Responses to Reviewers' Critiques

MOLECULAR-CELL-D-12-01793: "An Ancient Transcription Factor Initiates the Burst of piRNA Production During Meiosis in the Mouse Testis"

MOLECULAR-CELL-D-12-01792: "Redefining the piRNA-Producing Loci of the Mouse Testis as Genes"

We would like to thank the Reviewers for rapidly and thoughtfully evaluating our two manuscripts. We are grateful for their enthusiasm for our A-MYB story, and we have now incorporated the relevant data from the second manuscript into a single revised A-MYB paper. We also thank the Reviewers for their favorable comments on the importance of our discovery that A-MYB regulates transcription of pachytene piRNA genes and on the quality of our data and analyses.

Both Reviewers urged us to combine the two manuscripts. We concur with the view that a single manuscript focused on the role of A-MYB in pachytene piRNA biogenesis is more effective. Our second manuscript presented experiments and analyses that underpin the A-MYB story as well as more tangential findings about the promoter architecture, genic structure, and transposon content of piRNA-producing loci. To produce a single, unified manuscript about A-MYB regulation of pachytene piRNA production, we have included in the revised manuscript some of the figures previously in the Resource paper:

- Figure 1 (now Figure 1A), which describes how we defined piRNA genes and Figure S1 which shows the location of these genes on the chromosomes;
- a revised version of Figure 2 (now incorporated into Figure 1 as panel B), which presents our aggregated data showing that piRNA genes are transcribed by RNA polymerase II and not by RNA polymerase III (formerly Figure S4C);
- a new Figure 3 and accompanying Figure S3 presenting examples of piRNA genes defined by our data sets and analytical methods;
- and Figure S2B, which show that the piRNA genes we define more efficiently account for the piRNAs in mouse testis than previous piRNA clusters (now S1B), and a new Figure S1C which shows that our piRNA genes have more accurate 5′ and 3′ ends than the previous piRNA clusters.

Figures 4–7 (and related supplementary material) of the original Resource manuscript enumerated the detailed properties of piRNA-producing genes.

These do not directly contribute to the A-MYB story, and we have not included them in the unified manuscript.

We have added two replicate RNA-seq data sets for *A-Myb* mutant and heterozygous testes at 14.5 dpp and 17.5 dpp, bringing the total depth of our data to 404 Million (14.5 dpp) and 246 Million (17.5 dpp) genome-mapping reads. These critical analyses are now based on three biologically independent replicates each for mutant and control. This has allowed us to focus more narrowly on those piRNA biogenesis proteins most significantly regulated by A-MYB. In the process of adding these replicates, we improved our RNA-seq normalization procedure for all data sets, resulting in minor changes in rpkm values (e.g., Figure 2B, middle). In rooster, we detected an additional piRNA pathway protein bound by A-MYB, *TDRD1* (added to Figure 8E). We also identified the previously unannotated rooster *PIWIL2* gene, allowing us to determine that it is not bound by A-MYB.

Reviewer #1

The only major criticism that I have is the claim of the feed forward loop.

I simply do not follow the author's argument. For me, A-MYB is simply a master regulator of the pachytene piRNA program. It is essential to provide the transcripts for pachytene piRNAs and also for MIWI (both are essentially not expressed at earlier stages) and it boosts the production of several piRNA pathway genes, maybe to make piRNA biogenesis more efficient to cope with the massive increase in piRNA levels. In my opinion, there is simply no loop as claimed by the authors. The only feed forward loop is that A-MYB seems to positively control its own transcription, a scenario well known for many TF. So while the findings are very interesting, I do not support the claim that this is a feed forward loop.

A coherent feed-forward loop is defined solely by its architecture. The wiring diagram describing A-MYB regulation of piRNA production (Figure 5C) corresponds precisely to the connectivity of a feed-forward loop as described by Uri Alon and colleagues (e.g., Figure 1a, Mangan and Alon, *PNAS* 2003).

In contrast, **A-MYB auto-regulation is not a feed-forward loop; it is a positive feed-back loop.** Positive feed-back loops are indeed common for transcription factors. Feed-forward and positive feed-back loops have quite different properties: positive feed-back loops amplify small increases in initiating signal, while coherent feed-forward loops reject such small fluctuations; positive feed-back loops can produce runaway behavior (a nuclear meltdown is an example of a positive feed-back loop), while feed-forward loops tend to produce

a rapid increase in output after an initial delay, then self-extinguish when the initiating signal decreases.

The authors pick this argument again up in the discussion when they write:

"Pachytene piRNA levels increase 75-fold (median for the 100 genes) from 10.5 to 12.5 dpp coincident with increased expression of A-Myb. However, from 12.5 to 14.5 dpp, pachytene piRNAs increase only 1.2-fold. Pachytene piRNAs subsequently resume their accumulation, increasing 65-fold from 14.5 to 17.5 dpp."

I have two concerns here: First, the piRNA increase from 10.5 to 12.5dpp deals with very low overall numbers and is therefore rather sensitive to over- or under estimations.

The numbers are not low; we simply report them in the standard unit ppm. Of course, our sequencing depth was much greater than 1 million reads and the actual values for C57BL/6J wild-type testes at 10.5 dpp and 12.5 dpp are 33- to 55-times higher:

time	total genome-mapping reads	total piRNA reads
10.5 dpp	68,434,206	52,289,600
12.5 dpp	41,941,594	33,879,779

Secondly, in the absence of biological replicates such a statement is simply difficult in my opinion.

While replicates would be ideal, our parallel data sets cross-confirm each other. For example, our wild-type oxidized piRNA data sets show a 65-fold increase in overall pachytene piRNA abundance from 14.5 to 17.5 dpp; our *Miwi* heterozygotes show a 68-fold increase over the same time period. Moreover, total miRNA hairpin-mapping reads, as a percent of total genome-mapping reads, differ only by ~11% between the 10.5 dpp and 12.5 dpp wild-type data sets. (This is true even if we exclude annotated ncRNAs from the total.)

Minor Comments:

1."The production of MIWI per se does not cause the shift of hybrid and pachytene piRNAs from MILI to MIWI, since pre-pachytene piRNAs retain their 27 nt modal length (Figure 6B) and predominant loading into MILI (Figure 6C) throughout post-natal spermatogenesis." can the authors really conclude this? This would suggest that the cell is able to discriminate which precursors feed into MILI and which into MIWI.

That is precisely the hypothesis we favor, particularly as we believe that a similar sorting process occurs in the germ-line compartment of the fly ovary, sending some transcripts (from dual-strand clusters marked by Rhino binding) to the Aub/Ago3 Ping-Pong pathway while restricting others to the Piwi pathway. Nonetheless, because we needed to combine two manuscripts into one, this section did not make it into the final, unified manuscript.

I appreciate the very careful analysis of the authors. But can we exclude that the pre-pachytene piRNA precursors are the predominant biogenesis substrate before the A-MYB switch and that after the switch, these precursors are not efficiently further processed (levels cannot compete with the other precursors, levels decrease in the pachytene stage)?

Meaning that both the pre-pachytene piRNA precursor transcripts and *Mili* are actively transcribed in pachytene spermatocytes, but the pre-pachytene piRNA precursors are not processed into piRNAs or that they are not loaded into MILI? Shouldn't this model predict that the transcripts from pre-pachytene piRNA genes would accumulate to *higher* levels in pachytene spermatocytes, which they do not? But formally, we cannot exclude the possibility raised by the Reviewer.

The fact that they are still detectable and have a MILI size profile might simply indicate that these MILI-piRNA complexes are stable. I think this statement requires to show that pre-pachytene piRNAs are still loaded into MILI at pachytene stages.

We agree that it would be ideal to show that pre-pachytene piRNAs are actively loaded into MILI in pachytene spermatocytes. We plan to develop methods to test this in purified pachytene spermatocytes, but such methods development may take years. Nonetheless, our data clearly show—by looking at purified spermatogonia versus 90% pure spermatocytes—that pre-pachytene piRNAs, persist at high levels in pachytene spermatocytes. We have now strengthened this conclusion using piRNA 95% pure pachytene spermatocytes and RNA-Seq data from 3 dpp and 8 dpp testes, purified leptotene/zygotene spermatocytes, and 95% pure pachytene spermatocytes: pre-pachytene loci are actively transcribed in purified pachytene spermatocytes.

However, we would like to note that the Reviewer's explanation, while formally possible, fails to explain why from 10.5 dpp to 17.5 dpp, the abundance of pre-pachytene piRNA precursor transcripts (measured by RNA-seq) was more correlated with pre-pachytene piRNA abundance at 17.5 dpp ($\rho = 0.47$) than it was at 10.5, 12.5, or 14.5 dpp ($0.32 \leq \rho \leq 0.40$; Figure S2C). We note that at 17.5 dpp the median abundance of pre-pachytene piRNA precursor transcripts is similar to that of pachytene piRNA precursor transcripts.

It is hard for us to envision a simple explanation for why at 17.5 dpp—when pachytene spermatocytes compose a larger fraction of the testis than at earlier times—the abundance of pre-pachytene precursor transcripts should be well correlated with pre-pachytene piRNA abundance. Our data sets and the data sets from Gan et al. and Modzelewski et al. (*Developmental Cell*, 2012) show that pre-pachytene piRNAs are present in in pachytene spermatocytes. If these were made in pre-pachytene cells and stably persist in pachytene spermatocytes, we would expect that pre-pachytene transcript abundance would be less well correlated with pre-pachytene piRNA abundance at 17.5 dpp than at earlier times.

2. "Second, a testis-specific A-Myb mutant, Mybl1repro9, which is caused by a cytosine-to-adenine transversion that changes alanine 213 to glutamic acid, leads to meiotic arrest at the pachytene stage with subtle defects in autosome synapsis" what is in this context a testes specific A-MYB mutant?

As described previously (Toscani et al., 2997), *A-Myb* null mutant mice have defects in multiple tissues, including both testis and breast. In contrast, the A-Myb point mutant *Mybl*^{repro9} shows phenotypic defects only in testis (Bolcun-Filas et al., 2011). Therefore, this mutant is testis-specific.

3. "However, our data suggest that the overall change in piRNA abundance caused by loss of MIWI is quite small: we detected almost no change at 14.5 dpp (change in total piRNA abundance = 1.1) and only a modest decrease at 17.5 dpp (change in total piRNA abundance = 0.58). Moreover, piRNAs from 16 pachytene piRNA genes decreased 2.7-fold ($p = 0.0046$) at 14.5 dpp and 3.5-fold ($p = 1.8 \times 10^{-6}$) at 17.5 dpp in Miwi mutant testes (Figure 3)"

I find these numbers rather difficult. it sounds like that this is carved in stone. First, the data is based on one biological replicate

The analysis for 14.5 dpp was based on two biologically independent RNA-seq replicates (two mutant and two heterozygous). We now indicate the number of replicates more clearly in the legends and text.

and secondly, it is clear that the miRNA normalization does lead to some errors.

If we understand the Reviewer correctly, the concern is that a systematic normalization artefact has caused us to incorrectly observe no change in piRNA abundance in the *Miwi* mutant. Given that the maximum difference in the percent of small RNAs mapping to miRNA hairpins either between biological replicates or between mutant and heterozygote at the same developmental stage was <15%, a normalization artefact seems very unlikely to explain our **failure to**

detect a meaningful change in piRNA abundance between *Miwi*$^{-/-}$ and *Miwi*$^{+/-}$. Isn't the simplest explanation that loss of MIWI does not alter piRNA abundance? In support of this view:

(1) the numbers are not in small; we report them using the standard IUPAC unit *ppm*. The 14.5 dpp data sets (two heterozygous and two homozygous) ranged from >10 to >100 million genome-mapping reads (>7.7 to >92 million piRNA reads), while the 17.5 dpp data ranged from >17 to >25 million genome-mapping reads (>14 to >24 million piRNA reads).

(2) we observed *no* meaningful change at **two** different times in testis development, 14.5 dpp and 17.5 dpp, (*Miwi* mutants arrest at ~20.5 dpp);

(3) experimentally determined numbers are reported throughout the manuscript using two significant digits. Numbers reported with only two significant digits cannot possibly convey great precision, but rather accurately report the *lack of precision* in the smaller digit of the two;

(4) we used two biologically independent replicates for the 14.5 dpp *Miwi*$^{-/-}$ and *Miwi*$^{+/-}$ data sets;

(5) could miRNA normalization really cause us to detect **no** substantive loss in piRNA abundance in the *Miwi* mutant, despite our having used data from three biologically independent sets (a pair of replicates at 14.5 dpp and a single data set at 17.5 dpp)? We normalized to the sum of all miRNAs in the unoxidized data set, then scaled the oxidized data to the unoxidized using shared piRNA species. For the mutant data to show normal piRNA levels artefactually would require a massive decrease in miRNA abundance in the *Miwi* mutant. But we did not observe a meaningful change in the percent of reads corresponding to miRNAs. We know of no evidence supporting such a change in miRNA expression in *Miwi* mutants. We really don't think we somehow failed to detect the collapse in piRNA abundance claimed in the literature. We believe that our reproducible and self-consistent deep sequencing data show that the literature claim of a dramatic collapse in piRNA abundance in *Miwi* null testes is simply wrong.

Reviewer #2

This is a very interesting study from the Zamore lab. The key finding in this manuscript is the identification of A-MYB as a transcription factor that binds to pachytene piRNA clusters. This factor also happens to be a master regulator of meiotic gene expression, so controls a lot of genes, including several of the piRNA pathway factors. This factor is conserved in birds.

(1) "…it is not clear why MYB would be needed at piRNA clusters as this manuscript does not reveal the nature of the transcribing polymerase for piRNA clusters."

We emphatically disagree. We show that piRNA precursor transcripts are capped and polyadenylated and that their promoters are occupied by RNA polymerase II but not by RNA polymerase III.

Some of the data analysis presented in the MYB manuscript is not entirely novel, for example Figure 6, showing that pachytene piRNAs sort into Miwi and Mili. This observation was made even as early as 2006 in the early papers where they noticed that piRNAs differed in their length at the 3' ends and hidden in supplementary figures of various papers that the authors did not cite.

Space constraints prevent us from including the sorting data in the final, unified manuscript, so this point is moot. But we would like to note that we know of no group that has claimed that piRNAs sort between MILI and MIWI. Just the opposite, the field generally insists that piRNAs distribute passively between the two proteins. Our data suggest that piRNAs are specifically enriched in MIWI by an active mechanism. We will continue to test the hypothesis that piRNAs are actively sorted according to their genomic origins, but not in this manuscript.

Some of the conclusions are drawn from datasets that have mixed cell populations, so claims of hybrid piRNAs and presence of pre-pachytene piRNAs throughout spermatogenesis etc. may not be entirely supported.
Given the constant cross-citation of data in the two manuscripts and shortcomings on some of the claims in the MYB study, I feel that one manuscript collecting the most supported aspects will be suitable for Mol Cell: a) What defines a cluster

We have deferred discussion of hybrid piRNAs to a future study (see below). We have combined the two manuscripts into a single paper that defines the piRNA-producing genes, with particular emphasis on the pachytene piRNA genes and the role of A-MYB in coordinating their expression and biogenesis.

Also reduce the constant use of numbers in every line, it breaks the flow.

It is hard to tell the story without the numbers, which are, of course, the actual biology. Moreover, the journal requires that *p*-values accompany every claim of significance. We hope that our revised manuscript strikes a successful balance between the biology (numbers) and its interpretation using words.

1. Abstract: "Here, we show that the transcription factor A-MYB initiates pachytene piRNA production. A-MYB drives transcription of both pachytene

piRNA loci and the mRNAs for core piRNA biogenesis factors, including MIWI, which binds pachytene piRNAs, several Tudor proteins, and MitoPLD."
Given that A-MYB drives transcription of all the other (100s of) genes required to kick-start the meiotic program, the selective reference to piRNA factors is misleading. As if A-MYB is just for the piRNA pathway.

The word "piRNA" never appears in the Bolcun-Filas et al. study. Surely since both Ewelina Bolcun-Filas and John Schimenti are authors on our study, we should be allowed in the short space of an abstract to focus on the novel aspects of the work?

Also in the previous study Bolcun-Filas et al, genes like Piwil1 (Miwi) is clearly mentioned as a target.

We clearly state this in our manuscript and then go on to describe our data as confirming the earlier Bolcun-Filas study.

2. How true is this statement in page 4: "A-MYB also initiates transcription of the genes encoding many piRNA biogenesis factors, including the pachytene-specific PIWI protein MIWI and the piRNA biogenesis factors TDRD1, TDRD5, TDRD6, TDRD12 and MitoPLD." Are Tdrd1 and MitoPLD not expressed already in embryonic stages where they should function in piRNA biogenesis? Immunofluorescence studies have localized Tdrd1 to fetal germ cells. Tdrd5 is also expressed early in fetal stages (Smith et al., 2004, Gene Expression Patterns and Yabuta et al., 2011). So A-MYB cannot INITIATE, perhaps REINITIATES their transcription?

Transcriptional initiation describes binding of RNA polymerase to a promoter followed by RNA synthesis from the transcriptional start site. Reinitiation imply that polymerase resumes transcription, having fallen off the template 3′ to the promoter, making new RNA joined to the original abortive transcript. To avoid confusion, we have revised the sentence:

> "In the juvenile testis, A-MYB also initiates transcription of the genes encoding many piRNA biogenesis factors…"

3. Can one claim that A-MYB orchestrates a feed-forward loop for piRNA generation, as A-MYB orchestrates the whole meiotic environment where pachytene piRNAs are also produced.

Yes, it is an accurate claim. The architecture of the A-MYB regulatory circuit in the pachytene piRNA pathway is unrelated to the architecture of transcriptional control for other biological pathways. This is common phenomenon in systems biology. Please see our response to Reviewer 2, below.

4. Fig. 1A. It is highly misleading to put the names of different spermatogenic stages on top of the bar plots, as it appears this information is specific to the indicated stage, which is not true. The age indication is sufficient and correct.

Our intent was certainly not to mislead, but rather to indicate the most advance stage of meiosis achieved by a specific time after birth. We have moved this information to the text.

5. Page. 5. 3RD para. Carmell et al., 2007 did not show piRNAs bound to Miwi2. It was Aravin et al. 2008.

We should have cited Aravin et al. (2008); we have added the reference.

Miwi2 is also detected till 3 days after birth (3dpp), so the correct wording would be "which binds piRNAs in perinatal testis".

We have revised the sentence to read "in perinatal testis."

"Mili which binds piRNAs throughout spermatogenesis". Can we say that? Spermatogenesis is also beyond round spermatids (when Miwi is last seen), into spermiogenesis etc.

We now write, "MILI, which binds piRNAs at least until the round spermatid stage of spermatogenesis…."

(when Miwi is last seen)

As a matter solely of intellectual discussion, this seems unlikely to be true: we and others find a peak of 30 nt piRNAs in oxidized deep sequencing data from mature, swimming sperm. Unless these piRNAs are somehow stable without being bound to a PIWI protein, there must be some MIWI (and perhaps MILI) beyond the round spermatid stage.

6. Identification of three types of piRNA clusters: Page 4. The authors sequence total small RNAs from different age testes, which should have a mixture of germ cells. They cite the work from Gan et al., 2011, where they purified specific germ cells. But as the authors might have noted, they (Gan et al) purified it using sedimentation gradients and these rarely give absolute purity obtained with FACS sorting.

We are surprised by this concern, since the literature suggests the opposite. FACS sorting, as reported by pioneers of the method such as the Singer-Sam lab, produces more highly purified sertoli cells, but less pure spermatogonia or spermatocytes than sedimentation gradient methods (Mays-Hoopes et al., *Biol Reprod* 1995). We originally used data from Gan et al., which correspond to >90%

pure spermatogonia or spermatocytes. We now add data from Modzelewski et al., which correspond to 95% pure pachytene spermatocytes.

So making a claim that pre-pachytene piRNAs continue to be expressed in all cells from pre-pachytene to pachytene and round spermatids is totally wrong. This is a major defect of the Figure.1A. I am totally for using such sedimentation methods as it is easy and gives material of reasonable purity, but to use this to make such a claim is not correct. If the authors insist on this, they should FACS sort pure populations and sequence piRNAs from these.

We do not understand why we should use a method that does not improve the purity of the desired cells. The published purity of pachytene spermatocytes that can be obtained by FACS is less than the purity of the cells used to generate the deep sequencing data sets we analyzed: the Gan et al. data was from >90% pure pachytene spermatocytes; the Modzelewski et al. data correspond to >95% pure pachytene spermatocytes.

Also, how a pachytene piRNA cluster will be different from a pre-pachytene cluster within in a pachytene spermatocyte or round spermatid.

This is a fascinating question. While we do not know the mechanism, our data suggest that, ***much as is the case in flies***, distinct chromosomal loci (i.e., piRNA genes) produce transcripts that are directed to load their piRNA products into specific PIWI proteins. In this view, pre-pachytene piRNA precursor transcripts load mainly MILI, whereas pachytene piRNA gene transcripts load mainly MIWI. Nonetheless, it has been quite challenging to incorporate all of our data into a single manuscript, forcing us to defer publication of the sorting data to a future manuscript.

Very least, I would re analyze this data and tone down the claim of continuous pre-pachytene cluster generation.

The data from Gan et al. were of defined purity. Those authors reported that, "The purity of the three cell types all exceeded 90% based on morphological evaluation and was confirmed by quantitative real-time RT-PCR evaluation of cell-type-specific marker genes."

Modzelewski et al. (*Developmental Cell*, 2012) also isolated specific spermatogenic cell types using the STA-PUT method based on separation by cell diameter/density at unit gravity (Bellvé, 1993). Those authors measured the purity of resulting fractions by microscopy based on cell diameter and morphology. They found that pachytene cells composed ~95% of the cells.

A 5–10% contamination of pre-pachytene cells in the gradient-purified pachytene spermatocytes would have little effect on our analyses. By way of

illustration, let us presume that no pre-pachytene piRNAs are actually present in pachytene spermatocytes. The maximum level of pre-pachytene piRNAs we would expect at 10% contamination would be one-tenth of the level in pre-pachytene spermatocytes. Yet, we detect **comparable** levels of pre-pachytene piRNAs in spermatocytes and spermatogonia.

Moreover, across the developmental time series in what is now Figure 2B, pre-pachytene cells constitute a *decreasing fraction* of the germ-line cells in the testis. If transcription of pre-pachytene genes and production of pre-pachytene piRNAs from these transcripts did not continue in pachytene spermatocytes, then the abundance of both the pre-pachytene transcripts and piRNAs should fall precipitously in the older juvenile testes. But it does not. Finally, we observe continued transcription of the pre-pachytene loci in RNA-seq data from the 95% pure pachytene spermatocytes.

7. Page. 9. Hybrid piRNAs: This is surely an artefact of mixed cell populations. If the authors want to keep this claim, they should use small RNA libraries from FACS-sorted cell populations.

Although we cannot understand how mixed cell populations could produce the effects we observe for a specific subset of piRNAs genes, we do not wish to create a controversy that distracts from the central message of our manuscript. We now present the hybrid category in Figure 2A, but delete all other analyses of those 30 piRNA-producing genes from the figures. We hope to present the evidence for the new, hybrid class of piRNA genes in a separate manuscript in the near future. For now, we would like to note that the properties of the hybrid class strongly support our view:

• 22/30 hybrid piRNA genes have a significant A-MYB ChIP peak < 1,000 bp ($q < 10^{-25}$) from their transcription start site: the median distance is 70 bp. The ChIP peaks can only come from pachytene cells, since A-MYB is not present in pre-pachytene spermatocytes. In the *A-Myb* mutants, transcript abundance for these 22 hybrid piRNA genes decreased >2-fold at 17.5 dpp (median $q = 8.3 \times 10^{-5}$) compared to their heterozygous littermates. The change in transcript abundance was measured using three biologically independent replicates for mutant and heterozygous testes. The testes were isolated and the libraries prepared at different times over the past year. Replicate #1 was sequenced in a different run from replicates #2 and #3. We conclude transcription of these loci in pachytene spermatocytes is partially dependent on A-MYB.

• Yet the median increase in piRNAs from these 22 piRNA genes from 10.5 dpp to 17.5 dpp is just 21-fold. In contrast, the median increase over the same period

for pachytene piRNAs is >8,600-fold. The most straightforward interpretation is that these loci produce piRNAs before the onset of the pachytene stage.

• The median increase in transcripts from these 22 piRNA genes from 10.5 dpp to 17.5 dpp is just 4.0-fold. The median increase for transcripts from pachytene piRNA genes over the same period is ~116-fold.

• At 10.5 dpp, pachytene spermatocytes are not present in the testis (e.g., Bellvé et al., 1977), yet the median rpkm value for the transcripts of these same 22 hybrid piRNA genes is 8.5 rpkm, a transcript abundance not terribly different from that of pre-pachytene piRNA genes (15 rpkm at 10.5 dpp and 12 rpkm at 17.5 dpp).

To us, these data strongly support the conclusion than that the hybrid piRNA genes combine properties of the pre-pachytene piRNA genes (i.e., expressed before the pachytene stage) with features of the pachytene piRNA genes (i.e., up-regulated at the pachytene, many by A-MYB).

Responses to Comments on the Resource Manuscript (now incorporated, in part, into a single unified manuscript)

Reviewer #1

My major reservations towards the presented data are:
1. The data is to the largest part a refinement of previously described loci and a careful, yet descriptive analysis of these loci in terms of transcription boundaries, intron-exon structure, transposon content and promoter architecture. All of these analyses are of high quality, but they really do not tell us anything significant new about mouse piRNA clusters.

First, our findings about the unusual promoter architecture of bidirectionally transcribed pachytene piRNA genes were new and unexpected. Second, no previous study has defined the precursor transcripts for mouse piRNAs and none has demonstrated that mammalian piRNA precursors are continuous transcripts rather than multiple, smaller transcripts. Given that each piRNA in worms is a separate RNA polymerase II transcript—a finding considered worthy of publication in *Cell*—this seems to us to be an important finding. We believe that the determinants that cause these transcripts to make piRNAs are hidden in their genic structure. We hope that armed with >150 independent high throughput sequencing experiments contained in our original Resource manuscript, we will be able to test this hypothesis soon.

It has been described previously that juvenile piRNAs are derived from pachytene piRNA clusters and also from genic mRNAs. This manuscript does refine the known loci, helps to define the clear boundaries, annotates their introns, but besides this does not add terribly much in terms of the biology of these loci. Of all the loci that the authors describe (intergenic and genic), how many have been described before? I also think more transparency should be given in terms of the genic piRNAs….How big is the overlap to the Lai study? what would happen if the cutoff in terms of piRNAs per kb or per transcript would be lowered? Do we then get dozens more genic piRNA loci?

Because we are merging these data into the A-MYB story, we have not performed the time-consuming analyses suggested by the Reviewer. We would of course get more loci if we lowered the cutoff of piRNA abundance or density per gene, but that seems to miss the point. Nearly all the piRNAs in the adult mouse testis can be explained with a *smaller* fraction of the genome than previously thought. At the very least, we hope that future authors will no longer claim that pachytene piRNAs originate from ~3,000 genomic clusters (Gan et al.,

RNA 2011; Zheng and Wang, *PLoS Genetics* 2012), a claim that inflates the number of mouse pachytene piRNA genes by 10- to 15-fold.

Are these (as published by Lai/Lau) predominantly from 3'UTRS?

To answer the Reviewers question for the sake of discussion only—as these analyses are not part of the unified manuscript resubmitted to the journal, the genic piRNAs are predominantly from 3′ UTRs. Since 3′ UTRs are nearly always the longest exon in a mouse transcript, we examined the *density* and not the abundance of piRNAs from the 5′ UTR + ORF versus the 3′ UTR. Overall, genic piRNAs (which include pre-pachytene, hybrid, and pachytene piRNAs) have six-times greater median density in the 3′ UTR compared to the rest of the mRNA. Among the genic piRNAs, the pre-pachytene have a nearly six-times greater median density in the 3′ UTR compared to the rest of the mRNA; the hybrid have a nearly nine-times greater median density in the 3′ UTR compared to the rest of the mRNA.

One conclusion of our analysis is that while 3′ UTR mapping piRNAs are common for genic piRNA-producing loci, 3′ UTRs do not necessarily produce more piRNAs than the rest of the message: four of the seven genic pachytene piRNA loci have a greater density of piRNAs outside the 3′ UTR than in the 3′ UTR. In fact, about 17% of genic, pre-pachytene loci produce a greater density of piRNAs from the 5′ UTR + ORF than the 3′ UTR. We do not know what determines whether piRNA production will favor the 3′ UTR of a particular transcript, although we are using our data to investigate this phenomenon.

2. The title "Redefining the piRNA-Producing Loci of the Mouse Testis as Genes" is rather bold. first this is only about juvenile piRNA clusters or loci. A complete resource on mouse piRNA producing loci (as indicated in the title) would include the embryonic sources and would not be restricted to the juvenile piRNA populations.

In retrospect, we completely agree. We are currently sequencing samples from 05 dpp perinatal mice in order to extend our redefinition of piRNA clusters as gene to include the embryonic piRNA loci currently defined solely by piRNA density (i.e., clusters). We also plan to do a more complete job of defining pre-pachytene loci. Those data will, of course, be presented elsewhere.

In addition, genic piRNAs have always defined as mRNA derived and I therefore really do not see the point in stressing that these loci are "genes". What else should they be?

The most reasonably explanation—and the one we had thought was embraced by the piRNA community—was that distinct transcripts produced the piRNA

and proteins. How else could it be that for some pre-pachytene loci the piRNAs map mainly to the 3′ UTR while for others this is not the case? Moreover, 89% (221/249) of those that "map to 3′ UTR regions" are in regions that are not annotated as part of the mRNA of the nearest gene. They only mapped to a protein-coding gene *after we had experimentally determined the 3′ ends of all the transcripts in the adult mouse testis by PAS sequencing*.

Even if the Reviewer does not believe that distinct transcripts make the proteins and the piRNAs, surely the hypothesis is reasonable? piRNA data alone cannot distinguish between (1) a single transcript making both piRNAs and proteins and (2) separate but overlapping transcripts serving as the sources of piRNAs and proteins. This discussion reminds us of early claims that intronic miRNAs derived from the pre-mRNA of the underlying gene. But for many such cases, the pri-miRNA is now thought to use a separate promoter from the pre-mRNA. When one does not know the transcript that produces a gene product, surely one cannot claim that they derive from specific genes.

3. The entire section about "transcriptional silencing" of piRNA producing loci in other cell types is over-sold in my opinion. It is per se interesting to see that the TSS regions of clusters are occupied to a low level with Pol II in non-testes tissues. But to conclude that this represents transcriptional silencing is an overstatement.

We agree that this section of the second manuscript was too preliminary and off-topic. We have not included it in the single, revised manuscript.

As the authors point out, A-MYB is missing in ES cells and is presumably also low/absent in many other somatic cell types. This by itself would be sufficient explanation that these loci are transcriptionally not active.

We agree that the absence of A-MYB in other tissues *should* suffice to explain the lack of transcription of intergenic piRNAs. But it doesn't: we can detect RNA pol II recruitment to the promoters of intergenic piRNAs in other tissues. GRO-seq data show that these polymerases produce nascent transcripts. The high pausing index of the loci, the presence of nascent transcripts despite low levels of steady-state full-length piRNA precursor RNAs, together suggest that in addition to being activated by A-MYB, intergenic piRNAs are also repressed by a polymerase pausing mechanism.

Transcriptional silencing, however, is an active process and it therefore needs to be demonstrated. The authors did not detect H3K27me3 marks but did not investigate any further into other silencing marks. In addition, the fact that testes

piRNA sources are not transcribed in other cell types does hardly add anything to the piRNA field.

It has been reported that piRNAs can be detected in tissues other than testis, and ectopic PIWI expression is associated with several types of cancer. In addition to testis, A-MYB is present in the spleen, mammary gland (the *A-Myb* null mutant disrupts breast development), and CNS, yet we and others cannot detect piRNAs in these tissues (old Figure 6A). For us, the activation of piRNA pathways in germ cells and the inactivation of this pathway in somatic cells are two sides of the same question, deserving equal attention.

on Pol II pausing: how would the pausing idea go hand in hand with the dependence on A-MYB? are all intergenic clusters in A-MYB mutants paused?

Our data explain the high level of transcripts from pachytene piRNA loci at the onset of the pachytene stage: these genes are activated by A-MYB. Our analyses suggest that these loci would be transcribed at lower levels in the absence of A-MYB (by MYB-independent or B- and C-MYB-dependent mechanisms) if they were not actively repressed by a polymerase pausing mechanism. We have not analyzed pausing in mutants, but it is a good suggestion for us to follow in the future.

Additional Points:

1. Figure S2B: how can the authors compare their data to Lau et al, who published rat piRNAs? Should this not be Aravin et al?

In addition to rat piRNAs, Lau et al. published mouse piRNA data and annotated mouse piRNA clusters (Lau et al., 2006; Supplemental Tables S3 and S4). Their mouse piRNA data sets are available from GenBank using accession numbers DQ684678 to DQ727400.

also: given that all three curves are essentially reaching the same total height, it follows that the previous studies already identified nearly all of the relevant loci. meaning (as mentioned above) that the new data is essentially a refinement but not a major change in piRNA cluster annotation.

Our point was not that previous studies identified too few loci but rather that they encompassed *too large* a fraction of the genome. The curve would also reach the same height if we assume that every base in the genome makes piRNAs. (In truth, this is almost the situation in flies, where we detect 30% of genomic nucleotides producing at least one piRNA in the adult ovary.)

Figure S1B shows that our piRNA genes explain more piRNAs with *fewer* genomic base pairs than previous studies. In other words, defining piRNA genes

using traditional methods—transcript sequence, histone marks, 5´ cap and 3´ cleavage and polyadenylation sites—reveals that piRNAs come from a *smaller* fraction of the genome than previously thought.

2. the statement that 41 loci escaped previous annotation only makes sense if the authors show the contribution of these clusters to the whole picture. are these genic piRNA precursors? Do they contribute only low levels of piRNAs?

The Reviewer's concern seems predicated on our defining too many loci. But despite having found 41 loci previously missed by approaches that relied solely on the density of piRNAs mapping to the genome, we explain more piRNAs with *fewer* genomic base pairs.

Of the 41 loci, three-quarters are pre-pachytene piRNA genes. For the remaining 10, three are hybrid loci and seven are pachytene loci that produce just small amounts of piRNAs (at least by the exuberant standards of pachytene piRNAs). Collectively, the 41 loci account for 2% of piRNAs at 10.5 dpp, when pre-pachytene piRNAs predominate, but only 0.36% in the adult testis, which makes mainly pachytene piRNAs. The 31 novel pre-pachytene loci account for >15% of the pre-pachytene piRNAs that map to our 84 pre-pachytene piRNA genes. We anticipate that there will be far more pre-pachytene loci, each expressing fewer piRNAs, than pachytene piRNAs.

what would happen if we lower the threshold? would then another set of 30 clusters emerge?

The threshold is not really the issue. We need to use 10.5 dpp (or earlier) CAGE, PAS, RNA, and piRNA sequencing data plus H3K4me3 and RNA pol II ChIP to identify and define additional pre-pachytene piRNA loci. We capture the overwhelming majority of pachytene piRNAs because our efforts have focused on stages where these predominate. Consequently, we only define the most highly transcribed pre-pachytene loci. Our efforts to more exhaustively define the pre-pachytene loci are nearly complete (the samples are being sequenced or analyzed), but these studies lie outside the scope of the unified manuscript.

3. Fig S2C states that this is Pol II occupancy, rather than Pol III occupancy

Thank you for catching this! We have corrected our error.

4. on the "unique" promoter features of piRNA loci (especially the divergently transcribed intergenic clusters: these observations are per se interesting but unfortunately purely descriptive. there is not a single follow up on this. what does this mean? is this an important feature of loci that produce high level piRNAs? Is

the low CpG content not in contradiction to their hypothesis that piRNA clusters are transcriptionally silenced in other tissues via DNA methylation?

We agree that these data, while potentially appropriate for a descriptive Resource paper, are too preliminary to merit inclusion in a unified paper on A-MYB. We note that low CpG promoters can be silenced by CpG methylation (Han et al. Hum Mol Genet 20:4299-4310, 2011).

5. on the observed Wdfy3 transcript isoforms:
"The abundance of long isoform transcripts did not decrease (2.08 rpkm in heterozygotes versus 3.86 rpkm in mutant), while the small isoforms became undetectable (10.0 rpkm versus 0 rpkm), consistent with the idea that the short, but not the long, Wdfy3 transcript is a piRNA precursor RNA."
It is rather likely that this locus drives the short isoform in germ cells but the long isoform in non-germ cell, non-piRNA producing cells. Therefore it is no surprise to see the piRNAs restricted to only the short isoform. The importance of this finding for the field is therefore questionable.

We can exclude the hypothesis that the long isoform is expressed only in the soma and the short, piRNA-producing isoform in the germ line: both isoforms are expressed in purified pachytene spermatocytes (95% purity; Modzelewski et al., 2012). Unfortunately, we don't have room for this example in the unified manuscript.

6. Figure 2:
first, it is not clear what aggregation charts are. are these "meta-profiles"?
second, are the clusters scaled or do we look at start plus minus 1kb and stop plus minus 1kb as indicated below the two ChIP seq tracks?

The RNA data are scaled from "Start" to "End" (a metagene profile), but the data 5′ to start and 3′ to end are not scaled. This is now more clearly indicated on the figure (now Figure 1B). This format has been used by others (e.g., Figure 2, Bonn et al., *Nature Genetics* 2012; Figure 6, Riddle et al., *Genome Research* 2011).

thirdly: I would find it very important to show two intergenic and two genic examples in detail across the entire locus. Especially for the intergenic clusters it would be great to show clusters that are rather large to show the proposed enrichment for full length poly-adenylated RNAs also for these more challenging examples.

We have added a new figure (Figure 3) and supplementary figure (Figure S3) with these illustrations.

For the intergenic clusters: would it not make more sense to split these up into bi-directional clusters and mono-clusters? otherwise the antisense portion appears to be much less pronounced but it might not be.

We now analyze the bidirectionally and unidirectionally transcribed piRNA genes separately in Figure 1B.

7. "accumulation of toxic RNAs"[:] what is a toxic RNA?

Typically, toxic RNAs contain simple repeat sequences. For example, myotonic dystrophy is caused by an expanded CUG or CCUG repeat in an RNA transcript. The RNA, rather than the protein, is thought to cause the disease. A high fraction of piRNAs, particularly in invertebrates, correspond to simple repeats; these likely silence loci that generate toxic RNAs, much as transposon-mapping piRNAs silence parasitic elements.

"This definite set of piRNA loci and primary piRNA transcripts should provide an invaluable resource to study piRNA biogenesis and function."
It is simply not clear to me where the invaluable resource would really be and how many questions can now be answered that could not be answered with the previous datasets. For example, it is likely that the TF motif for A-MYB would also have been extractable from the previous data sets.

We were unable to identify the A-MYB-binding site from previous data sets, and I am told by members of the Hannon lab that they also failed to find any motif common to pachytene piRNA genes defined by piRNA density. We have added Figure S1C which illustrates this point. Of the 214 piRNA genes we defined, 65% lie ≤ 200 bp from an A-MYB peak. We examined the annotated piRNA clusters of Lau et al., Aravin et al., and Girard et al. For the majority of Aravin et al. and Girard et al. clusters, the 5′ end of the annotated cluster lies >3,000 bp from an A-MYB peak; for Lau et al., the 5′ ends of about a quarter of annotated clusters lie >3,000 bp from an A-MYB peak. The 5′ ends of another quarter of Lau et al. clusters lie ≤ 200 bp from an A-MYB peak. However, Lau et al. annotated 20 pairs of piRNA genes as 20 clusters (they should be 40 individual clusters in pairs). Notwithstanding the annotation error, we automatically placed these clusters in the 0–200 bp bin, reasoning that, in theory, the Lau et al. data could be used to find the A-MYB binding site by looking in-between the two sets of piRNAs. In truth, this is an overly generous assumption: in the six years since these data were published, no one has used them in the published literature to identify the transcription factor regulating even this small set of clusters.

Reviewer #2: Use several high-throughput methods to clearly define mouse piRNA clusters. Define gene-overlapping and intergenic piRNA clusters. Identify mouse piRNA cluster transcripts to be spliced. The analysis presented in this manuscript is of very high quality and will be of interest to the field. The most interesting data in this MS is the Figure 2 and Fig. 3A. Much of the remaining data is a re-analysis or linked to the work being discussed in the MYB manuscript. So it falls short of making a separate manuscript at Mol Cell, even as a resource. It should go together with the Myb study where it will contribute main figures and supporting data for that manuscript.

1. Page 4. "Rhino binds the DNA of Drosophila...". Rhino cant bind DNA, it probably binds chromatin via specific marks.

This was a stupid error on our part. Of course, Rhino binds chromatin, not DNA! This text is not part of the revised manuscript.

2. The text is full of numbers in percentages and P values that breaks the flow and finally the reader loses focus of the interesting aspect being discussed.

We hope that the revised manuscript strikes a better balance of science and poetry.

3. Nobody is going to remember all these numbers, please clearly state the key biologically relevant findings without the clutter of numbers.

Numbers are beautiful; they are not clutter! The numbers *are* the actual biological findings.

"[W]hen you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of Science, whatever the matter may be." Lord Kelvin

4. Page 17. "During the pre-pachytene stage of spermatogenesis, piRNAs are amplified...." If this refers to mouse spermatogenesis one could cite Aravin et al., 2008 and De Fazio et al., 2011 instead of the fly papers.

While the analysis of mouse Ping-Pong amplification did not make it into the single, unified manuscript, we should have cited some mouse papers, in addition to the fly papers. But the original citations referred to the discovery of Ping-Pong amplification, "…a cycle of PIWI-protein–catalyzed cleavage called Ping-Pong amplification," which we correctly credited to the Hannon and Siomi labs.

Also, it is now clear that mouse piRNA amplification in embryonic stages is hardly cyclic, given that Miwi2 is not a slicer.

We know of no evidence supporting the assertion that embryonic piRNA amplification is not cyclic. De Fazio et al. interpreted their *Miwi2*^{DAH} mutant data as evidence that cycles of MILI:MILI Ping-Pong drive amplification of embryonic piRNAs . Moreover, they hypothesized that while MIWI2 is loaded with secondary piRNAs, it cannot produce additional secondary piRNAs because it is naturally inactive as an endonuclease. This conclusion is inferred from the absence of a molecular or fertility phenotype in the *Miwi2*^{DAH} mutant. The authors presume, but do not prove, that the DDH —> DAH mutation in MIWI2 blocks its endonuclease activity. The assumption relies on the unjustified view that the non-quantitative data in Figure 5a of Rivas et al. (2005)—a beautiful but early study that long preceded rigorous quantitative enzymology of mouse AGO2—can be extrapolated to all mouse Argonaute proteins. De Fazio et al. showed that mutating a single glutamine, presumed by homology to lie at the endonuclease active site, had no detectable effect in vivo. They presented no biochemical evidence that this mutation reduces or eliminates the putative MIWI2 endonuclease activity.