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CSR-1 RNAi Pathway Positively Regulates Histone Expression in C. elegans

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1st Editorial Decision 14 February 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while all three referees consider the study as interesting in principle, they all raise the concern that the link between CSR-1/RNAi and histone mRNA processing is not yet sufficiently established in a direct and causal manner at this stage of analysis. They all put forward a number of constructive suggestions for how this link could potentially be strengthened by further experimentation. On balance, and given the high interest expressed by the referees in principle, we should be able to consider a revised manuscripts that addresses the concerns of all three referees - in particular the one mentioned above - in an adequate manner and to their full satisfaction. Still, I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees. Please do not hesitate to contact me at any time in case you would like to consult further on any aspect of the revision.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Palani et al., present a novel model for histone biogenesis in C. elegans that involves endogenous small interfering RNAs (endo-siRNAs) and the CSR-1 Argonaute protein. Using published data, they observed that mRNA levels for most histone genes were down-regulated in csr-1 mutant animals, which led them to hypothesize that decreased histone expression could contribute to the chromosome segregation and sterility phenotypes seen in these mutants. Notably, endo-siRNAs complementary to core histone mRNA sequences are enriched in CSR-1 IP libraries and some of these siRNAs map to the 3' ends of mature histone mRNAs. The authors propose that the endo-siRNAs guide CSR-1 to positions between the stem-loop and polyadenylation signal (PAS) of histone pre-mRNAs to direct cleavage and produce mature mRNAs that end in the stem-loop, instead of the less efficiently expressed polyadenylated forms. This model is supported by the demonstration of increased histone pre-mRNA levels in csr-1 and other mutants (ego-1, drh-3) that disrupt accumulation of endo-siRNAs bound by CSR-1. A strong effect on histone protein levels was also confirmed through western blotting and immunostaining. Finally, they authors demonstrate that overexpression of histone genes lacking the endo-siRNA target site and PAS rescues the embryonic lethality of csr-1 and ego-1 mutants. This study is very novel and reveals an entirely new and surprising function for siRNAs in histone biogenesis. It is of general interest because it exemplifies the utility and diversity of small RNAs in regulating gene expression. Overall, the experiments are well done, with appropriate controls, leading to clear cut conclusions. The following suggestions would further strengthen the work, but, in my opinion, are not necessary to support the authors' model:

1. Does CSR-1 co-IP with histone pre-mRNAs in an endo-siRNA dependent manner? This would confirm that CSR-1 directly binds these RNAs.

2. Comparison of transgenes that maintained the PAS versus those without seems like a stronger experiment than analyzing high and low expressing transgenes lacking the PAS. The presence of the PAS should make the transgenes dependent on the CSR-1 pathway and thus rescue would be lost. I imagine the generation of such transgenic strains is not trivial and but if the authors already have such strains, I encourage them to include the data.

3. Why is the total histone mRNA lower in csr-1(RNAi), ego-1(RNAi) and drh-3 mutants (Fig. 2)? If it is not processed, is it destabilized?

Referee #2

"CSR-1 RNAi pathway positively regulates histone expression in C. elegans" by Palani et al

Previous work has identified an Argonaute protein in C. elegans CSR-1 that forms complex with endogenous siRNAs produced by RNA-dependent RNA polymerase EGO-1 and dicer related helicase DRH-3. Depletion of siRNAs in complex with CSR-1 causes sterility and defects in chromosome segregation. The results presented in this paper show that CSR-1, EGO-1 or DRH-3 are required for proper processing of histone mRNAs. The authors show that endo-siRNAs, which require EGO-1 for their production, match 3'-UTR of histone mRNAs in antisense orientation at the cleavage site. Depletion of RNAi factors causes accumulation of unprocessed histone mRNAs and decrease in levels of histones at a stage prior to emergence of sterility and chromosome segregation defects. Importantly, the introduction of additional histone genes, which lack endo-siRNA target sites and polyadenylation signals, could rescue the developmental phenotypes of RNAi mutants. Based on these and other results, the authors suggest a model for RNAi-mediated processing of histone mRNAs.

Critique: The results presented in the paper are interesting and suggest a role for the RNAi machinery in proper processing of histone mRNAs. The work described in the paper does not directly link the CSR-1 (or other RNAi factors) to histone transcripts except that endo-siRNAs map to the 3'-UTR of histone mRNAs. However, the result showing that additional histone genes can rescue the effects of RNAi mutants is very interesting and fully supports the authors' conclusion that the changes in histone proteins levels are likely main cause of segregation defects in the csr-1 mutant. The authors' shall consider addressing following points before the paper is considered for publication in the EMBO Journal.

(1) It seems important to know whether CSR-1 is directly involved in processing histone mRNAs. To address this issue, the authors shall consider performing RNA-IP experiments to test if CSR-1 binds histone mRNAs. The loss of factors required for the production of endo-siRNA is expected to affect CSR-1 binding to histone mRNAs.

(2) To establish direct link between RNAi and histone mRNA processing, the authors might be able to modify sequence at 3'-end of histone gene construct and ask whether artificial production of siRNA matching this sequence would promote target mRNA processing.

(3) The model suggested in the abstract and in the paper that polyadenylation of transcripts is followed by endo-siRNA production by EGO-1 and siRNA-mediated cleavage of mature histone mRNA remains largely speculative. The authors should consider providing some insights into how polyadenylation would stimulate EGO-1 activity and the cleavage of histone mRNAs by the RNAi machinery.

Referee #3

Review Palani et al.

The manuscript by Palani et al. describes a number of studies that suggest a role for the Argonaute protein CSR-1 in histone mRNA maturation in C. elegans. This is based on the observation that histones are depleted when csr-1 is lacking and that histone mRNAs contain sites that match endogenous 22G RNAs. The most convincing part of the study is the description of rescue of the embryonic lethal phenotype of csr-1 mutants by expressing histones 2A/B, 3 and 4 from a transgenic array. The model the authors propose is that CSR-1 would induce cleavage in between the stem loop structure and the PAS sequence and that this would be followed by poly-adenylation.

Although intriguing, I don't think the data are very compelling. It will require significant work to reach conclusive statements. At this point I cannot recommend publication. Below I will list my concerns/suggestions.

1) In the abstract the authors make a giant leap. They write "Since nematodes lack U& snRNA it appears that endo-siRNAs assume its role in guiding histone mRNA processing in C. elegans". This is simply a major overstatement. This is a possibility, nothing more, nothing less.

2) The authors do not provide any evidence that CSR-1 endonucleolytic activity is required during histone mRNA processing. Can the authors demonstrate the cleavage products that they predict in wild-type, but not in csr-1 mutant animals. This is hard, but I guess to make this claim, it would be required to do so. Especially since the 22G RNAs do not really align nicely to the site that needs to be cut. Argonautes cleave precisely in between bases 10 and 11 from the 5' side of the siRNA. Although not shown for CSR-1, one would expect this property to be conserved in CSR-1. Is the DDH motif of CSR-1 required for its function?

3) The authors rescue csr-1 lethality with transgenic histone mRNA lacking the PAS sequence. PAS-containing sequences should not rescue. This should be tested.

4) The normally present sequence that is presumably cut by CSR-1 should be replaceable with sequences matching other CSR-1 bound 22G RNAs, whereas introducing sequences not represented among CSR-1 22G RNAs should block processing of transgenic histone mRNAs. Such experiments should be performed in wild-type animals, just to see whether the correlation is stronger than just the observation described here.

5) Do authors do not provide an explanation for how histone mRNAs are processed in the soma, where csr-1 is absent. Are histones also lower in glp-4; csr-1 mutant animals compared to glp-4 alone? (glp-4 mutation results in a virtual lack of germ cells)

6) The authors show 22G RNAs matching the histone mRNAs in between the stem loop and the PAS. How specific are the 22G RNAs for this part of the histone mRNA? Are there no matches to other regions? The authors show that these 22G RNAs are enriched in CSR-1 IPseq data (from Claycomb et al). How do these enrichments compare to the typical enrichments for the top targets of the CSR-1 pathway? I seem to recall that these enrichments are much higher than what is described here for the histone 22Gs.

7) The authors rescue with a combination of H2A, H2B, H3 and H4. Why include H3 and H4, since these two are not really changed. Also, why are H3 and H4 not affected? The authors give a hypothetical explanation, but this is not really satisfying. If this is a true function for CSR-1 it should affect H3 and H4 as well.

8) In general, the rescue experiments should be repeated with genetic mutants. Histone overexpression may affect RNAi and thus result in rescue. The authors do try to control for this (cdl-1 RNAi), but they cannot exclude that the effect imposed by cdl-1 RNAi is just too strong to be rescued by histone overexpression, while RNAi of the (RNAi components!) csr-1 and ego-1 can be rescued by a potential RNAi defect induced by histone overexpression. I think this is an important issue as a recent paper by the Fire lab has shown that histone modification is part of regular RNAi (Nat.Genet, Dec 2011). Given that histones may exchange readily, overexpression of histones may cripple RNAi by preventing the build-up of proper silencing marks on the chromatin.

9) I am wondering whether the effects described by these authors just reflect correlation and do not hold a direct mechanistic link. The germline is clearly affected by loss of CSR-1 (and EGO-1, DRH-3) and this may lead to all sorts of secondary effects, including defects in histone production. Perhaps germline identity is partially lost in these mutants and it's because of this that histone production drops? There is a fourth player in this scene, CDE-1. Mutants for cde-1 apparently still contain CSR-1 and its 22G RNAs, but still chromosome segregation defects are observed in cde-1 mutants. Is there a defect in histone mRNA production in cde-1 mutants as well? If so, that would not be consistent with the proposed model. The same holds true for other RNAi mutants, many of which show chromosome segregation defects (although much less than that observed in CSR-1).
10) Finally, it is strange to see an paper submitted to EMBO J, totally formatted for another journal. The authors could have done themselves a favor to expand their introduction and discussion in order to present the work in a more balanced manner.

1st Revision - authors' response 15 May 2012

Response to the referees' comments

Referee #1

Palani et al., present a novel model for histone biogenesis in C. elegans that involves endogenous small interfering RNAs (endo-siRNAs) and the CSR-1 Argonaute protein. Using published data, they observed that mRNA levels for most histone genes were down-regulated in csr-1 mutant animals, which led them to hypothesize that decreased histone expression could contribute to the chromosome segregation and sterility phenotypes seen in these mutants. Notably, endo-siRNAs complementary to core histone mRNA sequences are enriched in CSR-1 IP libraries and some of these siRNAs map to the 3' ends of mature histone mRNAs. The authors propose that the endo-siRNAs guide CSR-1 to positions between the stem-loop and polyadenylation signal (PAS) of histone pre-mRNAs to direct cleavage and produce mature mRNAs that end in the stem-loop, instead of the less efficiently expressed polyadenylated forms. This model is supported by the demonstration of increased histone pre-mRNA levels in csr-1 and other mutants (ego-1, drh-3) that disrupt accumulation of endo-siRNAs bound by CSR-1. A strong effect on histone protein levels was also confirmed through western blotting and immunostaining. Finally, they authors demonstrate that overexpression of histone genes lacking the endo-siRNA target site and PAS rescues the embryonic lethality of csr-1 and ego-1 mutants. This study is very novel and reveals an entirely new and surprising function for siRNAs in histone biogenesis. It is of general interest because it exemplifies the utility and diversity of small RNAs in regulating gene expression. Overall, the experiments are well done, with appropriate controls, leading to clear cut conclusions. The following suggestions would further strengthen the work, but, in my opinion, are not necessary to support the authors' model:

1. Does CSR-1 co-IP with histone pre-mRNAs in an endo-siRNA dependent manner? This would confirm that CSR-1 directly binds these RNAs.

To address this question we performed pull-down experiments that employ biotinylated 2′-O-methyl oligonucleotides and were successfully used in C. elegans for studies of miRNA and siRNA targets (Hutvágner et al., PLoS Biol, 2004), (Aoki at al., EMBO J, 2007). We designed an oligo that mimics 34 nucleotides present between the stem-loop and PAS in the H2A pre-mRNA 3′ UTR. By pulling down this biotynilated oligo with streptavidin beads we detected an

enrichment of CSR-1, which was lost after depletion of EGO-1 RdRP, i.e. secondary siRNAs (see Figure 2A). This experiment directly supports the existence of CSR-1-bound ego-1-dependent siRNAs complementary to the pre-mRNA region within which the cleavage occurs. In a complementary approach, we designed oligos antisense to the coding region of H2A mRNA. These pull down experiments revealed a direct, ego-1-dependent interaction between CSR-1 and H2A mRNA, consistent with the presence of CSR-1-bound endo-siRNAs targeting histone messages, see Figure 2B and Supplementary Figure S1. We conclude that CSR-1 directly binds both histone mRNA and pre-mRNA.

2. Comparison of transgenes that maintained the PAS versus those without seems like a stronger experiment than analyzing high and low expressing transgenes lacking the PAS. The presence of the PAS should make the transgenes dependent on the CSR-1 pathway and thus rescue would be lost. I imagine the generation of such transgenic strains is not trivial and but if the authors already have such strains, I encourage them to include the data.

Initially, we used a plasmid containing endogenous sequences of histone genes ending after the stem-loop in 3′UTR. We re-introduced a PAS sequence downstream of the stem-loops of histone H2A and H2B genes in the previously designed plasmid. Therefore, in these modified histone genes, plasmid sequences were present between the stem-loop and PAS instead of sequences normally targeted by siRNAs. None of the five transgenic lines generated with this plasmid showed an mRNA expression level comparable to the rescuing line with truncated 3′UTR (Supplementary Figure S7) and none showed a comparable level of rescue of the lethality caused by csr-1(RNAi) (Figure 7A). However, the PAS-containing transgenes in two lines (armEx180 and armEx184) were expressed ~2-fold higher compared to the germline-silenced line (armEx152) used as a control in the initial experiments, but this expression did not contribute to any noticeable increase in histone protein levels (compare Supplementary Figure S7 and Figure 7B). These results are consistent with the lower expression and utilization of histone messages that cannot be processed.

Reviewer #1 brought up an important point (see point #3) about the observed decrease in the total levels of histone mRNA (processed and unprocessed) in samples enriched with unprocessed messages. This decrease was evident from the analysis of published csr-1(-/-) microarray data

(Figure 1) and also shown in the current Figure 3 (former Figure 2). Studies of maternal effect mutations in the stem-loop-binding protein (SLBP) in flies revealed the existence of a mechanism promoting destabilization of the unprocessed messages in mutant oocytes (Sullivan et al., G&D, 2001). We believe that this finding is very similar to what we observe in C. elegans when the maternal function of cdl-1(SLBP), csr-1 or ego-1 is inhibited by RNAi. Therefore, in experiments where the artificial histone pre-mRNA is designed to be expressed from a transgene in the germline of C. elegans, it may be very difficult to achieve the desired high levels of expression. It may be possible in theory but certainly not easy in practice.

3. Why is the total histone mRNA lower in csr-1(RNAi), ego-1(RNAi) and drh-3 mutants (Fig. 2)? If it is not processed, is it destabilized?

Yes, we believe so; see above. The mechanism responsible for this effect is not known yet and would be interesting to investigate in the future.

Referee #2

"CSR-1 RNAi pathway positively regulates histone expression in C. elegans" by Palani et al

Previous work has identified an Argonaute protein in C. elegans CSR-1 that forms complex with endogenous siRNAs produced by RNA-dependent RNA polymerase EGO-1 and dicer related helicase DRH-3. Depletion of siRNAs in complex with CSR-1 causes sterility and defects in chromosome segregation. The results presented in this paper show that CSR-1, EGO-1 or DRH-3 are required for proper processing of histone mRNAs. The authors show that endo-siRNAs, which require EGO-1 for their production, match 3'-UTR of histone mRNAs in antisense orientation at the cleavage site. Depletion of RNAi factors causes accumulation of unprocessed histone mRNAs and decrease in levels of histones at a stage prior to emergence of sterility and chromosome segregation defects. Importantly, the introduction of additional histone genes, which lack endo-siRNA target sites and polyadenylation signals, could rescue the developmental phenotypes of RNAi mutants. Based on these and other results, the authors suggest a model for RNAi-mediated processing of histone mRNAs.

Critique: The results presented in the paper are interesting and suggest a role for the RNAi machinery in proper processing of histone mRNAs. The work described in the paper does not directly link the CSR-1 (or other RNAi factors) to histone transcripts except that endo-siRNAs map to the 3'-UTR of histone mRNAs. However, the result showing that additional histone genes can rescue the effects of RNAi mutants is very interesting and fully supports the authors' conclusion that the changes in histone proteins levels are likely main cause of segregation defects in the csr-1 mutant. The authors' shall consider addressing following points before the paper is considered for publication in the EMBO Journal.

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*We were not able to efficiently carry out RNA-IP experiments since 1) we do not have enough antibody to the endogenous CSR-1 to use for IP and 2) CSR-1::GFP expression in the existing transgenic line created by Claycomb et al, Cell, 2009, which was produced by using heterologous germline-specific pie-1 promoter, is very low. Furthermore, it was noted in Gu and Claycomb et al, Methods Mol Biol, 2011, that the rescuing fusion proteins of CSR-1 may not be functional and that IP efficiencies in these lines may be low: "*Interestingly, a 3XFlag epitope inserted 28 amino acids N-terminal of the PIWI domain of CSR-1 did not rescue the *csr-1(tm892)* mutant phenotype, suggesting that the fusion protein is non-functional. Furthermore, the IP efficiency of the rescuing RDE-1 and CSR-1 fusions may be low." *Although we attempted the RNA-IP using anti-GFP antibodies in the CSR-1::GFP rescued strain, we were unable to get efficient immunoprecipitation of the protein.*

To address this question we used an alternative strategy described earlier, see the response to point #1 of reviewer #1.

(2) To establish direct link between RNAi and histone mRNA processing, the authors might be able to modify sequence at 3'-end of histone gene construct and ask whether artificial production of siRNA matching this sequence would promote target mRNA processing.

We designed GFP reporter constructs for these types of experiments but were not able to get transgenic lines with germline expression with these constructs.

(3) The model suggested in the abstract and in the paper that polyadenylation of transcripts is followed by endo-siRNA production by EGO-1 and siRNA-mediated cleavage of mature histone mRNA remains largely speculative. The authors should consider providing some insights into how polyadenylation would stimulate EGO-1 activity and the cleavage of histone mRNAs by the RNAi machinery.

The model suggesting initial polyadenylation of the histone transcripts followed by EGO-1-dependent production of siRNAs and finally siRNA-based processing is indeed speculative and was removed from the abstract. We present this possibility in the "discussion" section now. We also discuss alternative possibilities for the role of polyadenylation: "The production of the polyadenylated histone pre-mRNA intermediate could help in recruiting factors required for further processing or, alternatively, could attract proteins promoting pre-mRNA degradation in oocytes, such that only properly processed messages ready for translation are deposited into the zygote and early embryo".

The proof of the model above was not intended in this manuscript. Instead, we provide the following new findings that are novel and significantly contribute to the progress of the field:

1) *We identify the cause of the severe germline and early embryo defects in the RNAi mutants, this question has remained unresolved for 12 years;*
2) *We provide evidence that the Argonaute protein CSR-1 directly binds to histone messages in an siRNA-dependent manner and that histone protein levels are severely reduced in the knockdown of csr-1. This confirms a direct positive effect of endogenous RNAi on gene expression, which has not been previously reported.*
3) *We provide results that support a model in which CSR-1-bound siRNAs complementary to the region between the stem-loop and PAS in the 3′ UTR are important for proper processing of histone messages: we show that CSR-1 binds to the sequences present in this region in an siRNA-dependent manner, and that expression of histone messages that do not require processing are not dependent on the RNAi pathway since their expression allows rescue of the lethality caused by the depletion of RNAi components. We believe*

that the model discussed here is well supported by our data, although we cannot exclude that CSR-1 affects histone mRNA processing in a direct but different way.

Referee #3

Review Palani et al.

The manuscript by Palani et al. describes a number of studies that suggest a role for the Argonaute protein CSR-1 in histone mRNA maturation in C. elegans. This is based on the observation that histones are depleted when csr-1 is lacking and that histone mRNAs contain sites that match endogenous 22G RNAs. The most convincing part of the study is the description of rescue of the embryonic lethal phenotype of csr-1 mutants by expressing histones 2A/B, 3 and 4 from a transgenic array. The model the authors propose is that CSR-1 would induce cleavage in between the stem loop structure and the PAS sequence and that this would be followed by poly-adenylation.

Although intriguing, I don't think the data are very compelling. It will require significant work to reach conclusive statements. At this point I cannot recommend publication. Below I will list my concerns/suggestions.

1) In the abstract the authors make a giant leap. They write "Since nematodes lack U& snRNA it appears that endo-siRNAs assume its role in guiding histone mRNA processing in C. elegans". This is simply a major overstatement. This is a possibility, nothing more, nothing less.

This sentence was removed from the abstract and the possibility is discussed in the Discussion section.

2) The authors do not provide any evidence that CSR-1 endonucleolytic activity is required during histone mRNA processing. Can the authors demonstrate the cleavage products that they predict in wild-type, but not in csr-1 mutant animals. This is hard, but I guess to make this claim, it would be required to do so. Especially since the 22G RNAs do not really align nicely to the site that needs to be cut. Argonautes cleave precisely in between bases 10 and 11 from the 5' side

of the siRNA. Although not shown for CSR-1, one would expect this property to be conserved in CSR-1. Is the DDH motif of CSR-1 required for its function?

We show that CSR-1 binds to the sequences present in the region between the stem-loop and PAS in the 3' UTR in siRNA-dependent manner, see the response to point #1 of reviewer #1. The ends of the mature histone messages map to this region, but they do not align perfectly to the positions opposite of bases 10 and 11 in the matching siRNAs as would be expected for a "classical" Argonaute. This could mean that 1) CSR-1, which is guided by secondary siRNAs containing triphosphates at the 5′ ends, may have a different pattern of cleavage as compared to Argonautes loaded with 5′ monophosphate-bearing siRNAs; 2) CSR-1 cleaves in a traditional way but the end of the message gets trimmed by exonucleases; 3) CSR-1 acts to recruit some other factors mediating the cleavage. Please note that we discuss possibility #3 in the text since there are no reagents available for testing the requirement of the DDH motif for CSR-1 function; the existing CSR-1::GFP and CSR-1::FLAG lines show poor rescue and it is notoriously difficult to generate germline expressing CSR-1 transgenic lines according to meeting presentations from the Mello lab.

*The reviewer is certain that since "*Argonautes cleave precisely in between bases 10 and 11 from the 5' side of the siRNA. …, one would expect this property to be conserved in CSR-1". *However, this statement is speculative and is actually not consistent with the published data. The study that describes the endonucleolytic activity of CSR-1 (Aoki at al., EMBO J, 2007) did present data on the cleavage preferences of CSR-1, although only for one siRNA, which had either a 5′ mono- or 5′ triphosphate. None of the mapped cleavage sites for either of these siRNA types matched bases 10 and 11 but instead were skewed further, to the 3′ half of the siRNA sequence. Notably, cleavage sites for the siRNA with a 5′ triphosphate were located closer to the 3′end of siRNA, resembling what we show in Figure 1F. The protein sequence of the 5′ monophosphate binding pocket present in Argonautes (see Figure 3a in Ma et al, Nature, 2005) is conserved in RDE-1 working with primary siRNAs but not conserved in CSR-1, which loads secondary siRNAs with 5′ triphosphates. There is a lot of room for deviation from the "norm", and our study would certainly promote these lines of inquiry.*

3) The authors rescue csr-1 lethality with transgenic histone mRNA lacking the PAS sequence. PAS-containing sequences should not rescue. This should be tested.

See response to reviewer #1, point #2.

4) The normally present sequence that is presumably cut by CSR-1 should be replaceable with sequences matching other CSR-1 bound 22G RNAs, whereas introducing sequences not represented among CSR-1 22G RNAs should block processing of transgenic histone mRNAs. Such experiments should be performed in wild-type animals, just to see whether the correlation is stronger than just the observation described here.

See response to reviewer #2, point #2. Also, our new results with transgenic lines where the sequences between the stem-loop and PAS have no matching siRNAs are consistent with the lower expression and utilization of such messages, see response to reviewer #1, point #2.

5) Do authors do not provide an explanation for how histone mRNAs are processed in the soma, where csr-1 is absent. Are histones also lower in glp-4; csr-1 mutant animals compared to glp-4 alone? (glp-4 mutation results in a virtual lack of germ cells)

CSR-1 is not absent from the soma, see results Figure 3A and Figure S4B in Claycomb et al., 2009: the longer isoform of CSR-1 is soma-enriched. Also, we present CSR-1 immunostaining highlighting its expression in intestinal nuclei in Figure S2. In this tissue, H2B is also depleted upon csr-1(RNAi) (see Figure S4), indicating that the dependence of histone processing on RNAi machinery is not limited to the germline.

6) The authors show 22G RNAs matching the histone mRNAs in between the stem loop and the PAS. How specific are the 22G RNAs for this part of the histone mRNA? Are there no matches to other regions? The authors show that these 22G RNAs are enriched in CSR-1 IPseq data (from Claycomb et al). How do these enrichments compare to the typical enrichments for the top targets of the CSR-1 pathway? I seem to recall that these enrichments are much higher than what is described here for the histone 22Gs.

There are siRNAs complementary to the entire length of the histone genes, although none have been cloned further downstream of the PAS. Also, other genes have higher enrichment of siRNAs, but their function is beyond the scope of this paper. We are providing an explanation for the specific phenotypes caused by knockdown of the RNAi machinery in the germline, not the function of all CSR-1-bound 22G RNAs. It is not necessary for any specific siRNA to be most abundant in order to be most functionally relevant for a given phenotype.

We do have a manuscript under review concerning the global role of csr-1-dependent siRNAs in the transcriptional regulation in the soma. siRNAs complementary to the coding regions of histone genes may have a role in this type of regulation, as we point out in the Discussion section. We do not think that providing this manuscript is necessary for the evaluation of this work, but we can make it available to reviewers upon request.

7) The authors rescue with a combination of H2A, H2B, H3 and H4. Why include H3 and H4, since these two are not really changed. Also, why are H3 and H4 not affected? The authors give a hypothetical explanation, but this is not really satisfying. If this is a true function for CSR-1 it should affect H3 and H4 as well.

Our colleagues studying U7 snRNA in mammals have observed a decrease in histone H4 expression upon Lsm11 knock-down. We noted that they used different anti-H4 antibodies from the ones we had used. We exchanged antibodies and indeed found a significant depletion of H4 in the same csr-1(RNAi) and ego-1(RNAi) samples that we had tested previously, see Figure S3. We believe that very sensitive antibodies do not allow for the detection of a difference in signal due to saturation of the signal. Careful dilution experiments are needed to reveal such differences. We were not carrying out dilutions of wild type samples routinely, only in repeat experiments for cases where we had seen a difference and wanted to estimate the level of depletion, this did not allow us to detect changes in H3 and H4 earlier.

8) In general, the rescue experiments should be repeated with genetic mutants. Histone overexpression may affect RNAi and thus result in rescue. The authors do try to control for this (cdl-1 RNAi), but they cannot exclude that the effect imposed by cdl-1 RNAi is just too strong to be rescued by histone overexpression, while RNAi of the (RNAi components!) csr-1 and ego-1 can be rescued by a potential RNAi defect induced by histone overexpression. I think this is an

important issue as a recent paper by the Fire lab has shown that histone modification is part of regular RNAi (Nat.Genet, Dec 2011). Given that histones may exchange readily, overexpression of histones may cripple RNAi by preventing the build-up of proper silencing marks on the chromatin.

i) *Yes, ideally, one would like to see the rescue of the genetic mutants. However, the rescue we achieved is dependent on a balance between histone knockdown and histone overexpression. In the case of csr-1(RNAi) and ego-1(RNAi) treatment, the depletion of these factors in maternal germlines leads to severe embryonic phenotypes in the progeny. However, these phenotypes are less severe than the sterility of the genetic mutants that produce far fewer progeny, if any. Thus, a higher level of histone overexpression from a transgene is likely to be needed for rescue of the genetic mutants. Furthermore, there are several histone-producing loci in the C. elegans genome, but our lines are overexpressing only one locus with one of each histone gene. Additionally, the rescuing transgenic line is getting progressively silenced in the germline, which is common in many transgenic C. elegans lines. We were in the process of attempting this rescue experiment when we determined that our rescuing transgenic line, armEx149, was becoming progressively silenced. Nevertheless, lack of rescue of the genetic mutants with our transgene would only mean that the expression is not high enough, i.e. the result is likely to be inconclusive.*

ii) *We confirmed that knockdown of csr-1 and ego-1 is achieved in the rescuing line (see Supplementary Figure S5B), which indicates that the rescue is not due to inefficient RNAi. Furthermore, we performed RNAi against smc-4 and pos-1 in this line, which resulted in the expected phenotypes (see Supplementary Figure S5A). This shows that RNAi is functioning in the germline in the rescuing line.*

iii) "….a recent paper by the Fire lab has shown that histone modification is part of regular RNAi (Nat.Genet, Dec 2011). Given that histones may exchange readily, overexpression of histones may cripple RNAi by preventing the build-up of proper silencing marks on the chromatin". *This is not a correct interpretation of the published study, which did not provide any functional data regarding the requirement*

of histone modifications for regular RNAi. In fact, RNAi targeting germline genes is effective within hours after injection or feeding of dsRNA, consistent with the same time-frame of siRNA accumulation, see the citation from the Fire 2011 paper below. However, histone modifications occur much later; they are intriguing, but nothing is really known about their role. "We observed a high level of *smg-1* siRNA at the 4-h time point, with even higher *smg-1* siRNA levels at the 24-h time point (Fig. 5c), indicating an ongoing RNAi process. In contrast, we did not observe any accumulation of H3K9me3 at the *smg-1* locus at either 4 or 24 h after RNAi induction (Fig. 5b)."

9) I am wondering whether the effects described by these authors just reflect correlation and do not hold a direct mechanistic link. The germline is clearly affected by loss of CSR-1 (and EGO-1, DRH-3) and this may lead to all sorts of secondary effects, including defects in histone production. Perhaps germline identity is partially lost in these mutants and it's because of this that histone production drops? There is a fourth player in this scene, CDE-1. Mutants for cde-1 apparently still contain CSR-1 and its 22G RNAs, but still chromosome segregation defects are observed in cde-1 mutants. Is there a defect in histone mRNA production in cde-1 mutants as well? If so, that would not be consistent with the proposed model. The same holds true for other RNAi mutants, many of which show chromosome segregation defects (although much less than that observed in CSR-1).

We provide results and arguments in support of histone depletion as the main cause of csr-1 and ego-1 mutant phenotypes. This is more than correlation since increasing histone production actually rescues the phenotypes. Our findings are significant because not many phenotypes of the RNAi pathway mutants are clearly linked to the misregulation of specific genes, whether through a direct or indirect effect. Data in Figure 2A,B and Figure S1, together with previously discussed findings of CSR-1-bound siRNAs complementary to histone mRNAs strongly support the direct effect of RNAi.

The fact that cde-1 mutants have siRNAs normally loaded by CSR-1, even more siRNAs than wild type worms, but show a phenotype similar to a mutant with depletion of these

siRNAs is not explained mechanistically in the original publication on cde-1 (van Wolfswinkel et al., Cell, 2009). It is proposed that: " Elevated siRNA levels are associated with erroneous gene silencing, most likely through the inappropriate loading of CSR-1 siRNAs into other Argonaute proteins. We propose a model in which CDE-1 restricts specific EGO-1-generated siRNAs to the CSR-1 mediated, chromosome associated RNAi pathway, thus separating it from other endogenous RNAi pathways *".* *Essentially, in cde-1 mutants siRNAs are present but they are less functional because they are loaded onto other Argonautes competing with CSR-1, thus the mutant phenotype is similar to csr-1. We would expect histone mRNA production to be defective in cde-1, this would be consistent with our model, not the reverse as suggested by the reviewer. We do not think that experiments with cde-1 mutant are necessary.*

10) Finally, it is strange to see an paper submitted to EMBO J, totally formatted for another journal. The authors could have done themselves a favor to expand their introduction and discussion in order to present the work in a more balanced manner.

The EMBO Journal is a part of Nature Publishing Group and allows the direct transfer of manuscripts submitted to other journals. We agree that Article presentation fits the revised version of our manuscript better.

2nd Editorial Decision 01 June 2012

Thank you for sending us your revised manuscript. In the meantime, referee 3 has seen it again. He/she still raises a number of concerns with respect to the conclusiveness of the dataset, but puts forward suggestions how to address these. Given the more positive assessment of the other two referees in the first round and given that referee 3 points to a clear way forward, we would be prepared to allow an exceptional second round of revision in this case. However, the points raised by referee 3 need to be addressed to his/her satisfaction.

We are looking forward to the further revised manuscript in due course.

Sincerely yours,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3

The authors made several attempts to strengthen the story and I appreciate the problems they encounter upon transgenesis. However, technical difficulties are no excuse to publish data that do not allow strong conclusions. I am of the opinion that the presented data are not particularly strong in supporting a role for CSR-1 in processing histone mRNA. Some of my concerns were rightly rebutted (e.g. the cde-1 case and the 10-nucleotide off-set), although one cannot take the presented data as a support to suggest that CSR-1 cleaves non-canonically. At best the data suggest it but it has to be acknowledged that this is a strong statement that needs experimental testing. More importantly, the most important concerns remain. These deal with demonstrating that the effects observed really are guided by siRNAs in CSR-1.

Making transgenes lacking the PAS seems not possible. In other words, the transgenesis appears to be not reproducible. This makes me worry that the transgenes that are presented may have passed through a significant bottleneck and behave non-representative. I am completely aware of transgene problems in the germline, but that's a problem many worm workers have to deal with. I am afraid that sometimes it just prevents meaningful experiments.

I appreciate the authors efforts to by-pass this problem using the 2'OMe oligo pull-down. This is a good idea, but this experiment is not well controlled. And given the strong phenotype of ego-1 is will be hard to control no matter what one does. But at the very least the following should be done/clarified before I am convinced that this experiment demonstrates what is claimed by the authors:

-Determine H2A mRNA levels in wild-type and ego-19RNAi) animals. If ego-1 RNAi results in lower H2A mRNA levels than a general, a-specific binding of CSR-1 to it will give the same result. Given the very high background in the experiment this is a serious concern.
-Less CSR-1 may be present in ego-1 RNAi animals, again confounding the results.
-Does the unrelated control oligo pull down any mRNA? It should, otherwise it will not be a good control as the background will be completely different in both pull-down conditions.

General comment: the Westerns all seem to have extreme contrast. Please provide less-edited versions. One can be easily fooled by playing with contrast and brightness issues.

2nd Revision - authors' response 18 June 2012

Referee #3

I am of the opinion that the presented data are not particularly strong in supporting a role for CSR-1 in processing histone mRNA.

*We assume that the reviewer is not convinced with the data addressing the **direct catalytic** role of CSR-1. The affect of the csr-1 mutation on histone mRNA processing and histone protein accumulation is the same as that of cdl-1(SLBP), and this argument (see Figures 3,4) cannot be any stronger. The connection between the csr-1 phenotype and histone misprocessing is very solid, in our opinion, and this result alone is a significant contribution to the field. We recognize that the details of the mechanism need to be worked out, although our data do support a direct binding of CSR-1 to histone mRNA.*

Some of my concerns were rightly rebutted (e.g. the cde-1 case and the 10-nucleotide offset), although one cannot take the presented data **as a support to suggest that CSR-1 cleaves non-canonically**

(We do not say that CSR-1 cleaves non-canonically in the text, we merely highlight this possibility in the rebuttal.).

At best the data suggest it but it has to be acknowledged that this is a strong statement that needs experimental testing.

We modified the text in several places to acknowledge that the catalytic activity of CSR-1 in histone mRNA processing remains to be confirmed. It is important to note that it is not trivial to achieve this level of conclusiveness: 1) rescue with CSR-1 transgenes is notoriously difficult and 2) loss of rescue with catalytically inactive CSR-1 would not prove that CSR-1 cleaves histone mRNA precursor. Instead, our experiment using 2'OMe oligos identical to the sequence between the stem-loop and PAS, thus mimicking the H2A pre-mRNA to pull down CSR-1 in ego-1-dependent manner, places CSR-1 directly at the region that needs to be cleaved. Given previously published in vitro evidence of CSR-1's catalytic activity along with our in vivo phenotypic and rescue data, it is not a big stretch to suggest that CSR-1 may act directly.
Importantly, the initial evidence for the role of Dicer in let-7 and lin-4 precursor processing consisted of strong genetic and phenotypic analysis supplemented by data showing accumulation of the precursors in the dcr-1 mutant (Grishok et al., 2001). That evidence rightly convinced the reviewers who strongly supported the publication, and the following work in the field is fully consistent with this initial study.

More importantly, the most important concerns remain. These deal with demonstrating that the effects observed really are guided by siRNAs in CSR-1.
Making transgenes lacking the PAS seems not possible. In other words, the transgenesis appears to be not reproducible. This makes me worry that the transgenes that are presented may have passed through a significant bottleneck and behave nonrepresentative.

The fact is that increased histone expression levels from a transgene allow rescue of the csr-1 and ego-1(RNAi)-induced phenotypes. We maintain that this result can be used to prove that histone depletion is responsible for the phenotype.

I am completely aware of transgene problems in the germline, but that's a problem many worm workers have to deal with. I am afraid that sometimes it just prevents meaningful experiments.

We acknowledge that it is difficult and may be not possible to use transgenes for addressing mechanistic questions related to histone mRNA processing, but the rescue experiment is certainly not meaningless.

I appreciate the authors efforts to by-pass this problem using the 2'OMe oligo pull-down. This is a good idea, but this experiment is not well controlled. And given the strong phenotype of ego-1 is will be hard to control no matter what one does.

There are two 2'OMe experiments that are testing different things. Additional controls are not impossible and have been included in the new Figure S1.

But at the very least the following should be done/clarified before I am convinced that this experiment demonstrates what is claimed by the authors:
-Determine H2A mRNA levels in wild-type and ego-19RNAi) animals. If ego-1 RNAi results in lower H2A mRNA levels than a general, a-specific binding of CSR-1 to it will give the same result. Given the very high background in the experiment this is a serious concern.

This argument does not apply to the experiment shown in Figure 2A, in which we use 2'OMe oligos to mimic the H2A pre-mRNA, and there is no H2A mRNA involved in the pull-down. The amount of H2A 2'OMe oligo used with the wild-type and ego-1(RNAi) extracts for pull-down is the same.
Regarding Figure 2B: The total H2A mRNA is decreased in ego-1(RNAi) as shown in Figure 3, but it does not disappear. The relevant measure is the enrichment of the mRNA in the pull down compared to Input. We used RT-qPCR to quantify this enrichment, and found that the efficiency of H2A mRNA pull down by a specific antisense oligo in ego-1(RNAi) samples is similar to that in wild type, and significantly higher than non-specific background (Figure S1A). However, this is not accompanied by a similar enrichment of the CSR-1 protein compared to input or pull-down with unrelated oligo (Figure 2).

-Less CSR-1 may be present in ego-1 RNAi animals, again confounding the results.

It was shown by Claycomb et al 2009, Supplemental Figure S5, that the levels of CSR-1 protein are not changing in drh-3(tm1217) or drh-3(ne4253) worms that are depleted of siRNAs. Consistently, we find that CSR-1 protein levels do not change in ego-1(RNAi) (Figure S1B).

-Does the unrelated control oligo pull down any mRNA? It should, otherwise it will not be a good control as the background will be completely different in both pull-down conditions.

CSR-1-bound siRNAs target many messages, at least ~ 4,000 were identified in the CSR-1 IP. In fact, the real number of CSR-1 targets is likely to be higher since an arbitrary cut-off was used and it is very likely that some CSR-1-bound siRNAs have not yet been cloned. Therefore, one would not be able to choose, with certainty, a control mRNA that is not targeted by CSR-1. This is the reason that we used a plasmid sequence not present in the C. elegans genome as a control. Similar oligos not related to C. elegans mRNAs were used as a control in the original publication describing the application of this technique in C. elegans (Hutvágner et al., 2004). We performed the experiments precisely following the protocol described in "Argonaute pull-down and RISC analysis using 2'-O-methylated oligonucleotides affinity matrices". Jannot G, Vasquez-Rifo A, Simard MJ., Methods Mol Biol. 2011;725:233-49.

General comment: the Westerns all seem to have extreme contrast. Please provide less edited versions. One can be easily fooled by playing with contrast and brightness issues.

We provide darker images of Westerns. Notably, the depletion of histone proteins upon csr-1 and ego-1(RNAi) was discovered by Daphne Avgousti accidentally when she was looking for changes in specific histone modifications. These results were repeated numerous times to exclude possible artifacts. Although we knew about siRNAs targeting histone messages for a while, the discovery of histone protein depletion in RNAi mutants prompted us for further investigation described in this manuscript.

3rd Editorial Decision 25 June 2012

Thank you for sending us your re-revised manuscript. Referee 3 has now seen it again, and now supports publication, even though he/she still disagrees with a number of points you make (please see below). You may wish to respond to or address his/her comments.

Prior to acceptance of the paper, there are also a number of editorial issues that need further attention:

* Please add additional statistical details (incl. number of independent repeats and error bars with explanations) to the legends on figures 6B, 7A, S1, S5, S6, S7.

* Please add scale bars and/or explanations to figures S2, S4

* Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) and/or explanation to ask for the original scans. In the case of the present submission there are a number of panels that do not fully meet these requirements: Figure 7B, S3A, S3B.

I therefore like to kindly ask you include suitably amended versions of these figures in the final version of the manuscript and to explain in the figure legend that (whether) all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question.

As also mentioned by referee 3, it would also be good to include panels for the western blots included in the paper (or the respective source data) that do not have such a strong contrast. Maybe a different exposure would be helpful.

* In addition and as part of a new initiative, we now generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in

the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3

I will comment on the various reactions provided by the authors below, following citations from their rebuttal.

Citation:
Importantly, the initial evidence for the role of Dicer in let-7 and lin-4 precursor processing consisted of strong genetic and phenotypic analysis supplemented by data showing accumulation of the precursors in the dcr-1 mutant (Grishok et al., 2001). That evidence rightly convinced the reviewers who strongly supported the publication, and the following work in the field is fully consistent with this initial study.

This is not really relevant. We are discussing another protein and it is 11 years of small RNA research later. I do want to make one comment: the beautiful dicer work referred to was backed-up by strong biochemistry from earlier work, making the next step much more convincing. The biochemistry matched the products, something that cannot be stated (yet) in the case of CSR-1 and histon mRNAs.

Citation:
The fact is that increased histone expression levels from a transgene allow rescue of the csr-1 and ego-1(RNAi)-induced phenotypes. We maintain that this result can be used to prove that histone depletion is responsible for the phenotype.

Given the problems, I say it can be used to suggest it, not to prove it.

Citation:
We acknowledge that it is difficult and may be not possible to use transgenes for addressing mechanistic questions related to histone mRNA processing, but the rescue experiment is certainly not meaningless.

I did not intend to bring across that the experiments are meaningless. My apologies. However, given the difficulties surrounding this issue the reader should be made fully aware of potential problems that we do not have clearly on our radar.

Citation:
Regarding Figure 2B: The total H2A mRNA is decreased in ego-1(RNAi) as shown in Figure 3, but it does not disappear. The relevant measure is the enrichment of the mRNA in the pull down compared to Input.

I do not agree. If one considers non-specific binding of CSR-1 to mRNAs, the relevant measure is how much mRNA molecules are loaded in a lane, as that will set the CSR-1 signal. The authors show that in both wild-type and ego-1 mutants around 5% of the input is retrieved. Given the lower H2A mRNA levels in ego-1 mutants this has to result in fewer mRNA molecules in the ego-1 mutant IP sample. This may well

explain the result, independent of the effect of ego-1 on CSR-1 loading.

Citation:
It was shown by Claycomb et al 2009, Supplemental Figure S5, that the levels of CSR-1 protein are not changing in drh-3(tm1217) or drh-3(ne4253) worms that are depleted of siRNAs. Consistently, we find that CSR-1 protein levels do not change in ego-1(RNAi) (Figure S1B).

Good to have this data in.

Citation:
CSR-1-bound siRNAs target many messages, at least ~ 4,000 were identified in the CSR- 1 IP. In fact, the real number of CSR-1 targets is likely to be higher since an arbitrary cut-off was used and it is very likely that some CSR-1-bound siRNAs have not yet been cloned. Therefore, one would not be able to choose, with certainty, a control mRNA that is not targeted by CSR-1. This is the reason that we used a plasmid sequence not present in the C. elegans genome as a control. Similar oligos not related to C. elegans mRNAs were used as a control in the original publication describing the application of this technique in C. elegans (Hutvágner et al., 2004). We performed the experiments precisely following the protocol described in "Argonaute pull-down and RISC analysis using 2'-O-methylated oligonucleotides affinity matrices". Jannot G, Vasquez-Rifo A, Simard MJ., Methods Mol Biol. 2011;725:233-49.

I do not agree with the arguments. One could certainly use oligo's designed to pull down endogenous mRNAs that are unlikely to be bound by CSR-1 and a number that pull down heavily targeted mRNAs. If all 'non-targets' pull down CSR-1 I would consider the possibility that CSR-1 is pulled down rather non-specifically. An oligo that does not bind any mRNA is likely to give a totally different background. All considered, I am just not convinced by these experiments.

Citation:
We provide darker images of Westerns.

Personally, I would want to see 'softer' images as they often give a more honest representation of the background. But I trust that the authors know what they are doing.

In conclusion, two of my concerns remain. These make me doubt whether the proposed model is correct. However, it may also be good to make people aware of these data as they may open up an unexpected area of small RNA biology. And as long as the 'direct' model is not advertised as 'proven' EMBO J. may be a good venue to bring this work out in the open.

3rd Revision - authors' response 12 July 2012

Referee #3

I will comment on the various reactions provided by the authors below, following citations from their rebuttal.

Citation:
Importantly, the initial evidence for the role of Dicer in let-7 and lin-4 precursor processing consisted of strong genetic and phenotypic analysis supplemented by data showing accumulation of the precursors in the dcr-1 mutant (Grishok et al., 2001). That evidence rightly convinced the reviewers who strongly supported the publication, and the following work in the field is fully consistent with this initial study.

This is not really relevant. We are discussing another protein and it is 11 years of small RNA research later. I do want to make one comment: the beautiful dicer work referred to was backed-up by strong biochemistry from earlier work, making the next step much more convincing. The biochemistry matched the products, something that cannot be stated (yet) in the case of CSR-1 and histon mRNAs.

Both papers describe the effect of RNAi factors on RNA processing. Only one publication on Dicer (Bernstein et al., 2001) preceded (Grishok et al., 2001) study. After 11 years, there are now over 1200 papers on Argonaute proteins and their potential for slicer activity has been proven beyond any doubt. The novelty of our research is not in discovering a new biochemical property of Argonaute CSR-1, but in finding biologically relevant target RNAs whose maturation is very likely dependent on the direct role of CSR-1 either as a Slicer or recruiter of other proteins.

Citation:
The fact is that increased histone expression levels from a transgene allow rescue of the csr-1 and ego-1(RNAi)-induced phenotypes. We maintain that this result can be used to prove that histone depletion is responsible for the phenotype.

Given the problems, I say it can be used to suggest it, not to prove it.

We have softened the language to emphasize the consistency of our data with this model, however we do not state that this is the only possible model.

Citation:
We acknowledge that it is difficult and may be not possible to use transgenes for addressing mechanistic questions related to histone mRNA processing, but the rescue experiment is certainly not meaningless.

I did not intend to bring across that the experiments are meaningless. My apologies. However, given the difficulties surrounding this issue the reader should be made fully aware of potential problems that we do not have clearly on our radar.

We have made the caveats of the levels of expression of the transgenic lines clear, any conclusions from our data will have to be taken with this caveat in the mind of the reader.
Citation from text:
"The caveat of these experiments is that none of the transgenic lines designed to express histone mRNA with the stem loop and PAS separated by plasmid sequences produced mRNA at the levels similar to that of armEx149."

Citation:
Regarding Figure 2B: The total H2A mRNA is decreased in ego-1(RNAi) as shown in Figure 3, but it does not disappear. The relevant measure is the enrichment of the mRNA in the pull down compared to Input.

I do not agree. If one considers non-specific binding of CSR-1 to mRNAs, the relevant measure is how much mRNA molecules are loaded in a lane, as that will set the CSR-1 signal. The authors show that in both wild-type and ego-1 mutants around 5% of the input is retrieved. Given the lower H2A mRNA levels in ego-1 mutants this has to result in fewer mRNA molecules in the ego-1 mutant IP sample. This may well explain the result, independent of the effect of ego-1 on CSR-1 loading.

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Good to have this data in.

Citation:
CSR-1-bound siRNAs target many messages, at least ~ 4,000 were identified in the CSR- 1 IP. In fact, the real number of CSR-1 targets is likely to be higher since an arbitrary cut-off was used and it is very likely

that some CSR-1-bound siRNAs have not yet been cloned. Therefore, one would not be able to choose, with certainty, a control mRNA that is not targeted by CSR-1. This is the reason that we used a plasmid sequence not present in the C. elegans genome as a control. Similar oligos not related to C. elegans mRNAs were used as a control in the original publication describing the application of this technique in C. elegans (Hutvágner et al., 2004). We performed the experiments precisely following the protocol described in "Argonaute pull-down and RISC analysis using 2'-O-methylated oligonucleotides affinity matrices". Jannot G, Vasquez-Rifo A, Simard MJ., Methods Mol Biol. 2011;725:233-49.

I do not agree with the arguments. One could certainly use oligo's designed to pull down endogenous mRNAs that are unlikely to be bound by CSR-1 and a number that pull down heavily targeted mRNAs. If all 'non-targets' pull down CSR-1 I would consider the possibility that CSR-1 is pulled down rather non-specifically. An oligo that does not bind any mRNA is likely to give a totally different background. All considered, I am just not convinced by these experiments.

It would be possible to conduct further experiments on potential non-targets. However, any prediction of the likelihood that any given mRNA may be bound by CSR-1 would be arbitrary. These types of experiments would not allow making such strong conclusions as suggested by the reviewer. The data would be difficult to interpret if indeed CSR-1 is bound to the 'unlikely' targets. Our study is the first one to connect a specific group of CSR-1 mRNA targets to the csr-1 mutant phenotype. No other functional connections have been made thus far. Strictly speaking, there are no validated mRNA targets regulated by CSR-1.

Citation:

We provide darker images of Westerns.

Personally, I would want to see 'softer' images as they often give a more honest representation of the background. But I trust that the authors know what they are doing.

We have changed all Westerns to the 'softer' images and included the original scans as Source data to remove any doubts about the signals and backgrounds. Also, we included re-probes of membranes used for anti-histone Westerns with anti-actin antibodies when possible. For many experiments we routinely did this in addition to running separate gels with the same samples for anti-actin Westerns.

In conclusion, two of my concerns remain. These make me doubt whether the proposed model is correct. However, it may also be good to make people aware of these data as they may open up an unexpected area of small RNA biology. And as long as the 'direct' model is not advertised as 'proven' EMBO J. may be a good venue to bring this work out in the open.

The direct model has been suggested as a possible model that is consistent with the data, without indicating that it is the only possible model.