molecular systems biology

Programmed fluctuations in sense/antisense transcript ratios drive sexual differentiation in S. *pombe*

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1st Editorial Decision 22 December 2010

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise several concerns, which should be convincingly addressed in a major revision of the present work.

The major concerns raised by the three reviewers refer to the need to analyze further the impact of components of the RNAi pathway on the effect of antisense RNA (over)expression and the need to assess the effect of antisense RNA when expressed from a genomic location (as opposed to overexpressed from a plasmid). The recommendations provided by the reviewers are very clear in this regard.

We would also kindly ask you to make sure that all the datasets presented in this study are deposited in the appropriate major public databases and that the respective accession numbers are included in the Materials & Methods section.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office

msb@embo.org.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

Thank you again for submitting this work to Molecular Systems Biology and I look forward to receiving your revised work.

Best wishes,

Editor
Molecular Systems Biology

Referee reports

Reviewer #1 (Remarks to the Author):

This manuscript uses high throughput RNA sequencing to examine changes in transcription during S. pombe sexual differentiation. In addition to providing a comprehensive picture of the S. pombe transcriptme, the results reveal the occurrence of widespread antisense transcription that appears to be linked to repression of meiotic genes during vegetative growth. The widespread role for antisense transcription suggested by the RNA-seq data is potentially important and of great interest. However, experimental tests of the importance of antisense transcription seem preliminary and incomplete. Additional control experiments and better presentation of the data are required before this manuscript can be considered for publication.

Specific comments.

1. The antisense experiments with pREP1 plasmids presented in Figures 6 and 7 are potentially interesting, but it is difficult to ascertain how the experiments were performed. In the methods section, the authors state that pREP1 antisense plasmids were transformed into cells to assess effects on sense transcription. At another point for dis1+, they state that the antisense nmt1 cassette was inserted into the genome. These experiments need to be more clearly described. Are the authors looking at antisense trans effects, with the antisense transcripts coming from the prep plasmid or are they looking at cis effects with nmt1 promoter always inserted downstream of the appropriate gene?

2. For the data in Figures 6 and 7, the authors should quantify the effect of antisense transcription on the levels of the appropriate sense transcripts (using qRT-PCR). Similarly, in Figure 8, for the Kan-nmt1 insertions downstream of dis1+, the authors should quantify effects on sense dis1+ RNA. These RNA quantifications would verify that antisense transcription is direcly affecting sense RNA levels.

3. The ago1+ deletion effects (Figure 6 and 7C) are potentially very interesting but appear preliminary. The effect of other major components of the RNAi pathway, especially dcr1+, rdp1+, and clr4+ should also be tested. Does ago1+ deletion affect antisense-mediated changes in sense RNA levels?

4. In general, the presentation of the RNA-seq data in the Figures (in particular Figures 3 and 4) and the description in the text is difficult to follow and should be improved.

Reviewer #2 (Remarks to the Author):

Review of "Programmed fluctuations in sense/anti-sense transcripts ratios drive sexual differentiation in S. pombe" by Bitton et al

In this paper, the authors show that the sexual differentiation in fission yeast S. pombe is accompanied by elaborate non-coding RNA expression that is mediated by transcription factors Atf21 and Atf31. They provide evidence showing that the constitutive expression of the spk1, spo4, spo6 and dis1 antisense transcripts disrupt the functions of these genes. Importantly, the functional consequence of antisense RNA expression requires Argonuate protein that has been previously implicated in RNAi-related processes. The authors conclude that programmed generation of non-coding RNA is an important component of regulatory system that drives sexual differentiation in S. pombe.

Critique: The results described in the paper are interesting and conclusions presented are in general supported by the results presented. The data included in the paper will be an important resource for the S. pombe community. The following concerns need to be addressed before the paper can be considered for publication.

Specific comments:

(1) In Fig.6 B.C,F,G, the authors show that the ncRNAs expressed from the plasmid can affect the expression on the chromosome in an ago1-dependent manner. This indicates that the observed effect occurs in trans. Considering that RNAi in S. pombe has been suggested to largely act in cis, this is an important finding. The authors should investigate whether other components of RNAi-machinery are involved in this pathway. For example, dcr1D, rdp1D.

(2) The overexpression of antisense from a plasmid could result in artifacts. It is important that the authors perform additional experiment by disrupting antisense transcripts at the endogenous genomic location. This experiment is essential to support the main conclusion that ncRNA transcripts represent "genuine" components in the system controlling sexual differentiation.

(3) Do antisense RNA affect the RNA polymerase directly? If yes, does this effect require Ago1.

(4) How do effects of dis1 antisense RNA expression compare with phenotype caused by dis1D? The quality of the Western data in Figure 7 (and other figs) need to be improved to draw definitive conclusions.

(5) Figure 2 legend is not adequate. There is a lot of information in Figure 2, however, the significance is lost upon the reader due to lack of adequate description and explanation.

(6) The authors also mention general loss of antisense expression in crs1+, bqt1+, and meu1+. It would be worthwhile to show this as additional supplementary figure.

(7) In the section that discusses "Anti-sense ncRNA control of Pheromone signaling via modulation of Spk1", the text refers to Fig. 5J-k. But figure 5 has only 4 panels that are not numbered.

Reviewer #3 (Remarks to the Author):

The manuscript describes the strand-specific analysis of the fission yeast transcriptome in vegetative and meiotic cells. The data are used to comprehensively annotate UTRs in the fission yeast genome, which will b extremely useful for fission yeast researchers. The data also reveal the existence of large amounts of antisense transcription, which is often regulated during meiosis. Follow-up experiments show that anti-sense expression can silence meiotically-expressed genes in trans, in a manner dependent on a component of the RNAi machinery. These observations are of general interest.

Overall this is a very good and interesting piece of work. An extensive analysis of the fission yeast transcriptome has been published recently (Wilhelm et al, see reference list in the paper). However, this manuscript goes further by using strand-specific sequencing (note that Wilhelm et al used tiling arrays to obtain sequencing-specific information). This is a substantial advance, as it allows the specific detection of antisense transcription. The sequencing data appear to be good, although there are a number of issues that should be addressed regarding their analysis and presentation. The authors characterise the biological effect of antisense transcription convincingly, but there are a number of essential controls missing. I consider that the paper merits publication in MSB subject to the authors addressing the points listed below.

[1] The pages of the manuscript are not numbered, which makes the task of commenting on the manuscript rather difficult. For the comments below I have numbered pages starting with the page containing the introduction.

[2] Page 3 (and methods in p. 18). The pat1 experiments have been done using asynchronous diploid cells in the absence of nitrogen starvation. In an alternative protocol, diploids are homozygous for the mating type locus and are starved for nitrogen to block cells in G1 before pat1 is inactivated. The first protocol is used in some studies (Averbeck, Moldon) and the second in others (Mata, Wilhelm). The results using the two protocols are likely to differ substantially, as nitrogen starvation induces very strong changes in gene expression. Given this, it would be important to discuss this issue briefly in the main text.

[3] Page 3. It is not completely clear from the text which samples were used for the assembly of the TBlocks. If they were pooled, this would create problems when they are used for defining UTRs, as these regions can vary in meiotic cells (for example, through the use of alternative promoters). An extreme example of this phenomenon is shown in Wilhelm et al Figure 3d. Ideally each sample should be analysed independently and the results compared with each other. If this is not possible due to insufficient coverage, the limitations of the approach taken should be discussed.

[4] Page 4. Some of the experiments (vegetative cells and pat1 time course) have been done in duplicate using independent biological samples. It would be very important to get some measurement of the reproducibility of the experiments by comparing the replicates with each other. A simple way would be to show scatter plots of estimated expression levels between replicate experiments.

[5] Table S4. It should be indicated that the data are log-transformed (if this is what they are). It is unclear what the numbers represent: average between both repeats or a single experiment? It would be important to show [1] the results from both experiments and [2] the results for all genes, not just changing significantly. [2] could be done as a separate supplementary figure, and it is important because it contains very useful information (such as expression levels). Finally, the table contains information of TBlocks expression levels, but this cannot be interpreted as the position of the TBlocks is not given anywhere (should be included as supplementary information).

[6] Page 4. '162 of the 4,231 statistically significant genes have been previously described as up-regulated in meiosis'. I don't think the figure of 162 can be correct. Mata et al. identified over 1,000 genes up-regulated during meiosis. If the overlap between these two gene sets were only 162 it would be mean that previously identified meiotically-induced genes are hugely under-represented in the set identified by this study.

[7] Page 5/ figure 2: There are huge changes in gene expression (over a thousand genes) between haploid and diploid cells (regardless of the pat1 allele). As far as I know a comparison between gene expression in haploids and diploids has not been published in fission yeast, but this is very surprising - I think this deserves some discussion.

[8] Figure 3 is extremely difficult to see (even when amplified on a computer screen). There are simply too many data on it. Also, what are the -1 after some of the systematic names? This figure should be completely redrawn; I would suggest moving some of the examples to a supplementary figure and showing the different time points in different panels (rather than overlaid in different colours). The same applies to figure S2.

[9] Page 7. It would be useful to have a direct comparison of the results of the atf21/31 experiments with those genes identified by Mata et al (this would validate both experiments)

[10] Figure 4 legend - The legend is very confusing - there are no genes displayed in grey (as stated in the legend), also no description of panel VI

[11] Figure 4: The 'trimodal model' - I would not use the word trimodal, as it gives the impression of three discrete states; in fact, the idea is of a continuous distribution of sense/antisense levels.

[12] The authors show that expression of antisense RNA in trans causes phenotypes similar to the deletion of the corresponding gene (for spk1, spo4, spo6 and dis1). Moreover, they show that this effect is dependent of a component of the RNAi pathway (ago1). The authors do not speculate on the possible mechanism by which argonaute and the antisense RNAs silence gene expression. However, as this is one of the most important observations of this paper, it would be essential (and easy) to measure the effects of the antisense transcription on RNA levels. This could be done by quantifying the levels of sense and antisense transcript in wild type cells and in cells over expressing the antisense RNA. In addition, it would be essential to verify that no antisense RNA is produced from the transgene (by measuring the expression of the boundary between the end of the gene in the transgene and nmt1 3' UTR).

[13] Although the experiments discussed in the previous point are clear, they are all based on ectopic overexpression of the antisense RNA, and do not directly address the question of whether regulation by the antisense RNA is relevant in vivo. The authors study this by inserting a cassette (in both orientations) directly upstream of the start of the dis1AS. The results show that protein levels are affected when the cassette is inserted in one orientation, but not in the other. The assumption here is that the insertion blocks the production of the antisense, but this is not formally proven. As this experiment is crucial for the model (it is the only one that looks directly at the in vivo importance of the antisense RNA), I think that the assumption should be tested rigorously by quantifying the levels of sense and antisense RNAs in wild type strains and in cells carrying the insertion in both orientations.

[14] It would be interesting to discuss the meiotic phenotypes of ago1 mutants and how they fit the authors' model.

Minor comments
[1] 'To gain greatest insight' - should it be 'greater'?
[2] Page 6, 'Sense-antisense ratio close to zero' - should be 'close to 1'
[3] Page 6, 'We performed functional term enrichment analysis... for each of the 354 genes' - should be 'for the 354 genes' (the analysis is applied to the genes as a set)
[4] Page 7, ' A total of 12 samples were pooled and sequenced' - surely the samples were not pooled
[5] Page 16, line 9; should be 'at least'
[6] Antisense regulation in trans have been previously shown to be possible in S. pombe using reporters (Arndt et al, Molecular & General Genetics 1995, 248(3):293-300); this paper should be cited. (Note Arndt et al. did not address the possibility that this kind of regulation occurs in vivo or whether it has any practical importance).
[7] I am not sure what MSB policy is for the deposition of this type of data in public databases, but as far as I can see the data have not been deposited.

1st Revision - authors' response 23 March 2011

We thank the referees for such a warm and supportive response to our manuscript. We have dealt with the two major requests that you highlighted in your letter of 22nd December and have addressed the majority of the other issues raised by the referees in the time you have allocated for our re-submission. We have dealt with the referees' comments point by point below. We are also grateful to the referees for their suggestions, as we feel that the revised manuscript has been greatly strengthened by following these, and hope that you will be able to consider our manuscript favourably in light of these modifications.

We look forward to hearing from you in due course.

Reviewer 1

This manuscript uses high throughput RNA sequencing to examine changes in transcription during S. pombe sexual differentiation. In addition to providing a comprehensive picture of the S. pombe transcriptme, the results reveal the occurrence of widespread antisense transcription that appears to be linked to repression of meiotic genes during vegetative growth. The widespread role for antisense transcription suggested by the RNA-seq data is potentially important and of great interest. However, experimental tests of the importance of antisense transcription seem preliminary and incomplete. Additional control experiments and better presentation of the data are required before this manuscript can be considered for publication.

Specific comments.

1. The antisense experiments with pREP1 plasmids presented in Figures 6 and 7 are potentially interesting, but it is difficult to ascertain how the experiments were performed. In the methods section, the authors state that pREP1 antisense plasmids were transformed into cells to assess effects on sense transcription. At another point for dis1+, they state that the antisense nmt1 cassette was inserted into the genome. These experiments need to be more clearly described. Are the authors looking at antisense trans effects, with the antisense transcripts coming from the prep plasmid or are they looking at cis effects with nmt1 promoter always inserted downstream of the appropriate gene?

a) We apologise for the confusion over the approaches used to produce the antisense transcripts in the previous manuscript. The confusion arose from our poor explanations and the use of two approaches in the one study: the ectopic expression of a copy of the anti-sense transcript and the insertion of a marker downstream of the *dis1*$^{+}$ gene. We have added a cartoon in Figure 4D to summarise how we cloned the anti-sense constructs under the control of *nmt1*$^{+}$ promoter. This cloning strategy completely replaced the *nmt1*$^{+}$ transcript from the start of the 5' UTR to the very end of the 3'UTR with the antisense transcript.

b) At the suggestion of all three referees we have now integrated this induction cassette into the genome in order to ensure that the effects that we are looking at arise from expression *in trans* from a single heterologous locus, rather than from swamping the system with pREP1 plasmids as in the initial study. Integration had no impact upon the outcome of anti-sense production reported from the multi-copy plasmid, and we still find that the function of the target locus is abolished.

c) In each case expression from the integrated locus blocked the function of the target locus in an Ago1, Dcr1, Rdp1 dependent manner (and in the case for *dis1*$^{+}$, a Clr4 dependent manner – we were unable to test for the impact of Clr4 abolition on the other loci as we were unable to generate the strains, despite many attempts).

Time pressures meant that some experiments that rely upon pREP1 plasmids remain in the manuscript. These are:

- the demonstration in Figure S6A that both the short and the long Dis1 anti-sense transcripts reduce Dis1 protein levels (after which we use the long transcript for integration for all further studies).

- the demonstration in Figure 6K-P that *spk1* anti-sense has no impact upon meiotic progression if meiosis is triggered, independently of a requirement for conjugation, by temperature shift of a *pat1.114* diploid.

- the demonstration in Supplementary Figure S6C that expression of *dis1*$^{+}$ sense transcripts a) enhances the number of zygotes with horsetail nuclei and b) abolishes the ability of *dis1*^{AS} anti-sense transcripts to block horsetail movement.

2. For the data in Figures 6 and 7, the authors should quantify the effect of antisense transcription on the levels of the appropriate sense transcripts (using qRT-PCR). Similarly, in Figure 8, for the Kan-nmt1 insertions downstream of dis1+, the authors should quantify effects on sense dis1+ RNA. These RNA quantifications would verify that antisense transcription is direcly affecting sense RNA levels.

At the suggestion of the referees we have initiated the study of transcript levels with strand specific quantitative PCR, however, while we are able to detect trends in transcript production that are consistent with the manipulations that we have imposed, such as a consistent increase in anti-sense transcripts for the loci upon induction in multiple experiments, the results are yet to be entirely reproducible between technical and biological replicates. We are therefore nervous about committing to the results at the time of submission as we clearly require more time to set this technology up in our laboratories. We do however, hope that the enhancement of the cell biology within the study, such as the demonstration that either antisense production or anti-sense marker insertion phenocopy the complete loss of the *dis1*$^{+}$ gene, reassures the reviewer that the manipulations arise from RNAi control of gene function.

3. The ago1+ deletion effects (Figure 6 and 7C) are potentially very interesting but appear preliminary. The effect of other major components of the RNAi pathway, especially dcr1+, rdp1+, and clr4+ should also be tested. Does ago1+ deletion affect antisense-mediated changes in sense RNA levels?

We have now extended the antisense manipulations to assess the impact of deletion of *dcr1*$^{+}$, *ago1*$^{+}$ and *rdp1*$^{+}$ for all loci presented. In each case the phenotype arising from antisense production was abolished by removal of the RITS associated component (Figure 6F-J; Figure 7; Figure S6B, Figure S7A-J). Our attempts to introduce the *clr4*.Δ mutation into the genetic backgrounds to address the impact of Clr4 upon the phenotypes we studied were only successful with the *dis1*$^{+}$ locus. For other crosses

the inherent sickness of the *clr4*.Δ strain meant that we could not recover sufficient viable spores to isolate the relevant strain. For *dis1* however, we now show in Figure 7 C and D that the impact of antisense is dependent upon Clr4 function.

4. In general, the presentation of the RNA-seq data in the Figures (in particular Figures 3 and 4) and the description in the text is difficult to follow and should be improved.

We thank the reviewer for pointing out these issues with the graphics and now use a completely different representation to present the RNA-seq data in Figure 3, Figure S3 and Figure S8. We hope that this form of presentation makes the data more accessible.

Reviewer 2

In this paper, the authors show that the sexual differentiation in fission yeast S. pombe is accompanied by elaborate non-coding RNA expression that is mediated by transcription factors Atf21 and Atf31. They provide evidence showing that the constitutive expression of the spk1, spo4, spo6 and dis1 antisense transcripts disrupt the functions of these genes. Importantly, the functional consequence of antisense RNA expression requires Argonuate protein that has been previously implicated in RNAi-related processes. The authors conclude that programmed generation of non-coding RNA is an important component of regulatory system that drives sexual differentiation in S. pombe.

Critique: The results described in the paper are interesting and conclusions presented are in general supported by the results presented. The data included in the paper will be an important resource for the S. pombe community. The following concerns need to be addressed before the paper can be considered for publication.

Specific comments:

(1) In Fig.6 B.C,F,G, the authors show that the ncRNAs expressed from the plasmid can affect the expression on the chromosome in an ago1-dependent manner. This indicates that the observed effect occurs in trans. Considering that RNAi in S. pombe has been suggested to largely act in cis, this is an important finding. The authors should investigate whether other components of RNAi-machinery are involved in this pathway. For example, dcr1D, rdp1D.

We now show that the other components of the RNAi machinery are indeed required for the antisense manipulations to have any phenotype – please see more detailed response to Reviewer 1 point 3

(2) The overexpression of antisense from a plasmid could result in artifacts. It is important that the authors perform additional experiment by disrupting antisense transcripts at the endogenous genomic location. This experiment is essential to support the main conclusion that ncRNA transcripts represent "genuine" components in the system controlling sexual differentiation.

We share the enthusiasm of the referee for the disruption of the production of anti-sense transcripts at an endogenous locus. This was the rationale for the selection of the *dis1*$^{+}$ locus for gene targeting. It is an isolated gene for which the antisense transcript does not arise from the extension of a 3'UTR. We reasoned that if the anti-sense transcript arose from pol II or polII type 3 mediated transcription, then insertion

of a marker upstream of the anti-sense start site should abolish transcript production. We therefore performed the experiments in Figure 8. The analysis of meiotic progression in Figure 7 panel B shows that the marker insertion did not perturb Dis1 function if it was inserted in the sense direction. Thus our anticipated block to anti-sense production did not occur. We now highlight this point in the revised manuscript and draw the readers attention to the ability of RNA polIII to utilize gene internal promoters, although we feel that establishing the mechanism of antisense production is beyond the scope of this article. Rather, a detailed molecular analysis of antisense production at the *dis1*$^{+}$ locus promises to be an informative study in its own right.

(3) Do antisense RNA affect the RNA polymerase directly? If yes, does this effect require Ago1.

We could not understand the key point being raised here and so were unsure of the experiments required to address this issue.

(4) How do effects of dis1 antisense RNA expression compare with phenotype caused by dis1D? The quality of the Western data in Figure 7 (and other figs) need to be improved to draw definitive conclusions.

We now include a phenotypic characterization of meiotic progression in *dis1*.Δ *pat1.114* homozygous diploids in Figure 7 panel B. This analysis establishes that deletion of the *dis1*$^{+}$ gene, like the antisense manipulations that we report, abolishes horsetail movement. In a complementary experiment in Figure S5C, we show that the overproduction of *dis1*$^{+}$ transcripts increases the frequency of zygotes with horsetail nuclei.

The quality of the blots reflects in part our need to repeatedly re-use a small aliquot of Dis1 antibodies and our use of the BCIP chromogenic substrate. While not giving as spectacular black and white read outs of blots as the more sensitive ECL, we have found this, less sensitive form of developing to give more reproducible quantitation. We have however, repeated key blots where possible and hope that the referee agrees with our interpretation that the insertion of a marker into the 3' end of the *dis1*$^{+}$ gene leads to a significant reduction in Dis1 protein levels when inserted in the sense, but not antisense orientation.

(5) Figure 2 legend is not adequate. There is a lot of information in Figure 2, however, the significance is lost upon the reader due to lack of adequate description and explanation.

We have re-written the legend to Figure 2

(6) The authors also mention general loss of antisense expression in crs1+, bqt1+, and meu1+. It would be worthwhile to show this as additional supplementary figure.

We now include this additional supplementary figure (supplementary Figure S8 in the revised manuscript), following the same form as the new Figure 3.

(7) In the section that discusses "Anti-sense ncRNA control of Pheromone signaling via modulation of Spk1", the text refers to Fig. 5J-k. But figure 5 has only 4 panels that are not numbered.

7) We apologise for this error in compiling the manuscript and have amended the text with the incorporation of the new data in the revised manuscript.

Reviewer #3 (Remarks to the Author):

The manuscript describes the strand-specific analysis of the fission yeast transcriptome in vegetative and meiotic cells. The data are used to comprehensively annotate UTRs in the fission yeast genome, which will b extremely useful for fission yeast researchers. The data also reveal the existence of large amounts of antisense transcription, which is often regulated during meiosis. Follow-up experiments show that anti-sense expression can silence meiotically-expressed genes in trans, in a manner dependent on a component of the RNAi machinery. These observations are of general interest.

Overall this is a very good and interesting piece of work. An extensive analysis of the fission yeast transcriptome has been published recently (Wilhelm et al, see reference list in the paper). However, this manuscript goes further by using strand-specific sequencing (note that Wilhelm et al used tiling arrays to obtain sequencing-specific information). This is a substantial advance, as it allows the specific detection of antisense transcription. The sequencing data appear to be good, although there are a number of issues that should be addressed regarding their analysis and presentation. The authors characterise the biological effect of antisense transcription convincingly, but there are a number of essential controls missing. I consider that the paper merits publication in MSB subject to the authors addressing the points listed below.

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We apologise for this error in assembling the previous submission.

[2] Page 3 (and methods in p. 18). The pat1 experiments have been done using asynchronous diploid cells in the absence of nitrogen starvation. In an alternative protocol, diploids are homozygous for the mating type locus and are starved for nitrogen to block cells in G1 before pat1 is inactivated. The first protocol is used in some studies (Averbeck, Moldon) and the second in others (Mata, Wilhelm). The results using the two protocols are likely to differ substantially, as nitrogen starvation induces very strong changes in gene expression. Given this, it would be important to discuss this issue briefly in the main text.

We thank the referee for pointing this out and now discuss this issue in the discussion section of the revised manuscript on page 17.

[3] Page 3. It is not completely clear from the text which samples were used for the assembly of the TBlocks. If they were pooled, this would create problems when they are used for defining UTRs, as these regions can vary in meiotic cells (for example, through the use of alternative promoters). An extreme example of this phenomenon is shown in Wilhelm et al Figure 3d. Ideally each sample should be analysed independently and the results compared with each other. If this is not possible due to insufficient coverage, the limitations of the approach taken should be discussed.

We have amended the manuscript to describe the approach in detail. We defined the UTRs using the pooled data, but we agree with the referee that the dynamics of the UTRs are both interesting and important – so we have also predicted transcription start- and stop- sites for each locus independently for each sample, and have included these data in the supplement (Table S2).

[4] Page 4. Some of the experiments (vegetative cells and pat1 time course) have been done in duplicate using independent biological samples. It would be very important to get some measurement of the reproducibility of the experiments by comparing the replicates with each other. A simple way would be to show scatter plots of estimated expression levels between replicate experiments.

We were reluctant to combine the data since the first five samples were processed with different preparation kits to the second, and were subjected to sequencing on a single slide, whereas the second dataset was a subset of the 14 samples that were barcoded, pooled and sequenced on two slides at later stage (replicates of the 5 samples above, WT diploid at vegetative growth and the *atf21*.Δ and *atf31*.Δ datasets). For these reasons we did not feel it was appropriate to treat these samples as true replicates. This is, of course, a consequence of the rapid advances being made in the field: even over a relatively short amount of time, the protocols and capabilities of the platform are advancing so fast that it would have been wrong not to use the latest protocols for the second round of sequencing. However, correspondence between the two sets of samples is high – as seen from scatter plots requested by the reviewer, which we have now included in the supplemental data to the manuscript (Figure S5).

[5] Table S4. It should be indicated that the data are log-transformed (if this is what they are). It is unclear what the numbers represent: average between both repeats or a single experiment? It would be important to show [1] the results from both experiments and [2] the results for all genes, not just changing significantly. [2] could be done as a separate supplementary figure, and it is important because it contains very useful information (such as expression levels). Finally, the table contains information of TBlocks expression levels, but this cannot be interpreted as the position of the TBlocks is not given anywhere (should be included as supplementary information).

We apologise for not being as clear as we should have been about the contents of the table. We're also committed towards making the data easily accessible to others. To this end:

- We have amended the table (Table S4) to make it clear data are $\log_2$, and we have added genome coordinates, as requested.
- We have also included an extra table (Table S12) containing expression data from all samples, for all loci, interrogated in the study including TBlocks.
- We have submitted the raw (as .bam files) and processed data to the Gene Expression Omnibus.
- We will also make the data available via our own website and genome browser (xmap.picr.man.ac.uk).

[6] Page 4. '162 of the 4,231 statistically significant genes have been previously described as up-regulated in meiosis'. I don't think the figure of 162 can be correct. Mata et al. identified over 1,000 genes up-regulated during meiosis. If the overlap between these two gene sets

were only 162 it would be mean that previously identified meiotically-induced genes are hugely under-represented in the set identified by this study.

We apologise for our lack of clarity. We meant 162 out of the 176 genes currently annotated as MUGs (Meiotically Upregulated Genes) in GeneDB. We have amended the manuscript to clarify this (see also point 9, below).

[7] Page 5/ figure 2: There are huge changes in gene expression (over a thousand genes) between haploid and diploid cells (regardless of the pat1 allele). As far as I know a comparison between gene expression in haploids and diploids has not been published in fission yeast, but this is very surprising - I think this deserves some discussion.

We share the reviewer's interest in these distinctions between haploid and diploid cells and now highlight the differences on page 8 and also in the discussion section, page 18. In addition, we have modified Table S5 to include fold changes between haploid and diploid for all loci interrogated in the study along with their corresponding p-values and FDR. We also provide all differentially expressed loci corresponding to the data in the Venn diagram, (rather than just the differentially expressed protein coding genes).

[8] Figure 3 is extremely difficult to see (even when amplified on a computer screen). There are simply too many data on it. Also, what are the -1 after some of the systematic names? This figure should be completely redrawn; I would suggest moving some of the examples to a supplementary figure and showing the different time points in different panels (rather than overlaid in different colours). The same applies to figure S2.

In retrospect, these figures were a poor visualisation. We have redrawn them from scratch. We hope the referee agrees that they are substantially improved.

[9] Page 7. It would be useful to have a direct comparison of the results of the atf21/31 experiments with those genes identified by Mata et al (this would validate both experiments)

We have now added some discussion of the Mata et al, paper to the manuscript and show that despite the technical and biological differences between the two studies there is significant concordance in results. In addition, we have tried to make all the relevant gene/transcript lists available, with corresponding fold-changes, and provide the expression data for all loci, as well as making the raw data available as .bam files, through GEO, should others wish to conduct their own more in-depth analyses.

[10] Figure 4 legend - The legend is very confusing - there are no genes displayed in grey (as stated in the legend), also no description of panel VI

We have removed this figure from the manuscript.

[11] Figure 4: The 'trimodal model' - I would not use the word trimodal, as it gives the impression of three discrete states; in fact, the idea is of a continuous distribution of sense/antisense levels.

(as above).

[12] The authors show that expression of antisense RNA in trans causes phenotypes similar to the deletion of the corresponding gene (for spk1, spo4, spo6 and dis1). Moreover, they show that this effect is dependent of a component of the RNAi pathway (ago1). The authors do not speculate on the possible mechanism by which argonaute and the antisense RNAs silence gene expression. However, as this is one of the most important observations of this paper, it would be essential (and easy) to measure the effects of the antisense transcription on RNA levels. This could be done by quantifying the levels of sense and antisense transcript in wild type cells and in cells over expressing the antisense RNA. In addition, it would be essential to verify that no antisense RNA is produced from the transgene (by measuring the expression of the boundary between the end of the gene in the transgene and nmt1 3' UTR).

Please see response to Reviewer 1 point 2 for our attempts to set up the strand specific quantitative PCR technologies in our labs over the past three months. We have clarified the methods used to produce the antisense transcripts from the heterologous loci with the cartoon in Figure 4D. We hope that this clarifies the issue surrounding the potential of antisense production from the 3'UTR of the *nmt1*$^{+}$ locus used to generate the antisense transcript: we have completely replaced the entire *nmt1*$^{+}$ transcript with the antisense transcript, so no 3'UTR remains. Furthermore no antisense transcripts are produced at the *nmt1*$^{+}$ locus (Figure S3C).

[13] Although the experiments discussed in the previous point are clear, they are all based on ectopic overexpression of the antisense RNA, and do not directly address the question of whether regulation by the antisense RNA is relevant in vivo. The authors study this by inserting a cassette (in both orientations) directly upstream of the start of the dis1AS. The results show that protein levels are affected when the cassette is inserted in one orientation, but not in the other. The assumption here is that the insertion blocks the production of the antisense, but this is not formally proven. As this experiment is crucial for the model (it is the only one that looks directly at the in vivo importance of the antisense RNA), I think that the assumption should be tested rigorously by quantifying the levels of sense and antisense RNAs in wild type strains and in cells carrying the insertion in both orientations.

As indicated above we have been unable to establish the technology to address the point in the time allocated for revision.

[14] It would be interesting to discuss the meiotic phenotypes of ago1 mutants and how they fit the authors' model.

We now include a characterization of the meiotic phenotypes of *ago1*.Δ, *dcr1*.Δ, *rdp1*.Δ and *clr4*.Δ h^{90} strains in Figure 5. We show that all mutations reduce the frequency of mating, generate some abnormal asci and have a significant impact upon spore viability, suggesting that these molecules do indeed play key roles in ensuring the fidelity of sexual differentiation.

Minor comments

[1] 'To gain greatest insight' - should it be 'greater'?

[2] Page 6, 'Sense-antisense ratio close to zero' - should be 'close to 1'

[3] Page 6, 'We performed functional term enrichment analysis... for each of the 354 genes' - should be 'for the 354 genes' (the analysis is applied to the genes as a set)

[4] Page 7, ' A total of 12 samples were pooled and sequenced' - surely the samples were not pooled

[5] Page 16, line 9; should be 'at least'

[6] Antisense regulation in trans have been previously shown to be possible in S. pombe using reporters (Arndt et al, Molecular & General Genetics 1995, 248(3):293-300); this paper should be cited. (Note Arndt et al. did not address the possibility that this kind of regulation occurs in vivo or whether it has any practical importance).

[7] I am not sure what MSB policy is for the deposition of this type of data in public databases, but as far as I can see the data have not been deposited.

We have addressed all the minor comments including the insertion of a reference to the Arndt study at the start of the discussion.

2nd Editorial Decision 05 May 2011

Thank you again for submitting your revised work to Molecular Systems Biology. We have now heard back from the three referees. As you will see from the reports below, the referees acknowledge that the manuscript has been improved. They raise however still concerns with regard to the conclusiveness of the demonstration of an impact of AS transcription on the expression levels of sense transcripts. As such, the reviewers feel that it is essential to provide the respective qPCR quantifications requested in the initial round of review.

Please include the accession number to your data directly in the Material & Methods section.
I look forward to receiving your revised work.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

The revised manuscript is improved. The authors now examine the role of other RNAi genes in regulation of silencing by antisense transcripts and present their strand-specific RNA-seq data more clearly. The latter is still the strength of the paper as it would become an important resource for the pombe community. There is room for improvement and in particular some of the claims with regards to mechanisms of regulation that still lack strong evidence (and may represent indirect effects) should be softened.

1. The authors report difficulty in obtaining convincing qRT-PCR and RNA quantification for the experiments in Figure 6, which determine the effect of antisense transcription on target gene expression. This information and ChIP analysis of chromatin modifications at the target locus are critical for any conclusions about transcriptional gene silencing. If the authors could include this information, the manuscript would be greatly improved, otherwise, the conclusions should be stated more cautiously.

In this regard, the authors conclude that antisense transcription induces heterochromatin formation at the target locus based on their observation that Clr4 is required for some of the antisense effects. This ignores two recent publications that show a role for Clr4 in regulation of RNAi independently of histone H3 lysine 9 methylation (Gerace et al., Mol Cell, 2010 and Zhang et al, Science, 2011).

2. The conclusion that Atf21 and 31 regulate antisense transcription directly should be softened. Page 12. The authors present no evidence that the changes observed in atf21delta and atf31deleta cells are direct. For example, no data on putative predicted or experimentally determined Atf21/31 binding sites associated with antisense transcription is presented.

3. The data in Figure 6 should be quantified and reported as percentage of cells that mate and forms spores.

4. The western blot in Figure 7A is of poor quality. It is difficult to tell whether Dis1 levels are changing or not with the introduction of antisense RNA. It is unclear how quantification was performed. Also, why are Dis1 protein levels reduced in ago1delta cells in the absence of antisense RNA?

Reviewer #2 (Remarks to the Author):

I have read the revised version of the paper by Bitton et al. The authors have performed additional experiments to address concerns raised by the reviewers. For example, they have included results of the experiments performed using yeast stains containing an induction cassette integrated into the genome to study the effects of antisense RNA expressed in trans. Evidence included in the paper

also suggests that phenotypes caused by the expression of antisense RNA requires dcr1, rdp1 and clr4, in addition to ago1. The inclusion of new results has improved the quality of the paper.

However, I still feel that the main conclusion that non-coding transcripts and RNA interference proteins represent genuine components of the system controlling sexual differentiation is not fully supported by experimental evidence. This important conclusion is based largely on experiments involving ectopic expression of antisense RNAs. The possibilities such as that antisense RNA expressed from a strong promoter cause indirect effects or sequester RNAi components (e.g. see Buhler NSMB 2008) cannot be ruled out. There is no indication that antisense RNAs (e.g. dis1AS) specifically affect the expression of the targeted locus or cause broader non-specific effects. The results of experiments performed using Kan marker insertion fit with the authors' model but these data lack quantification and it is not clear if KanAS-induced effects require RNAi. PolIII can utilize internal promoters but is it capable of generating long antisense transcripts? The quality of the paper would be much improved if the authors disrupted the production of antisense transcripts at endogenous loci and tested their effects on the levels of sense gene transcripts by using quantitative assays.

As requested in the previous review, the quality of the western blot results remains poor. While this reviewer appreciates the difficulties in re-use of antibodies but the differences in proteins loaded in different lanes (e.g. see TAT1 signal in Fig. 7A and 8B) complicate interpretations of the results presented (also note that levels of Dis1 in RNAi mutants are already low). Considering this is the only data suggesting that antisense transcripts interfere with expression of dis1 expression, the authors shall consider improving these data or provide additional evidence (RT-PCR of sense transcript?) to support the conclusions.

As RNAi proteins have been shown to affect chromosome dynamics during meiosis, it might be interesting to discuss these results in light of the findings described in this paper.

Comment 3 in the previous review related to the possibility that antisense transcripts hinder production of sense transcripts by interfering with PolII transcription. If addressed, this could provide some insights into the mechanism by which antisense transcripts affect expression of meiotic genes.

Overall, this is an interesting study but the conclusions presented need to be fully supported by the evidence.

Reviewer #3 (Remarks to the Author):

In my first review of the manuscript I raised a number of points related to the presentation and analysis of the next-generation sequencing data. The authors have dealt with these issues, and I think the manuscript is now clearer and more solid.
I also requested some important controls related to the interpretation of the anti-sense expression. In particular, I mentioned that the authors should quantify the levels of sense and antisense transcripts in wild type cells and in cells overexpressing antisense RNAs or carrying insertions in the dis3 locus. Similar experiments were requested by another reviewer. The authors have not performed these experiments because they have not had time to set up the required methods before resubmitting their revision. I still think that these are basic controls (to prove that antisense transcription affects RNA levels) and that they should be carried out before publication. The authors mention that they have some preliminary results, although they are not yet consistent enough for publication. I recommend that the authors be granted more time to perform these experiments so that they can be included in the manuscript. Note that I still think that the paper merits publication in MSB when the additional controls are included.

Manuscript Number: MSB-10-2560R
Title: Programmed fluctuations in sense/antisense transcript ratios drive sexual differentiation in S. pombe
Author: Iain Hagan
Danny Bitton
Agnes Grallert
James Bradford
Yaoyong Li
Tim Yates
Paul Scutt
Yvonne Hey
Stuart Pepper
Crispin Miller

Dear Prof Hagan,

Thank you again for submitting your revised work to Molecular Systems Biology. We have now heard back from the three referees. As you will see from the reports below, the referees acknowledge that the manuscript has been improved. They raise however still concerns with regard to the conclusiveness of the demonstration of an impact of AS transcription on the expression levels of sense transcripts. As such, the reviewers feel that it is essential to provide the respective qPCR quantifications requested in the initial round of review.

Please include the accession number to your data directly in the Material & Methods section.

This is now included.

Reviewer #1 (Remarks to the Author):

The revised manuscript is improved. The authors now examine the role of other RNAi genes in regulation of silencing by antisense transcripts and present their strand-specific RNA-seq data more clearly. The latter is still the strength of the paper as it would become an important resource for the pombe community.
There is room for improvement and in particular some of the claims with regards to mechanisms of regulation that still lack strong evidence (and may represent indirect effects) should be softened.

As the field continues to advance rapidly, an increasing number short RNA driven gene regulatory mechanisms are becoming apparent. We have updated both the introduction and discussion of the manuscript to reflect the diversity of systems that operate within *S. pombe*, and we have softened the conclusions to steer away from making definitive statements about which of these mechanisms are likely to act at each locus (Pages 5,6; Page 18: 'Importantly, removal of Clr4 …'; throughout the Discussion).

1. The authors report difficulty in obtaining convincing qRT-PCR and RNA quantification for the experiments in Figure 6, which determine the effect of antisense transcription on target gene expression. This information and ChIP analysis of chromatin modifications at the target locus are critical for any conclusions about transcriptional gene silencing. If the authors could include this information, the manuscript would be greatly improved, otherwise, the conclusions should be stated more cautiously.

We have generated extensive qPCR and cell count data tracking meiotic progression to support each of the panels in Figure 6 in response both to this reviewer's requests and those of reviewer #3 (Figures 8B S6-S8 S10).

As outlined in the text on pages 19-20, we find three levels of response when the ncRNA is expressed *in trans*: for $spo4^{AS}$ and $spo6^{AS}$, clear induction of antisense and reduction of sense (Figure S7B, S8B), for $spk1^{AS}$ no apparent induction of antisense yet a clear reduction in sense (Figure S6B) and, for $dis1^{AS}$ no change in either sense or antisense (Figure S10), despite a phenotype that is indicative of a loss in protein function. The $spo4^{AS}$ and $spo6^{AS}$ data clearly meet the most parsimonious expectation of a direct positive correlation between antisense expression, accumulation and negative correlation with sense reduction. The $spk1^{AS}$ and $dis1^{AS}$ data however suggest that the respective antisense transcripts are unstable and do not accumulate, and yet there is still a clear phenocopy of the null.

In the case of $spk1^{AS}$ it is easy to see how this may arise because the sense transcripts have dipped, however with $dis1^{AS}$, the sense levels have not changed, and yet there is a phenotype. In the latter respect we feel that the observations of from the Allshire lab are particularly pertinent (Simmer *et al.* 2010 *EMBO Reports* 11(2):112-118). This study used hairpins to target *GFP* in a *GFP:ura4*$^{+}$ fusion. The resulting strains were able to grow on either plates containing FOA (kills cells harbouring the ornithine decarboxylase encoded by *ura4*$^{+}$) or on plates lacking uracil (absolutely requires the enzyme for viability). The key point is that there was only a marginal change in transcript levels. Thus, there maybe localized controls that are not detected by bulk analyses that are sufficient to impart a significant biological outcome.

The insertion of the markers at the *dis1* locus clearly shows a level of complexity that will not be resolved without a detailed molecular analysis of this one locus, which we feel is beyond the scope of the current study. We clearly find an elevation of antisense over sense at the phase of sexual differentiation at which Dis1 is required to generate the horsetail migrations in this *dis1:kan*^{R}AS that ablates gene function. However, there is also an up-regulation of the sense transcript at later time points. A diverse set of mechanisms could account for this. For example, it could be that the antisense transcription alters the histone phasing or heterochromatin composition across the locus, either increasing sense transcription or modulating the plasticity of the locus, priming it for transcription. The complexity increases when the marker is reading in the same orientation as the sense strand as: The antisense transcription is now enhanced and yet sense transcripts are now diminished, novel protein isoforms appear and yet there is no phenotype.

Thus the new data corroborate the previous results, however they highlight the complexity of the challenge of providing a full molecular account of ncRNA controls at each gene. We do agree with the reviewer that these are fascinating questions and that more extensive work would be required to definitively state which of the different mechanisms is in effect at each individual locus. However, the current standard of molecular interrogation of similar phenomena within the field suggests that a definitive characterization of each is likely to constitute an entire manuscript in its own right. We therefore deliberately take a step back from taking such a reductionist approach here, since to do so would be at the expense of the more global systems perspective of antisense regulation that we wish to present in this article. At this systems level we have proposed that the ncRNAs we identified play an important regulatory role in controlling sexual differentiation. We believe that we have shown this.

We therefore agree with the referees view of the pitfalls of over-interpretation of the data in the previous version of the manuscript and feel embarrassed to have appeared to be making such bold statements on the back of such little substance. We are grateful to the referee for pointing out this shortfall and hope that the toned down manuscript raises the pertinent points without over-interpreting the issues.

In this regard, the authors conclude that antisense transcription induces heterochromatin formation at the target locus based on their observation that Clr4 is required for some of the antisense effects.

This ignores two recent publications that show a role for Clr4 in regulation of RNAi independently of histone H3 lysine 9 methylation (Gerace et al., Mol Cell, 2010 and Zhang et al, Science, 2011).

We apologise for this oversight and have included citations to these papers (and others) to provide a more comprehensive review of the current (and rapidly progressing) state of the field (Pages 5-6) and have edited the manuscripts at multiple points in order to further emphasise the fact that a variety of different mechanisms are likely to be in effect at different loci. Given the rate of the expansion of Clr4 associated mechanisms appearing in the literature we anticipate that the list is, as yet, incomplete.

2. The conclusion that Atf21 and 31 regulate antisense transcription directly should be softened. Page 12. The authors present no evidence that the changes observed in atf21delta and atf31deleta cells are direct. For example, no data on putative predicted or experimentally determined Atf21/31 binding sites associated with antisense transcription is presented.

We thank the referee for pointing this out and apologise as we should have discussed all the potential interpretations in the original submission. We have edited the manuscript accordingly.

Abstract and Page 13:

> Rather, it shows that a considerable proportion of ncRNA production during sexual differentiation is dependent on Atf21 and Atf31.

3. The data in Figure 6 should be quantified and reported as percentage of cells that mate and forms spores.

This data is now provided in Supplementary Figures 6-8.

4. The western blot in Figure 7A is of poor quality. It is difficult to tell whether Dis1 levels are changing or not with the introduction of antisense RNA. It is unclear how quantification was performed. Also, why are Dis1 protein levels reduced in ago1delta cells in the absence of antisense RNA?

We have used fresh antibody to repeat all of the Dis1 western blots in the study (Fig. 7A) and hope that the improved figures meet the expectations of the referee. We have switched from alkaline phosphatase/BCIP based detection methods to use the fluorescence based ECF/molecular imaging approach for quantification of western blots. Additional statistics are also provided in Fig. S12, including further westerns to examine Dis1 levels with and without antisense induction, and in the *ago1.Δ*. The reduced expression in *ago1.Δ* cells is reproducible (c.f. Fig. 7A and Fig. S12C), but further work would be required to unpick this locus in depth. The smearing in Fig. S12D (see also Fig S8B) is also intriguing, suggesting further complexities at this locus. We prefer not to speculate at this point, since this is outside the scope of the current manuscript. As we remark in the discussion, the *dis1* locus may prove particularly useful for further studies. Arguably, the power of a genomic systems approach is that it allows the identification of these phenomena, before being able to confirm their importance. Antisense is clearly an area that warrants considerable scrutiny, and the next few years of research are likely to be incredibly exciting.

Reviewer #2 (Remarks to the Author):

I have read the revised version of the paper by Bitton et al. The authors have performed additional experments to address concerns raised by the reviewers. For example, they have included results of the experiments performed using yeast stains containing an induction cassette integrated into the genome to study the effects of antisense RNA expressed in trans. Evidence included in the paper also suggests that phenotypes caused by the expression of antisense RNA requires dcr1, rdp1 and clr4, in addition to ago1. The inclusion of new results has improved the quality of the paper.

However, I still feel that the main conclusion that non-coding transcripts and RNA interference proteins represent genuine components of the system controlling sexual differentiation is not fully supported by experimental evidence. This important conclusion is based largely on experiments involving ectopic expression of antisense RNAs. The possibilities such as that antisense RNA expressed from a strong promoter cause indirect effects or sequester RNAi components (e.g. see Buhler NSMB 2008) cannot be ruled out. There is no indication that antisense RNAs (e.g. dis1AS) specifically affect the expression of the targeted locus or cause broader non-specific effects.

The paper from the Bühler lab has indeed made an important contribution to our understanding of the competition between systems because it shows that TRAMP prevents the accumulation of rRNA fragments, which otherwise would become a substrate for the RNAi machinery, titrating it away from its proper target. Therefore we believe that the referee is suggesting that a similar swamping of the RNAi machinery may arise from the antisense inductions that we employ here. However, we anticipate that such a mechanism would result in similar phenotypes irrespective of whichever antisense transcript were to be produced. However, we show that for each of the four loci we investigate, a precise and specific phenotype, corresponding to deletion of the protein-coding gene opposite a given antisense transcript, is observed upon induction of that specific antisense molecule. Specifically:

- Spk1 is required for pheromone signaling to promote conjugation and sexual differentiation. Haploid h^{90} *spk1.Δ* cells are unable to mate and execute meiosis. *pat1.114 spk1.Δ* cells are able to complete meiosis because the pheromone signaling is no longer required to relieve the inhibition of Mei2. $spk1^{AS}$ blocks mating of h^{90} strains but has no impact upon a *pat1.114* induced meiosis. Thus, $spk1^{AS}$ induction completely phenocopies the *spk1.Δ* null in an identical context dependent manner.
- Dis1 is a spindle pole body associated microtubule polymerase that is required to create the microtubule array that drives horsetail migration. Horsetail migration is absent from *pat1.114 dis1.Δ* meioses. $dis1^{AS}$ induction in a *pat1.114* induced meiosis abolishes horsetail movement. Thus, $dis1^{AS}$ induction completely phenocopies the *dis1.Δ* null.
- Spo4/6 kinase is required for efficient mating of h^{90} strains. In the absence of the kinase the efficiency of mating is compromised. Absence of Spo4/6 from cells undergoing a *pat1.114* induced meiosis blocks meiotic progression after the first meiotic division. $spo4^{AS}$ and $spo6^{AS}$ induction compromises the mating efficiency of h^{90} cells to a similar degree as removal of either gene. $spo6^{AS}$ induction in a *pat1.114* meiotic context blocks meiotic progression after the first meiotic division. Thus, $spo6^{AS}$ induction completely phenocopies the *spo6.Δ* null in an identical context dependent manner.

We believe that the correlation between the null and ncRNA phenotypes and the acute context specificity suggest that the effects are direct rather than a genome wide re-balancing of the flux between the RNA surveillance and RNAi machinery, as *per* Bühler et al. (see also Zhang, 2011).

We have included further western analysis that demonstrates that Dis1 protein levels are unchanged by induction of either the $spo4^{AS}$, $spo6^{AS}$, $spk1^{AS}$ (either the antisense portion or the full length *ups1* sense transcript), but are reduced by induction of $dis1^{AS}$ (Figure 7A,B).

We have edited the results section at multiple points to make the experimental logic more clear:
Page 14:

> Importantly, induction of either *spk1*^{AS} or *ups1*^{sense} had no impact upon the ability of *pat1.114* cells to execute a haploid meiosis (Fig. 6K-P) indicating that *spk1*^{AS} production can specifically perturb the Spk1 pheromone response pathway, rather than blocking sexual differentiation *per se*.

Page 16:

> We conclude that the generation of *spo4/spo6* antisense transcripts had the same impact upon sexual differentiation as ablation of the kinase by gene deletion, indicating that they effectively abolished the function normally derived from these loci. The shared context specificity of the *spo6.D* and *spo6*^{AS} phenotypes strongly suggests that antisense production induces a locus dependent rather than generic, non-specific, modulation of gene function.

Page 17:

> Importantly, Dis1 protein levels were not affected by the production of antisense transcripts for all other loci tested in this study (Fig. 7B), indicating that the reduction in Dis1 protein levels arising from either ectopic expression from a ectopic locus or the insertion of a marker in the *dis1*$^{+}$ 3'UTR are the consequences of gene specific expression rather than a non-specific impact such as simple titration of the RNAi machinery (see below).

We also show that this effect is dependent upon components of the RNAi machinery. This would not be the case if the effects we were seeing were to be a consequence of their redirection away from their natural loci. If this were to be so, then we might have expected that deletion of the RNAi machinery would recapitulate the specific phenotypes we observe, rather than rescuing the cells from the impact of the antisense transcripts. We include additional data that shows that loss of Clr4, Dcr1, Rdp1 and Ago1 have little effect on meiosis either *in cis* or *in trans* (Page 18; Figure 6F-J; Fig. S11A-J; Fig. S6-S8), again supporting the hypothesis that this is not simply a non-specific (titration) effect.

The results of experiments performed using Kan marker insertion fit with the authors' model but these data lack quantification and it is not clear if KanAS-induced effects require RNAi. PolIII can utilize internal promoters but is it capable of generating long antisense transcripts? The quality of the paper would be much improved if the authors disrupted the production of antisense transcripts at endogenous loci and tested their effects on the levels of sense gene transcripts by using quantitative assays.

We have now included additional quantification (Fig. S12) and qPCR data, which further corroborate these results. Although the *kan*^{R} marker fails to ablate antisense expression in either orientation, it is clear that the natural regulatory programme of the locus is disrupted in both orientations. While at this stage, the molecular basis remains to be elucidated, it is clear that disruption to the natural-antisense levels lead to altered protein levels and severe disruption to the horsetail stage of meiosis. We now discuss this more fully in the manuscript.

Page 18:
However at this stage is not possible to distinguish whether this impact arises as a consequence of heterochromatin formation (Volpe et al 2002), possibly independently of H3K9 methylation (Gerace et al 2011), or Mlo3 directed switching between TRAMP and siRNA pathways (Zhang et al 2011).

We share the referees enthusiasm for specifically abolishing the production of an individual antisense transcript at a particular locus independently of any impact at any other loci. Indeed, this was the original motivation for the marker insertion experiments. However we believe that this goal can now only be achieved after the molecular basis for the production of antisense transcripts is established at a global level to identify the components that interact with particular loci to generate the ncRNAs. This will then enable us (or others) to abolish this specific interaction at this particular locus in isolation from any impact on any other loci in the genome. The point the referee raised could then be addressed. Our crude attempt of marker insertion did not achieve this goal. We therefore feel that this is likely to be a long-term goal for the field that will require considerable experimentation before this ambition can be realized. Consequently, we consider it to be beyond the scope of the current study.

As reiterated throughout these responses we have removed as much speculation as possible regarding the mechanism that may be operating at each of these loci and so longer raise the issue of the ability of polIII to recognize internal promoters. This is motivated by our belief that unveiling the molecular basis of ncRNA production at each locus and whether a single mechanism accounts for ncRNA production at all 600 loci we describe is a subject for future studies.

As requested in the previous review, the quality of the western blot results remains poor. While this reviewer appreciates the difficulties in re-use of antibodies but the differences in proteins loaded in different lanes (e.g. see TAT1 signal in Fig. 7A and 8B) complicate interpretations of the results presented (also note that levels of Dis1 in RNAi mutants are already low). Considering this is the only data suggesting that antisense transcripts interfere with expression of dis1 expression, the authors shall consider improving these data or provide additional evidence (RT-PCR of sense transcript?) to support the conclusions.

As stated above, we have repeated all of the western blots in the manuscript with fresh antibodies and a new detection system and included substantial amounts of qPCR data for the panels in Fig. 6 and Fig. 8.

As RNAi proteins have been shown to affect chromosome dynamics during meiosis, it might be interesting to discuss these results in light of the findings described in this paper. Comment 3 in the previous review related to the possibility that antisense transcripts hinder production of sense transcripts by interfering with PolII transcription. If addressed, this could provide some insights into the mechanism by which antisense transcripts affect expression of meiotic genes.

We now discuss this in the manuscript, but feel that this one of a number of competing possibilities. A full evaluation of this locus would need to be the subject of an entire manuscript in its own right. Here we focus on a systems' biology view of antisense, and thus consider a set of loci, without pursuing each one in quite such depth.

Overall, this is an interesting study but the conclusions presented need to be fully supported by the evidence.

In response to all three reviewers' comments we have emphasised that the mechanisms at each individual locus are likely to be diverse. However, we hope that the data are now persuasive in showing that they (a) involve antisense, and (b) are dependent on components of the RNAi machinery.

Reviewer #3 (Remarks to the Author):

In my first review of the manuscript I raised a number of points related to the presentation and analysis of the next-generation sequencing data. The authors have dealt with these issues, and I think the manuscript is now clearer and more solid.
I also requested some important controls related to the interpretation of the anti-sense expression. In particular, I mentioned that the authors should quantify the levels of sense and antisense transcripts in wild type cells and in cells overexpressing antisense RNAs or carrying insertions in the dis3 locus. Similar experiments were requested by another reviewer. The authors have not performed these experiments because they have not had time to set up the required methods before resubmitting their revision. I still think that these are basic controls (to prove that antisense transcription affects RNA levels) and that they should be carried out before publication. The authors mention that they have some preliminary results, although they are not yet consistent enough for publication. I recommend that the authors be granted more time to perform these experiments so that they can be included in the manuscript.

These data have now been included in Figures 8B S6-S8 S10

Note that I still think that the paper merits publication in MSB when the additional controls are included.

3rd Editorial Decision 28 October 2011

Thank you again for submitting your revised work to Molecular Systems Biology. We have now finally heard back from the three referees who accepted to evaluate your revision. As you will see, referee #1 and #3 are now supportive. Referee #2 acknowledges that the study has been improved but also continues to raise issues with regard to the mechanistic interpretation of the data.

In view of the support provided by the two other reviewers, which was confirmed by reviewer #3 when we circulated anonymously the reports to each referee, we feel that we can continue to consider the study. We would thus ask you to add a suitable critical discussion of the issues raised by referee #2 in the Discussion, in particular with regard to the impact of Ago1 on AS expression levels.

We would also kindly suggest to cite the recent work by Xu et al 2011 ("Antisense expression increases gene expression variability and locus interdependency" http://dx.doi.org/10.1038/msb.2011.1), for example on page 10 when mentioning the potential "switch" function of AS molecules.

Finally, we would ask you to add to the Materials & Method section a 'Data availability' sub-section where the accession numbers of the functional genomics datasets used in this study are listed (see http://www.nature.com/msb/authors/index.html#a3.5).

I look forward to receiving your revised work.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports

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The revised manuscript is improved. The authors now examine the role of other RNAi genes in regulation of silencing by antisense transcripts and present their strand-specific RNA-seq data more clearly. The latter is still the strength of the paper as it would become an important resource for the pombe community. There is room for improvement and in particular some of the claims with regards to mechanisms of regulation that still lack strong evidence (and may represent indirect effects) should be softened.

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4. The western blot in Figure 7A is of poor quality. It is difficult to tell whether Dis1 levels are changing or not with the introduction of antisense RNA. It is unclear how quantification was performed. Also, why are Dis1 protein levels reduced in ago1delta cells in the absence of antisense RNA?

Reviewer #2 (Remarks to the Author):

I have read the revised version of the paper by Bitton et al. The authors have performed additional experiments to address concerns raised by the reviewers. For example, they have included results of the experiments performed using yeast stains containing an induction cassette integrated into the genome to study the effects of antisense RNA expressed in trans. Evidence included in the paper also suggests that phenotypes caused by the expression of antisense RNA requires dcr1, rdp1 and clr4, in addition to ago1. The inclusion of new results has improved the quality of the paper.

However, I still feel that the main conclusion that non-coding transcripts and RNA interference proteins represent genuine components of the system controlling sexual differentiation is not fully supported by experimental evidence. This important conclusion is based largely on experiments involving ectopic expression of antisense RNAs. The possibilities such as that antisense RNA expressed from a strong promoter cause indirect effects or sequester RNAi components (e.g. see Buhler NSMB 2008) cannot be ruled out. There is no indication that antisense RNAs (e.g. dis1AS) specifically affect the expression of the targeted locus or cause broader non-specific effects. The results of experiments performed using Kan marker insertion fit with the authors' model but these data lack quantification and it is not clear if KanAS-induced effects require RNAi. PolIII can utilize internal promoters but is it capable of generating long antisense transcripts? The quality of the paper would be much improved if the authors disrupted the production of antisense transcripts at endogenous loci and tested their effects on the levels of sense gene transcripts by using quantitative assays.

As requested in the previous review, the quality of the western blot results remains poor. While this reviewer appreciates the difficulties in re-use of antibodies but the differences in proteins loaded in different lanes (e.g. see TAT1 signal in Fig. 7A and 8B) complicate interpretations of the results presented (also note that levels of Dis1 in RNAi mutants are already low). Considering this is the only data suggesting that antisense transcripts interfere with expression of dis1 expression, the authors shall consider improving these data or provide additional evidence (RT-PCR of sense transcript?) to support the conclusions.

As RNAi proteins have been shown to affect chromosome dynamics during meiosis, it might be interesting to discuss these results in light of the findings described in this paper.

Comment 3 in the previous review related to the possibility that antisense transcripts hinder production of sense transcripts by interfering with PolII transcription. If addressed, this could provide some insights into the mechanism by which antisense transcripts affect expression of meiotic genes.

Overall, this is an interesting study but the conclusions presented need to be fully supported by the evidence.

Reviewer #3 (Remarks to the Author):

In my first review of the manuscript I raised a number of points related to the presentation and analysis of the next-generation sequencing data. The authors have dealt with these issues, and I think the manuscript is now clearer and more solid.
I also requested some important controls related to the interpretation of the anti-sense expression. In particular, I mentioned that the authors should quantify the levels of sense and antisense transcripts in wild type cells and in cells overexpressing antisense RNAs or carrying insertions in the dis3

locus. Similar experiments were requested by another reviewer. The authors have not performed these experiments because they have not had time to set up the required methods before resubmitting their revision. I still think that these are basic controls (to prove that antisense transcription affects RNA levels) and that they should be carried out before publication. The authors mention that they have some preliminary results, although they are not yet consistent enough for publication. I recommend that the authors be granted more time to perform these experiments so that they can be included in the manuscript. Note that I still think that the paper merits publication in MSB when the additional controls are included.

3rd Revision - authors' response 02 November 2011

We are pleased to upload a revised version of our manuscript, in which we address the remaining comments.

In addition, we have:

1) Included the GEO accession for our data in a ëData availabilityí section in the methods as requested.

2) Added a reference to the Xu et al. manuscript, on page 10:

We therefore formed a tentative hypothesis that antisense molecules might act as ëswitchesí, suppressing protein production when in excess relative to their target mRNAs, while allowing production when under-expressed. A third class of antisense molecules maintained the sense/antisense ratio close to one (zero in log space). Although speculation, this would be in keeping with a role in maintaining protein homeostasis, in which antisense regulation is used to help keep protein abundance at an appropriate level (Fig. 4A-C). A similar model was proposed by Xu and colleagues in budding yeast while this manuscript was under review (Xu et al, 2011).
... and on Page 21: We show that an extensive and elaborate array of ncRNA production accompanies sexual differentiation in the fission yeast S. pombe. Experimental manipulation suggests that these transcripts specifically regulate the function of the target genes. While previous studies have demonstrated the principle of gene control by antisense expression in fission yeast using exogenous genes (Arndt et al, 1995), the ubiquity, and importance of the endogenous genes subjected to ncRNA expression in our datasets, means that antisense interactions now move from a theoretical possibility to an intrinsic part of the regulatory machinery, with the same status and importance as other levels of control. Encouragingly, a similar model of ncRNA control of gene regulation has recently been derived from the analysis of stress responses in budding yeast (Xu et al 2011).

We have also added additional paragraphs to the discussion to address the concerns of reviewer two. Detailed responses are included below.

MSB-10-2560RR Response to Referees comments:

We are grateful to all the reviewers for their detailed considerations of our work.

Reviewer #1 (Remarks to the Author):

> *The authors have satisfactorily addressed my comments and I am happy to recommend publication in MSB. This paper contains a huge amount of data and analysis. There are many some specific areas with regards to the role of antisense transcription that still remain ambiguous. However, I feel that the revised presentation is balanced and takes these issues into account.*

We thank the reviewer for the supportive comments.

Reviewer #2 (Remarks to the Author):

> *I have read the revised version of the paper by Bitton and colleagues. In addition to rewriting certain parts of the paper to remove speculations about possible roles of antisense RNAs, the authors have performed large number of additional experiments to improve the quality of the paper. In particular, they have improved the quality of the Western blot data and have provided qRT-PCR analysis of sense and antisense transcripts at different points during meiotic progression. The new Western blot results are consistent with the authors' view that antisense transcripts affect corresponding gene expression via mechanism(s) involving RNAi factors and Clr4, except that loss of Ago1 unexpectedly causes reduction in the levels of Dis1. The results of qRT-PCR analyses are however, extremely complex and it is difficult to draw definitive conclusions from these analyses about the functions of antisense transcripts during sexual differentiation.*
>
> *An important conclusion is that antisense RNAs silence genes and that this process requires the RNAi machinery. Indeed, induction of spo4 antisense RNA correlates with strong downregulation of sense RNA during sexual differentiation. However, this is not always the case. The authors observed only minor change in spo6 sense transcript upon the expression of spo6 antisense RNA (compare S7B and S8B), despite strong phenotypic effects. Paradoxically, the insertion of kan gene at the 3' end of the dis1 locus increases antisense RNA levels in both dis1:kanAS and dis1:kanS orientations but phenotypic changes are observed only in the dis1:kanAS cells. The lack of correlation between accumulation of antisense RNAs and silencing of gene as well as phenotypic changes appear to argue against the straightforward model about the function of antisense RNAs. The detailed understanding of antisense-mediated silencing of genes is beyond the scope of this study, but the results should support the conclusion that upregulation of antisense RNAs silence corresponding genes in a predictable manner.*
>
> *The results presented in figure 7 show that antisense expression has no effect on Dis1 levels in strains containing deletions of dcr1, clr4, and rdp1 genes. In contrast, the loss of ago1 paradoxically causes reduced expression of Dis1. This is surprising because the deletion of ago1 suppresses the phenotypes caused by the expression of dis1 antisense RNA similar to dcr1∆, clr4∆ and rdp1∆. Again, disconnect between the effects of ago1∆ on Dis1 expression and phenotypic changes, caused by the dis1AS expression appear to suggest a more complex mechanism.*
>
> *The levels of antisense RNAs are significantly lower in ago1∆ mutant, leading the authors to suggest that Ago1 function is required for the stability of antisense RNAs. However it is not clear whether Ago1 indeed affects the stability of RNAs or their*

expression. More importantly, this result raises the possibility that the suppression of phenotypic changes observed in RNAi mutant cells might actually be due to reduced expression of antisense RNAs in these mutant backgrounds. Considering the importance of conclusions from these experiments, the authors might want to take another look at these data.

I continue to feel that the manuscript describes interesting observations but in light of new data the exact nature of effects observed upon expression of antisense RNAs remains unclear.

The reviewer is right to suggest caution in ascribing specific mechanisms to the effects we see at each locus, and to consider the interplay between local and global effects upon Argonaute deletion. We have included additional text in the discussion to further consider these important issues (Page 23):

While the reliance of the specific phenotypes arising from ncRNA induction upon *ago1, dcr1* and *rdp1* indicates a key role for the RNAi machinery in implementing the control by ncRNA of gene function, it would be premature at this stage to draw precise conclusions about the level at which this control is executed at each particular locus. It will be important to disentangle genome wide consequences of the removal of the RNAi machinery upon meiotic progression from targeted controls at each specific locus. The loss of the more generic RNA processing factor, Argonaute, in particular, clearly has a global impact upon both sense and antisense levels. Thus, any consideration of specific effects at a target locus must be considered in the context of genome wide changes in the transcript profile. Until our understanding of the nature of such global changes improves, detailed interpretations of cause and effect, or even the relative contributions of one level of control over another, cannot be drawn. It is, however, clear that in every instance described here, the induction of ncRNA imposes a targeted attenuation of function that is both locus specific and RNAi dependent; it is simply not possible to infer the means by which the RNAi machinery exerts this control at present. Such detailed understanding can only arise from in-depth targeted analyses at key loci, such as *dis1*.

The reviewer also raises the complexity of the changes to sense abundance in response to over-expression of the different antisense molecules. Again, these are important points and we now discuss this in more detail (Page 26):

The complexity of these systems is also in keeping with the variety of expression patterns associated with antisense activity. Thus while for each of the four loci described here, a strong characteristic phenotype is observed as a consequence of perturbations to their natural antisense profiles, different patterns of sense expression arise.

In a system comprising a set of interacting regulatory pathways, involving both positive- and negative feedback loops, the lack of a simple relationship between sense and anti-

sense expression is unsurprising, particularly given the number of alternate mechanisms by which antisense itself may be operating. Such complexity raises both…

Reviewer #3 (Remarks to the Author):

> *The manuscript has been substantially improved after two rounds of revision. The authors have performed additional experiments to address my concerns. I consider that the latest version of the manuscript is appropriate for publication.*

We thank the reviewer for the supportive comments.