE shows the site of cleavage and polyadenylation near the annotated 3′ end of *Peony*. Scale bars: 100 µm. In D: *, $p<0.05$; ***, $p<10^{-3}$ as determined by chi-square analysis.

Carney_Figure_3

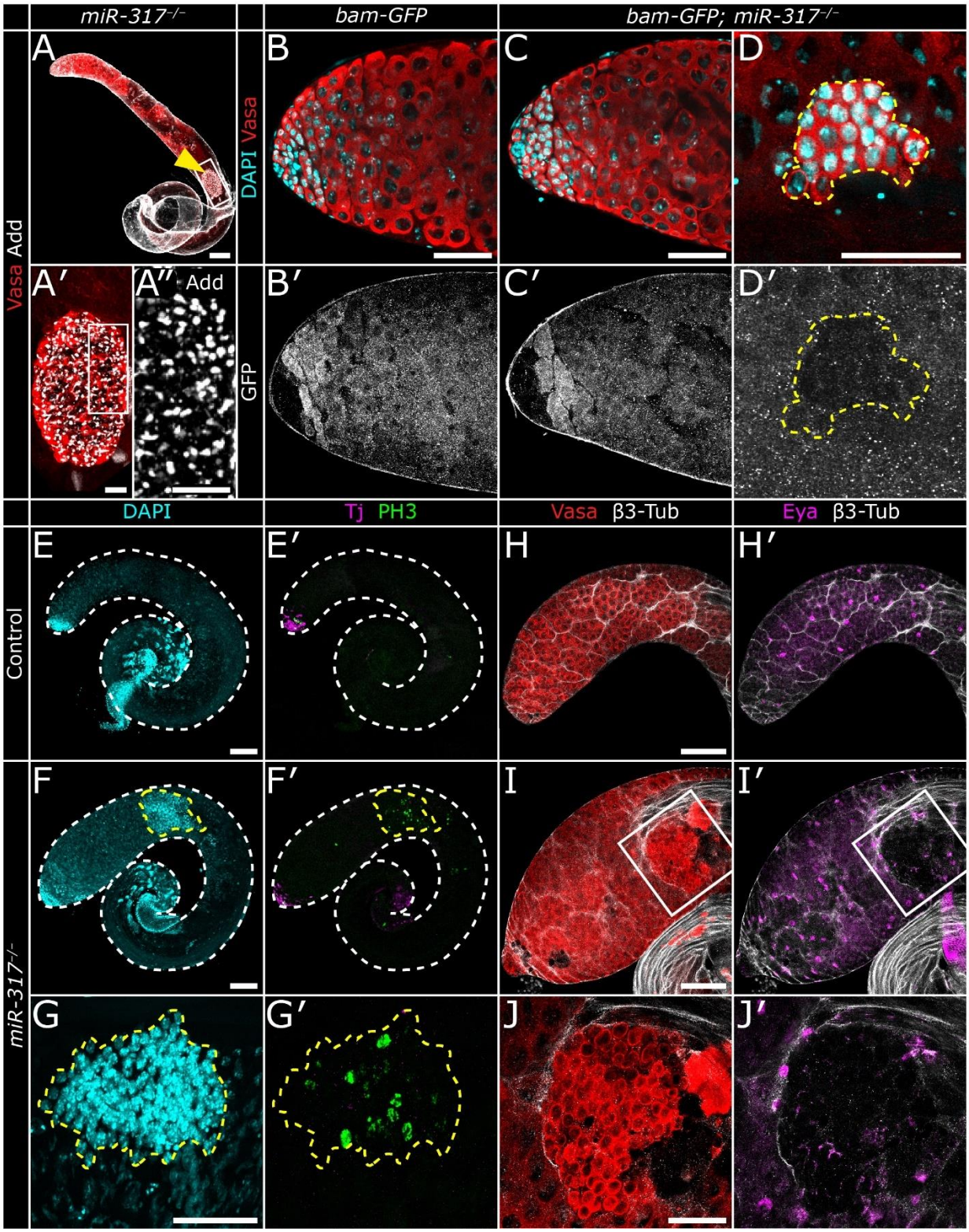


Figure 3. *miR-317* mutant testis tumors are composed of early germline cells

A *miR-317*$^{-/-}$ testis exhibiting a tumor (yellow arrowhead) with strong Vasa (red) and Adducin (Add; grayscale) staining. **A′** Enlargement of the tumor outlined with a white rectangle in A. All cells are Vasa-positive and Add-positive. **A″** Enlargement of the white rectangle in A′ shows Add staining exists mostly in spherical-shaped spectrosomes, with a minority exhibiting minimal branching. **B** Apex of testis with a *bam* transcriptional reporter (*bam-GFP*). GFP is shown in single panel in B′. **C** *bam-GFP* in a *miR-317*$^{-/-}$ background; GFP pattern (**C′**) does not appear different from control (**B′**). **D** Tumor in a *bam-GFP; miR-317*$^{-/-}$ testis; tumors of this genotype do not express *bam-GFP* (**D′**). **E** Control testis (outlined with white dashed line) exhibiting strong DAPI staining near the apex, in the region where germline cells undergo mitotic divisions. **E′** Tj-positive cyst cells reside near the testis apex, in proximity of the mitotic zone. PH3-positive cells mark mitotically active germline cells, which divide in synchrony within each germline cyst. **F** *miR-317*$^{-/-}$ testis with a large tumor, outlined by yellow dashed line and identified by region of bright DAPI-positive cells removed from the apex. **F′** Same testis as shown in F, now showing that while some cells in the tumor are PH3-positive, the Tj-positive cells are present only near the apex as in controls. **G** A *miR-317*$^{-/-}$ tumor with bright DAPI staining. **G′** Same tumor as shown in G. Some scattered cells are PH3-positive; tumor and surrounding cells are Tj-negative. (E,F, & G: DAPI, cyan; PH3, green; Tj, magenta). **H** Apex of control testis. Vasa marks germline cells, and β3-Tub marks cyst cell cortexes. **H′** Same testis as in H; Eya marks somatic cyst cell nuclei. **I** *miR-317*$^{-/-}$ testis stained with Vasa and β3-Tub and exhibiting a tumor (indicated by white box). **I′** Same testis as in I; β3-Tub and Eya mark cyst cell cortexes and nuclei, respectively. **J-J′** Enlargement of the tumor shown in I & I′ showing that while Vasa is strongly expressed in the tumor, β3-Tub and Eya are excluded. (H-J: Vasa, red; β3-Tub, grayscale; Eya, magenta). A, E, F, H, I: scale bars 100 µm. B, C, D, G, & J: scale bars 50 µm. A′, A″: scale bars 20 µm.

Carney_Figure_4

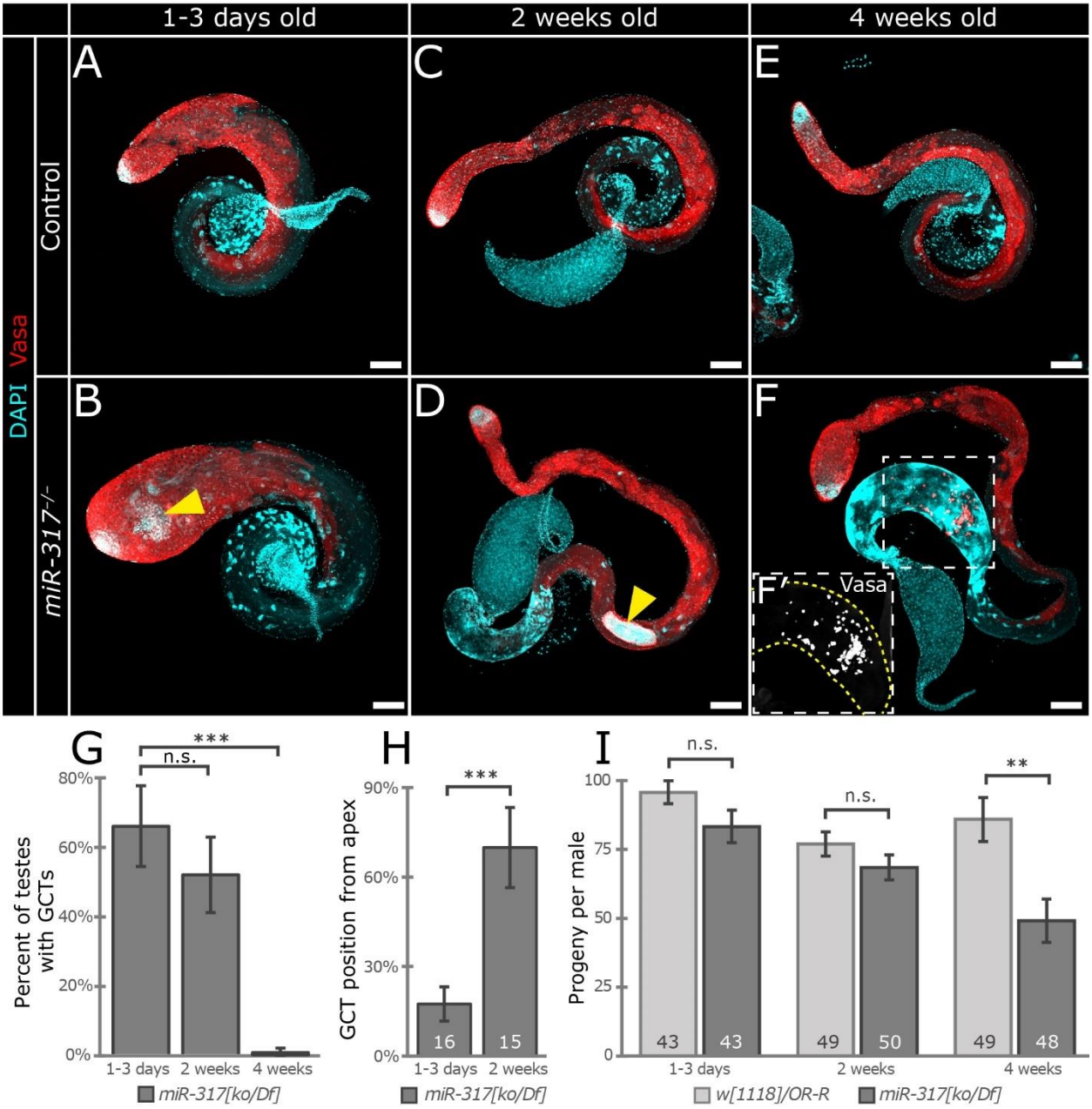


Figure 4. GCTs arise near the testis apex and disintegrate basally in aging testes

A Young, 1-3-day-old control testis stained for DAPI (cyan) and Vasa (red). **B** Young *miR-317*$^{-/-}$ testis exhibiting a GCT near the apex (yellow arrowhead) with strong Vasa and DAPI staining. **C** 2-week-old control testis. **D** 2-week-old *miR-317* mutant testis with a large GCT in the basal half of the testis (yellow arrowhead). **E** 4-week-old control testis. **F** 4-week-old *miR-317* mutant testis with the partially disintegrated remains of a GCT near the base of the testis (white square with dashed lines), discernible by strong Vasa staining. **F′** Enlargement of the box in F showing Vasa in grayscale. **G** Graph showing the incidence of GCTs in *miR-317*$^{-/-}$ testes over time. **H** Quantification of the average position of GCTs as a percentage from apex to testis base. **I** Fertility results reported as number of progeny produced per male for each genotype and time point. Error bars indicate standard deviation in G-H and SEM in I. Data in G based on 3 or 4 biological replicates. Sample sizes (given as numbers on each bar) indicate number of GCTs counted for each genotype (H) and number of males assayed (I). Student's t-test used to determine statistical significance. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s. = not significantly different. Scale bars 100 µm.

Carney_Figure_5

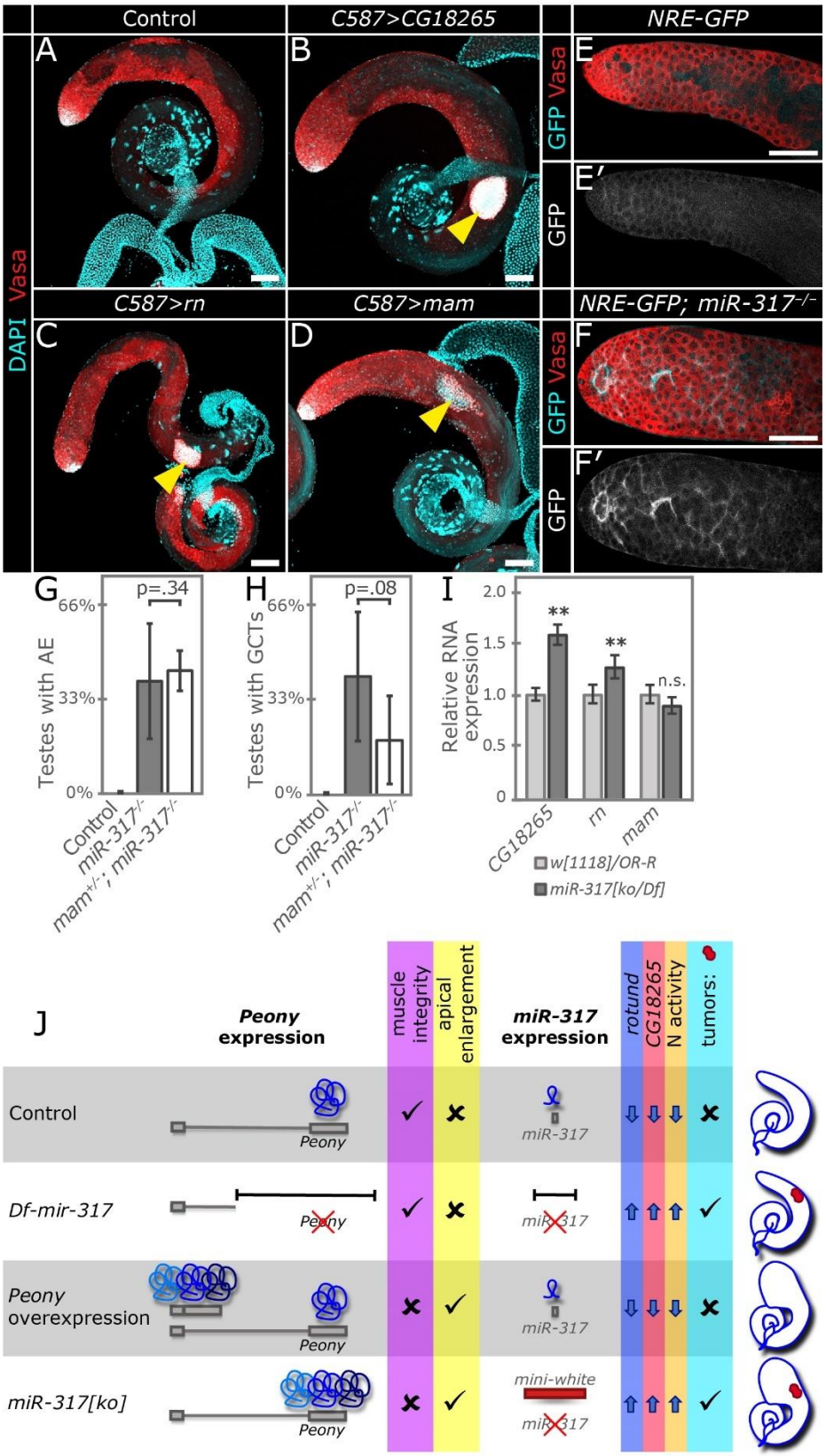
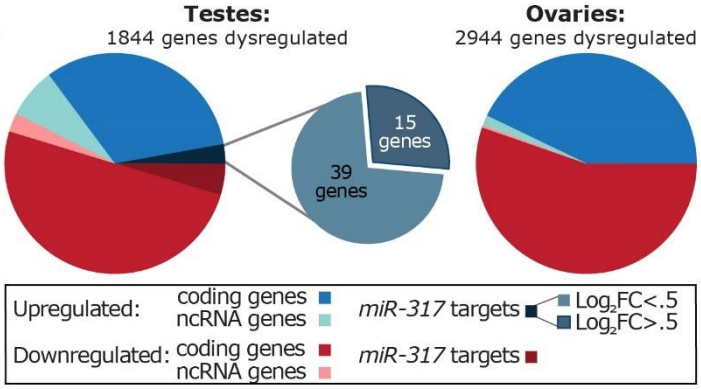


Figure 5. *miR-317* regulates multiple targets to prevent tumor formation

A-D Testes stained for DAPI (cyan) and Vasa (red). **A** Control testis. **B-D** Testes overexpressing *CG18265* (B), *rn* (C), or *mam* (D) under the control of the cyst-cell driver *C587-Gal4*, each exhibiting a GCT (yellow arrowhead). **E** Apex of control testis (*NRE-GFP*) in which GFP is expressed under the control of a Notch reporter element (NRE). GFP is nearly undetectable (GFP shown in grayscale in E′). **F** GFP expression is strongly upregulated in *NRE-GFP; miR-317*$^{-/-}$ testes (GFP shown in grayscale in F′). **G-H** Quantification of the percentage of testes that exhibit AE (G) or GCTs (H) in control, *miR-317*$^{-/-}$, or *mam*$^{+/-}$*; miR-317*$^{-/-}$ testes. Graphs depict mean ± standard deviation based on 3-7 biological replicates. **I** Results of qRT-PCR experiments assaying expression levels of *CG18265*, *rn*, and *mam* in male L3 larvae. Graph depicts mean ± confidence intervals based on 1 SEM. **J** Summary of our proposed model of the differential effects of *Peony* and *miR-317* on testes. In controls, *Peony* is expressed at its endogenous level, muscle integrity is intact, and AE is absent; *miR-317* is expressed, repressing target genes *rotund* and *CG18265* and Notch (N) activity, and tumors are absent. In *Df-miR-317* and *miR-317[ko]*, *miR-317* is absent, target genes are upregulated, N activity is increased, and GCTs (red clusters in testis schematics) form. In both *Peony* overexpression and *miR-317[ko], Peony* levels are high, causing loss of muscle integrity and the appearance of AE. **A-F**, scale bars 100 µm. **G-I**, statistical significance assessed using the Student's t-test; **, $p<.01$.

Carney_Supplemental_Figure_1

A



B

Testes			Ovaries	
GO#	p-value	Biological process:	GO#	p-value
GO:0002181	1.3E-16	translation	GO:0006412	3.9E-16
GO:0007165	6.9E-06	signal transduction	GO:0009966	1.2E-15
GO:0030154	8.8E-08	cell differentiation	GO:0030154	1.7E-18
GO:0007154	4.9E-06	regulation of cell communication	GO:0010646	1.2E-13
GO:0048858	6.5E-07	cell projection morphogenesis	GO:0048858	8.1E-07
GO:0016477	2.8E-07	cell migration	GO:0016477	1.4E-04
GO:0060541	6.8E-06	aerobic respiration	GO:0009060	1.8E-04
-	-	gene expression regulation	GO:0010468	1.2E-19
-	-	mitotic cell cycle	GO:0000278	3.5E-14
GO:0034330	1.2E-06	cell junction organization	-	-
GO:0030239	2.3E-04	myofibril assembly	-	-

C

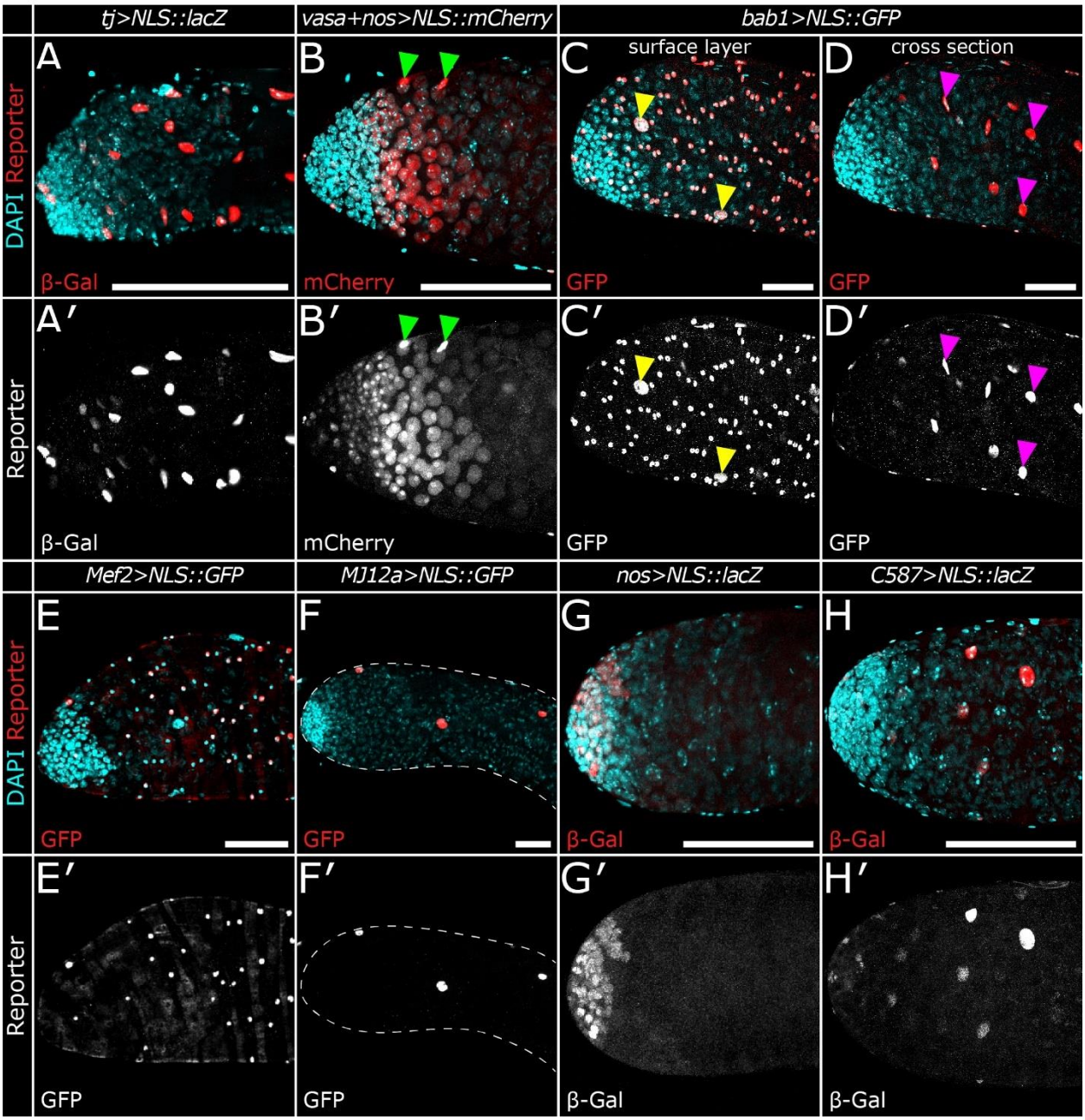
Testes - top dysregulated lncRNA genes		
Gene name	Log_2fold change	adjusted p-value
CR44178	5.37	4E-263
CR43258	4.33	2E-120
CR44674	4.21	5E-222
Peony	3.91	8E-105
CR46039	3.83	8E-87
CR44955	3.47	2E-105
CR44020	2.75	1E-49
CR43652	2.48	1E-32
CR44934	2.06	1E-43
CR43950	2.03	4E-21
CR46044	1.95	8E-20
CR45688	1.69	5E-16
CR45575	1.52	2E-12
CR43972	1.51	3E-14
CR43985	-1.53	5E-12
CR44033	-1.63	7E-18
CR43849	-1.83	2E-41
CR44941	-1.99	2E-22
CR44401	-2.07	3E-47
CR45598	-2.51	1E-50
CR44678	-2.51	6E-36
CR44349	-2.66	1E-38
CR41352	-3.36	1E-63
CR44333	-3.48	5E-67
CR45633	-3.77	1E-83
CR44335	-4.52	1E-133

Ovaries - top dysregulated lncRNA genes		
Gene name	Log_2fold change	adjusted p-value
Peony	4.16	3E-22
CR45631	2.39	4E-08
CR43626	2.25	9E-13
CR43973	2.02	4E-10
CR43857	2.00	4E-12
CR45466	1.81	2E-04
CR45596	1.66	4E-06
CR43975	1.57	2E-03
CR44923	1.54	1E-04
CR45171	1.52	4E-04
CR45756	-1.60	1E-03
CR45386	-2.12	2E-05

Supplemental Figure 1 (related to Figure 1): *miR-317[ko]* RNA-seq results from testes and ovaries

A Results of RNA-seq experiment comparing control (*w[1118]*) and mutant (*miR-317[ko]*) ovaries and testes. Genes downregulated in mutant tissues are indicated in shades of red; upregulated genes indicated in blues. More non-coding genes were dysregulated in testes than in ovaries (light blue and pink). For testes, *in-silico*-predicted *miR-317* targets are shown (dark blue and dark red); those predicted targets that were upregulated in miR-317 mutant testes were analyzed further regarding the magnitude of upregulation ($Log_2FC>0.5$ or $Log_2FC<0.5$; blue-gray pie chart). **B** Gene ontology (GO) analysis of the dysregulated genes in testes and ovaries; many of the most enriched 'biological process' GO terms were common between the two organs. For each process shown, the corresponding GO number is given as well as the significance of enrichment in each organ. **C** The most upregulated and downregulated lncRNAs are shown for both testes and ovaries (those with $Log_2FC>1.5$ or $Log_2FC<-1.5$). The Log_2FC values are shown in blue if upregulated, red if downregulated, as in Supplemental Tables. As indicated in A, more lncRNAs are dysregulated in testes than in ovaries. In both organs, *Peony* is one of the most upregulated lncRNAs in *miR-317* mutants (highlighted in green and blue for testes and ovaries, respectively).

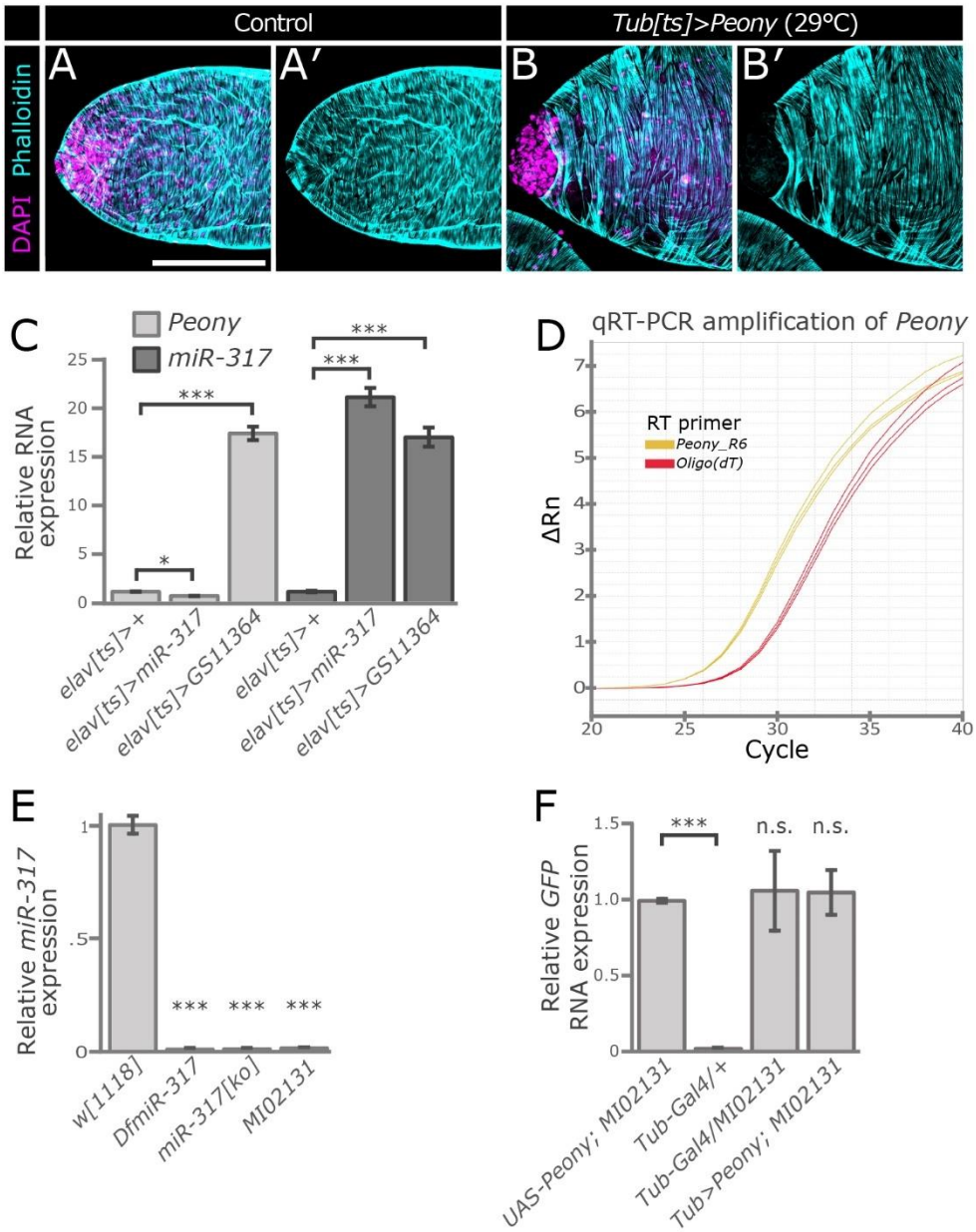
Carney_Supplemental_Figure_2



Supplemental Figure 2 (related to Figure 2): Testis expression patterns of Gal4 lines used in this study

A-D Expression patterns of Gal4 lines visualized by expression of reporters from UAS transgenes bearing a nuclear localization sequence (NLS): *UAS-NLS::lacZ* (A, G, & H); *UAS-NLS::mCherry* (B); and *UAS-NLS::GFP* (C-F). A′-H′ show reporter protein expression in single grayscale panels. **A** *traffic jam-Gal4* (*tj-Gal4*) is expressed in somatic cyst cells. **B** *vasa-Gal4; nos-Gal4* (*vasa+nos-Gal4*) is expressed primarily in germline cells but exhibits expression in some somatic cyst cells as well (examples: green arrowheads). **C-D** *bric a brac 1-Gal4* (*bab1-Gal4*) is expressed in somatic cells of the testis. Panel C shows a surface section in which muscle cell nuclei (numerous small nuclei) and pigment cell nuclei (yellow arrowheads) express GFP. D shows a deeper, internal cross section in which somatic cyst cell nuclei express GFP (examples: magenta arrowheads). **E** *Mef2-Gal4* drives expression in some, but not all, muscle cell nuclei. **F** *MJ12a-Gal4* is expressed only in pigment cells. **G** *nanos-Gal4* (*nos-Gal4*) is expressed in early germline cells near the apex. **H** *C587-Gal4* is expressed in somatic cyst cells. A, C, D, and F each depicts a single focal plane. Panels B, E, G, and H each depicts a z-projection of multiple focal planes to better illustrate expression patterns. All scale bars: 100 µm.

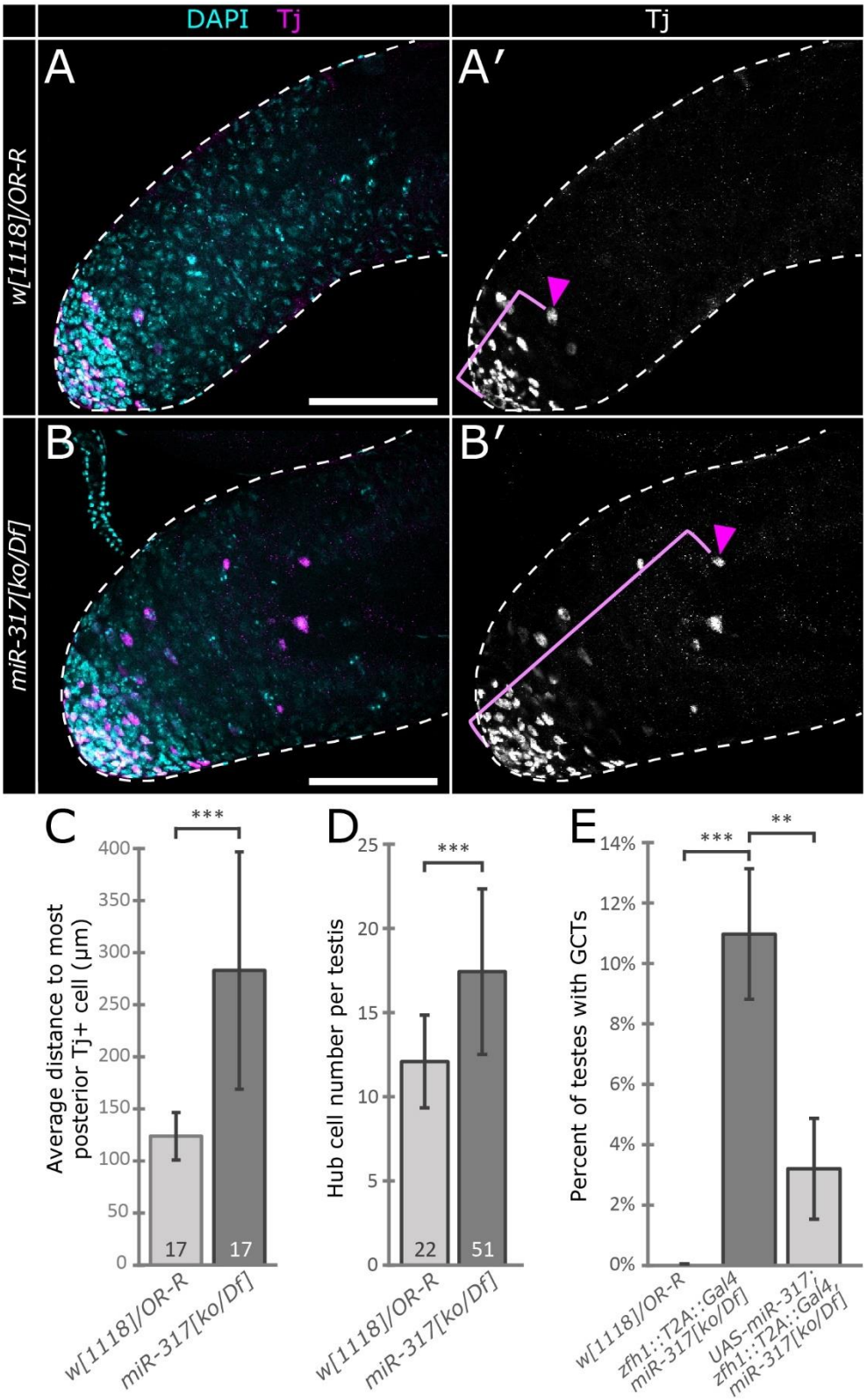
Carney_Supplemental_Figure_3



Supplemental Figure 3 (related to Figure 2): Regulation of the *Peony-miR-317* locus

A Apex of control testis stained for DAPI and phalloidin. Panel A′ depicts only phalloidin, showing an intact muscle sheath covering the apex. **B** Enlarged apex of testis overexpressing *Peony* under the control of *Tub-Gal80; Tub-Gal80[ts]* (*Tub[ts]>Peony*). Phalloidin stain (shown alone in panel B′) shows a broken muscle sheath at the apex. **C** Results of qRT-PCR using qPCR primers in *Peony* and pre-*miR-317.* Control expression levels were compared with overexpression using *elav-Gal4; Tub-Gal80[ts]* (*elav[ts]*). **D** Amplification curves from an RT-PCR experiment using downstream primers Peony_R6 or oligo-dT for RT followed by PCR using primers within *Peony*'s second exon. **E** Mature *miR-317* levels in control (*w[1118]*) and three mutant genotypes: *Df-miR-317, miR-317[ko],* and MiMIC transposon *MI02131*. **F** qRT-PCR results using primers targeting the GFP open reading frame in the *MI02131* transposon. *GFP* is absent from *Tub-Gal4/+* flies that lack the MiMIC insertion. *GFP* levels are not changed by overexpression of *Peony* (*Tub>Peony; MI02131*) compared to two controls: *UAS-Peony; MI02131* and *Tub-Gal4; MI02131*. Graphs show mean ± standard deviation. Statistical significance was assessed using Student's t-test based on technical triplicates (C&E) or biological triplicates (F). *, p<.05; ***, p<.001; n.s., not significantly different. Scale bar: 100 µm.

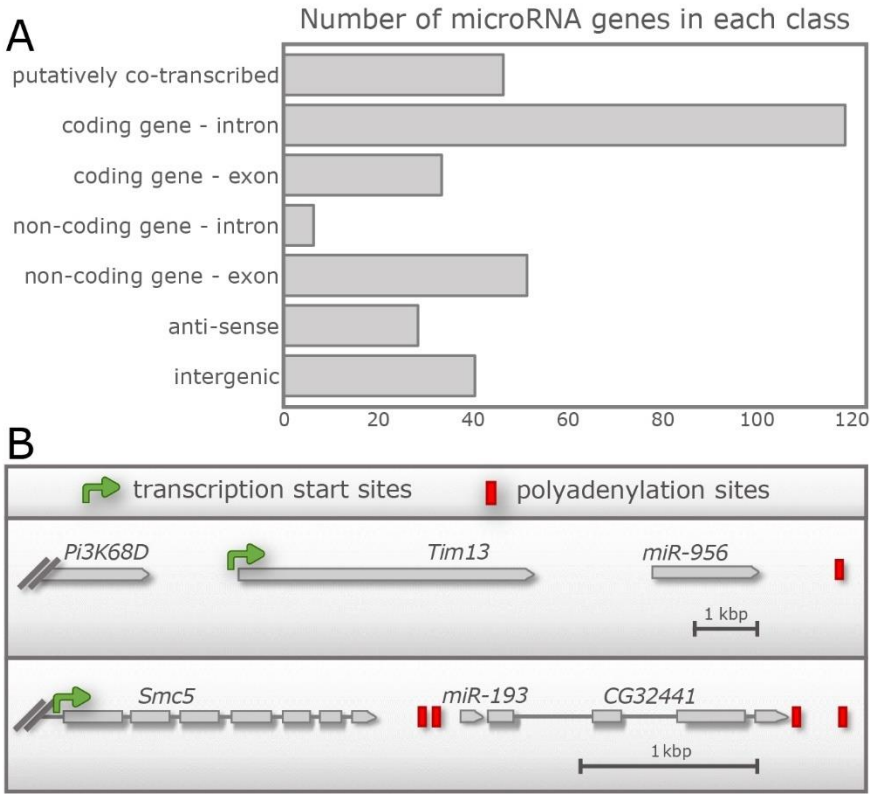
Carney_Supplemental_Figure_4



Supplemental Figure 4 (related to Figure 3): Effects of miR-317 on testis somatic cells

A Control (*w[1118]/OR-R*) testis apical region in which Tj^+ cells are clustered near the apex, reflecting the expression of Tj in hub cells, cyst stem cells, and early somatic cyst cells. **B** *miR-317[ko/Df]* testis apex exhibiting Tj^+ cells clustered at the apex as well as a small number of Tj+ cells located more posterior to the DAPI-bright spermatogonia. In A′ and B′, Tj is shown in grayscale. Magenta arrowheads indicate the most-distal Tj^+ cell, and brackets indicate distance from the apex. **C** Quantification of the average distance in µm from apex to most distal (posterior) Tj^+ cell. **D** Quantification of the average number of hub cells per testis in controls (*w[1118]/OR-R*) and mutants (*miR-317[ko/Df]*). **E** Quantification of percent of testes bearing GCTs in a rescue experiment using *zfh1::T2A::Gal4* to express *miR-317* in a mutant background. Controls exhibit no tumors. Overexpression of *miR-317* significantly rescues the mutant's tumor formation phenotype. Error bars indicate standard deviation from the mean; Student's unpaired t-test was utilized to test for statistical difference between genotypes based on number of testes (C) or hubs (D) quantified or biological duplicates (E); **, $p<.01$; ***, $p<.001$. Sample sizes given on bar graphs in C and D. Scale bars: 100 µm.

Carney_Supplemental_Figure_5



Supplemental Figure 5 (related to Discussion): Putatively co-transcribed gene pairs in Drosophila

A Graph showing the number of Drosophila miRNA genes in each of several major categories. Putatively co-transcribed miRNAs reside closely upstream or downstream of other genes in the same orientation. **B** Examples of two putatively co-transcribed miRNA genes. *miR-956* is downstream of *Tim13*, and *miR-193* is immediately upstream of *CG32441*.