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Nup358 binds to AGO proteins through its SUMO-interacting motifs and promotes the association of target mRNA with miRISC

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 April 2016

Thank you for the submission of your manuscript to our journal. We have now received the referee reports that are copied below.

As you will see, while the referees agree that the findings are potentially interesting, they also raise a number of concerns that would need to be addressed to strengthen the study. Both referees 1 and 2 point out several overstatements that need to be toned down, and that the effects of Nup358 might be indirect, eg through Dcp1a. Referees 1 and 3 also note that it should be investigated whether the interaction of Ago and GW with Nup358 is mediated by RNA. In general, the referees do not seem to be convinced by the co-localization studies of Nup358 with the ER and P-bodies and by the interaction of Nup358 with Ago. In addition, referee 3 remarks that the physiological relevance of the Nup358-Ago2 interaction remains unclear. All referees also pinpoint a number of important controls that are missing.

From these comments it is clear that publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will

therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

REFEREE REPORTS

Referee #1:

In this manuscript, the authors have identified and characterized Nup358 as factor acting in miRNA-guided gene silencing. Nup358 is part of the nuclear pore complex but is also found in specific ER structures termed annulate lamella (AL). The authors find that AL are often associated with P bodies and stress granules. Moreover, depletion of Nup358 disrupts P bodies. Furthermore, depletion of Nup358 also affects miRNA-guided gene silencing using reporter gene assays. Using several different approaches, the authors report that Nup358 is required for the interaction of RISC with specific target mRNAs. They further show that Nup358 interacts with Ago as well as GW proteins. A so-called SIM (SUMO-interacting motif) is required for the interaction with Ago proteins. When SIM is overexpressed alone, it disrupts miRNA-guided gene silencing potentially by a dominant negative effect. The authors propose a model in which exported mRNAs are sorted at the nuclear pore and Nup358 helps to establish a stable repressive mRNP.

This is a comprehensive analysis of a potential role of Nup358 in the miRNA pathway. However, many of the presented experiments are not as clear as the authors state in their text. A number of additional points are listed below.

1. Based on Figure S1B, the authors state that the Nup358 positive structures are associated with the ER. However, it is difficult to conclude anything from this image. A better image with would be needed. In addition, the fact that structures partially overlap does not necessarily mean that they are associated with each other. To claim this, addition biochemical experiments would be needed.

2. In Figure 1C, most of the Nup358 positive structures do not co-localize with P bodies or stress granules. Wouldn't a random co-localization be equally likely? Has confocal microscopy been used?

3. Why is Dcp1a also depleted when Nup358 is knocked down? Co-localization is also not apparent in the few cells that are shown in Figure 1D.

4. Generally, the authors should use at least two different siRNAs for functional experiments since siRNAs may also have off target effects and may therefore lead to miss interpretations.

5. Figure 2C: the authors should add the same controls that are used in Figure 2B.

6. Figure 3B, western blots for Ago2 should be added to show that similar amounts of proteins have been immunoprecipitated. For the mRNA co-IP shown in Figure 3D, an IgG control could be added.

7. Are miRNAs and miRNA target mRNAs co-immunoprecipitated with anti-Nup358 antibodies? This would strengthen the final model that is suggested by the authors.

8. Generally, the authors should go through their text and tone down their statements (e.g. ...these results establish Nup358 as an essential player in the miRNA pathway...etc.). SIM as general Ago

interaction motif needs to further functionally characterized in order to draw such strong conclusions.

9. Figure S3B attempts to analyze effects on global mRNA export. However not much can be seen in this figure and the authors should refrain from over-interpreting mild effects or low quality co-localization images.

10. Figure 3G: it is well possible that Ago and GW proteins interact with Nup358 via mRNA interactions. That would argue against a direct interaction with Ago2. Therefore, the authors should test whether the observed interaction is sensitive to RNase treatment.

11. Is Nup358 associated with components of the translation machinery, which could mediate binding to the miRNA pathway components?

12. miRNAs are also seen in polysomes and polysomes are not part of P bodies. Would that be consistent with the model presented in this study? Or are there different miRNA-guided regulatory pathways? This should be discussed.

13. Abstract: the sentence starting with "AGO family of proteins ..." needs to be corrected.

14. Annulate lamellae are not well introduced and characterized in this study. The authors state that these structures are 'pore-like structures'. However, Nup358 positive foci are found in the cytoplasm and are not associated with the nuclear envelope. So what 'pores' are formed? Furthermore, are there any other, more unrelated components of AL that could serve as control?

Referee #2:

In this manuscript, Sahoo et al. report that Nup358, a nucleoporin that localizes to the cytoplasmic side of the nuclear pore complex, is important for miRNA-mediated silencing. They show that Nup358 interacts with AGO subfamily proteins and propose that the interaction promotes the target recognition by miRISC. They further show that the SUMO-interacting motif of Nup358 acts as a binding site for Argonaute proteins, including AGO and PIWI subfamily proteins. Overall, the presented data themselves are generally sound and well performed, and this study may potentially provide an important insight in the RNA silencing field and. However, the data are sometimes over-interpreted toward a particular direction and the (direct or indirect) molecular mechanisms underlying the observed phenomena remains unclear. The following points should be carefully addressed before the manuscript is further considered.

Major points:

1. The major drawback of this study is the mechanistic ambiguity for how Nup358 promotes miRNA-mediated silencing. P-body was originally believed as a site for miRNA-mediated silencing, but it is now well accepted that P-body per se is dispensable for silencing (Chu and Rana, PLoS Biol. 2006; Eulalio et al., Mol. Cell. Biol. 2007). Thus, the proposed model for how Nup358 facilitates miRNA function appears somewhat superficial. The authors argue that Nup358 binds AGO2 and GW182 and mediates "the association of the target mRNA with miRISC." However, it is hard to imagine how, via such protein-protein interactions, Nup358 can exclusively facilitate the base pairing between the AGO-loaded miRNA and target RNA, but not the association between lambda-N-AGO2 and BoxB-RNA (Figure 3F), which is also in equilibrium and could theoretically be assisted by the protein interaction network.
2. In Figure 1C, the association of Nup358 with P-bodies should also be investigated in the normal condition. Also, the definition of "associated" and "free" should be clarified.
3. In Figure 1D, the expression level of Dcp1a should be quantified by Western blotting to clarify whether P-body formation per se or Dcp1a protein was destabilized by Nup358 depletion. If the level of Dcp1a (or other core P-body proteins) was in fact down-regulated by Nup358 depletion, the observed effects on miRNA-mediated silencing might all have to be attributed to Dcp1a (or others), not directly to Nup358.
4. Throughout the manuscript, the data of reporter assays (currently shown only in fold de-repression) should be presented in the Rluc/Fluc ratio. Representation by fold de-repression hides all the important information of the efficiency of miRNA-mediated silencing itself.

5. To evaluate the effect of Nup358 on general miRNA biogenesis, the authors performed deep sequencing using HEK293T cells. However, it is clear that depletion of Nup358 in HEK293T is inefficient (Figure S3A) and it is unclear why the authors did not choose HeLa cells, in which Nup358 knockdown is quite efficient (Figure 2A), for this important analysis.
6. To conclude "AGO2 interaction is mainly dependent on SIM1" (p11, line 4), the authors should investigate whether or not IR1+2 SIM2 interacts with Ago2.

Minor points:
1. The specificity of immunostaining of Nup358 should be validated using the siRNA against Nup358.
2. Page 3, line 22: The statement "Although many GW/WG sequences are present in GW182, two tryptophan residues (W623 and W634 in human TNRC6B isoform) that are interspersed by a minimum length of 10 amino acids majorly contribute to the AGO- GW182 interaction" is misleading. Although Pfaff et al. [9] reported that W623 and W634 are crucial for the interaction between AGO2 and a 83-aa short peptide derived from TNRC6B (positions 599-683), they clearly showed that full-length GW182 has many other Ago-binding tryptophan residues in the GW-rich repeat (positions 162-996).
3. Page 3, line 28: In general, siRNA triggers cleavage of mRNA, but not suppression of translation.
4. Several IP data are too faint to follow the authors' conclusion, e.g., Figures 4B, 4D, 5E. Western blotting data of long exposure should be added.
5. The coomassie staining gel in the direct binding assay (Figure 5H) should be presented without cropping in order to validate that the interaction is not mediated by other components.

Referee #3:

In this manuscript, Sahoo et al establish a relation between annular lamellae (AL), a so far relatively poorly understood subcellular structure deriving from the perinuclear ER, and miRNA silencing. They reveal that the nuclear pore complex protein Nup358 localizes to AL and plays a role in miRISC binding to target mRNA by direct interaction with Ago2, and that this interaction is required for target mRNA silencing. Finally the authors dissect the Nup358 domains relevant for the interaction and derive that Ago2 interacts with the SUMO binding domain of Nup358, whereas the interaction is conserved across species and maintained for different family members of Ago and Piwi proteins. This is an interesting study which is presented in a systematic manner that is easy to follow, and the main conclusions seem well supported by the data. The characterization of annular lamellae and the elucidation of their functional role in RNA silencing is an important contribution of relevance for a large audience not only in the RNA field but cell biology in general. Prior to publication I have only a few major and some technical comments:

1. The paper is lacking a conclusion in light of the physiological relevance of this newly found interaction, ie whether the Nup358-Ago2 interaction is required to increase quantitative efficiency of miRISC to encounter target mRNA molecules by compartmentalization, or whether the interaction is affecting miRISC - mRNA interaction at the molecular level (such as by eg altering Ago conformation, recruiting further factors etc). Related to this question the data in Figure 6B are curious: if loss of RNA silencing upon Nup358 knockdown can be rescued by tethering of N-HA-IR domains - which likely lack the AL localization moiety - to the target reporter, wouldn't this suggest that localization to AL is not the main relevance of Nup358 - Ago interaction? Where does this Nup358 truncation mutant (N-HA-IR1+2) localize? How does Ago localization change upon Nup358 knockdown? Does Nup358 depletion affect quantitative efficiency or result in a complete loss of RNA silencing?(which is impossible to assess since the data in Figure 2B for example show only a single condition/time point and dose which results in complete loss of silencing). Is the Ago2-Nup358 interaction RNA dependent? These are important questions that need to be addressed in order to understand the relevance of the presented findings.

2. Since the data in Figure 1B show that Nup358 is required for P-body formation, the rescue of silencing upon Nup358 knockdown with tethering Ago2 to the target mRNA reporter (Figure 3F) may suggest that P-body formation is not required for RNA silencing. This is an important point since there is still an unresolved controversy in the field about whether P-bodies are required for RNA silencing, or build up in consequence to RNAs being targeted for degradation. The authors

should test whether P-body formation is rescued together with the reporter knockdown upon Ago tethering and should comment on the implications of these data in light of the role of P bodies in RNA silencing in the discussion.

3. The authors mention in the introduction that Annular Lamellae are deriving from perinuclear ER but this information somehow gets lost throughout the elaboration of the conclusions and the discussion. However, this would be relevant information in order to relate this study to the emerging roles of the ER in RNA silencing, translation of non-secreted proteins as well as vesicular trafficking. Too often is there a disconnect in the literature due to a lack of emphasis of such relations - one prominent example being that Ago was initially discovered as Golgi/ER protein (GERP95, Cihaluk 1999) but it still took over ten years to recognize the role of these compartments in RNA silencing. Since many readers may be unfamiliar with AL biology, I would therefore encourage the authors to reiterate the relation of AL to the ER in more prominent passages of the manuscript, ie the abstract as well as comment on the findings of this study in light of emerging ER biology in the discussion.

Technical comments:

- The co-localization in Figure 1A and Figure S1A, C, F, G should be quantified
- Figure 1C and Figure S1D and E: The fraction of P-bodies or stress granules that do neither localize with NE nor AL should not be called 'free' (perhaps rather 'other'), since the authors do only look at those two compartments and cannot exclude that these granules associate with other structures
- Figure 1B, S1D and S1E lack scaling bars
- Figure 1C: the markers rather than the compartments should be specified in the image.
- For all co-localization studies, a positive control for alignment / chromatic aberration between the two channels (such as by TetraSpeck beads) should be shown as supplementary information.
- The experiment in Figure 1D lacks supplemental information on the efficiency of the knockdown.
- Page 7, line 7: '...HeLa cells have higher levels of endogenous let7a...' - this is unclear, higher compared to?
- The relatively modest effect of Dicer knockdown on the de-repression of the reporter in Figure 2B is curious - how are mature let7 levels affected?
- Figure S2A: which of the three Nup358 siRNAs are being used in all other experiments with Nup358 knockdown?
- The rescue data in Figure S2C are not entirely conclusive - also for control siRNA an increased level of repression is observed. Perhaps this indicates that overexpression of Nup358 in general enhances RNA silencing but the authors should perform experiments to address this issue (this may relate to my first point in the major comments: How is the quantitative RNA silencing efficiency and Ago2 subcellular distribution affected upon Nup358 overexpression?)
- How are the IP data in Figure 3B normalized - should be normalized to Ago2 levels which are pulled down, but this is unclear from the experimental description given.
- Page 9, line 1: there is an unspecified reference given
- The co-fractionation of Ago2 with Nup358 in Figure S4C is not convincing: since the two proteins both fractionate broadly and don't peak with each other, the fact that both proteins are found in some fractions together does not allow to make any conclusions about whether they are in the same complexes - as the authors suggest by the boxes in the figure and the wording in the legend and text. Unless the authors perform additional IPs in all fractions to assess interaction in each of these 'complexes' I would suggest to remove this data altogether.
- Which Ago2 structure is being used for the data in Figure 5J?
- The discussion is rather long and has several passages which appear redundant with the Results section - this should be shortened
- The model in Figure 6C is not instructive and in fact rather confusing (eg why are there two export paths shown for mRNAs? Why are there two alternate arrows from mRNAs to miRNA repression? Etc) - this should be improved. Also, again the relation of AL to the ER should be indicated.

1st Revision - authors' response 03 August 2016

We are submitting the revised manuscript entitled "Elucidation of Nup358's role in miRNA pathway reveals a new motif for interaction with AGO family of proteins" by Sahoo et al., for consideration for publication in the EMBO Reports as 'Research Article.' In the revised manuscript, we have addressed all the concerns raised by the reviewers and specific concerns pointed out by you. We have provided a point-by-point response to the reviewers' comments. The response to concerns pertaining to your comments is as follows.

Editor's comments:
As you will see, while the referees agree that the findings are potentially interesting, they also raise a number of concerns that would need to be addressed to strengthen the study. Both referees 1 and 2 point out several overstatements that need to be toned down, and that the effects of Nup358 might be indirect, e.g. through Dcp1a. Referees 1 and 3 also note that it should be investigated whether the interaction of Ago and GW with Nup358 is mediated by RNA. In general, the referees do not seem to be convinced by the co-localization studies of Nup358 with the ER and P-bodies and by the interaction of Nup358 with Ago. In addition, referee 3 remarks that the physiological relevance of the Nup358-Ago2 interaction remains unclear. All referees also pinpoint a number of important controls that are missing. Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

Our response: We thank the editor for the overall positive comments and the specific ones that we have addressed in the revised manuscript.

- The overall statements have been rewritten to tone down the conclusions as recommended by the reviewers.
- Indirect effect of Nup358 depletion on miRNA function through affecting P body formation has been ruled out by two means: Firstly, the level of Dcp1 a do not change between control and Nup358 depleted cells as shown by western analysis (Fig 1D, revised manuscript). Secondly, exogenous expression of AGO2 restored P body formation but not the miRNA defect in Nup358 depleted condition (Fig 7*C*, revised manuscript). These results show that P body disruption in Nup358 depleted condition does not indirectly cause effect on miRNA pathway, and support our conclusion that it plays an important role in coupling miRISC with the target mRNAs.
- We have shown that interaction of Nup358 with AGO2 and GW182 is RNA independent (Fig 3*E*, revised manuscript).
- We have provided more detailed data for the colocalization and interactions experiments, and they are discussed in detail under 'point-by-point response to reviewers comments'
- Potential relevance of Nup358-AGO2 interaction is discussed in the manuscript.
- Information pertaining to the number of experiments conducted (*n*), error bars (SD), *P* values and the method used to derive *P* values has been provided in all the relevant figure legends.
- All the microscopic images have been provided with the scale bars.
- We have provided the supplemental figures in the EV format as well.
- A potential cover page image has been submitted for consideration (under related to manuscript section).

We believe that the new data added has substantially strengthened our conclusions and improved the manuscript, and will now be suitable for publication in EMBO reports as a full article.

Referee #1:

In this manuscript, the authors have identified and characterized Nup358 as factor acting in miRNA-guided gene silencing. Nup358 is part of the nuclear pore complex but is also found in specific ER structures termed annulate lamella (AL). The authors find that AL are often associated with P bodies and stress granules. Moreover, depletion of Nup358 disrupts P bodies. Furthermore, depletion of Nup358 also affects miRNA-guided gene silencing using reporter gene assays. Using several different approaches, the authors report that Nup358 is required for the interaction of RISC with specific target mRNAs. They further show that Nup358 interacts with Ago as well as GW proteins. A so-called SIM (SUMO-interacting motif) is required for the interaction with Ago

proteins. When SIM is overexpressed alone, it disrupts miRNA-guided gene silencing potentially by a dominant negative effect. The authors propose a model in which exported mRNAs are sorted at the nuclear pore and Nup358 helps to establish a stable repressive mRNP. This is a comprehensive analysis of a potential role of Nup358 in the miRNA pathway. However, many of the presented experiments are not as clear as the authors state in their text. A number of additional points are listed below.

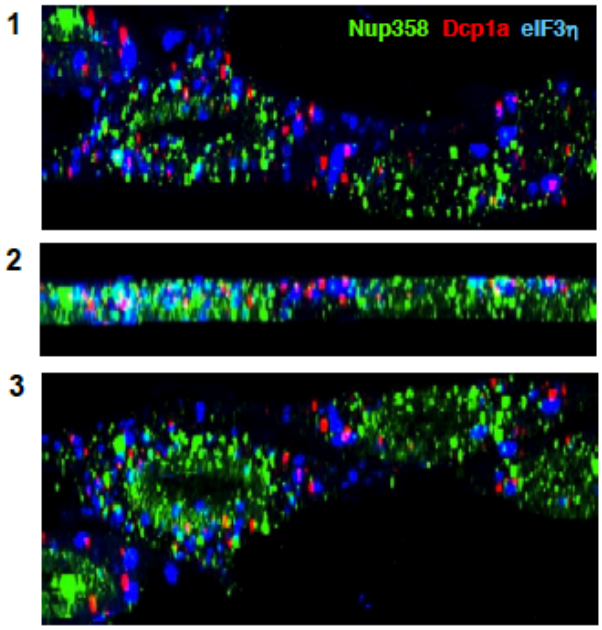
Our Response: We thank the reviewer for the positive comments, raising valid concerns and providing valuable suggestions. We have attempted addressing all the concerns raised by the reviewer, which has significantly strengthened and improved the manuscript.

1. Based on Figure S1B, the authors state that the Nup358 positive structures are associated with the ER. However, it is difficult to conclude anything from this image. A better image would be needed. In addition, the fact that structures partially overlap does not necessarily mean that they are associated with each other. To claim this, addition biochemical experiments would be needed.

Our Response: We thank the reviewer for the comments. The localization of Nup358 to AL has already been reported earlier [1,2]. We have provided better images to suggest the same (Fig EV1*B*, revised version). Furthermore, as documented earlier, we show that Nup358 positive cytoplasmic structures also co-localize with a subset of nucleoporins (Nup62, Nup214) and RanGAP1, that are previously shown to be a part of the AL [1-3]. These results clearly demonstrate that the cytoplasmic Nup358 positive structures are AL. As AL structures are well characterized, we have not performed the biochemical experiments.

2. In Figure 1C, most of the Nup358 positive structures do not co-localize with P bodies or stress granules. Wouldn't a random co-localization be equally likely? Has confocal microscopy been used?

Our Response: Confocal microscopy has been used for all the images. In few cases, 2D projection was obtained and provided in the manuscript to visualize the NE and AL structures better (e.g. Fig. 1*C*, revised manuscript). We have also confirmed the association by analysing the 3D reconstructions, for example refer to Reviewers' Fig 1. The extent of association between Nup358 and SGs or P bodies is comparable to the extent of associations between P bodies and SGs, which are well-established interacting structures. Moreover, the extent of interaction between these components goes up under overexpressed conditions. The relatively less association between these structures noticed under endogenous situation could reflect a dynamic and regulated interaction.



***Reviewers' Fig 1. Multidimensional Z-Stack images as 3D volumes of a HeLa cell showing relative localization of Nup358, Dcp1a and eIF3*h.** *HeLa cells were treated with 0.5 mM sodium arsenite for 30 min, fixed and stained for Nup358 (green), Dcp1a (red, P body marker) and eIF3*h *(blue, SG marker) using specific antibodies. Images were acquired using Zeiss 510 Meta laser scanning confocal microscope and 3D reconstruction of z-stack images was done with Zen software. The indicated pictures represent 3 (1,2,3) views of the 3D rendered image at different angles. Note that some of the Nup358 positive structures (green) are associated with P bodies (red) and SGs (green).*

3. Why is Dcp1a also depleted when Nup358 is knocked down? Co-localization is also not apparent in the few cells that are shown in Figure 1D.

Our Response: Dcp1a is not depleted in Nup358 knockdown cells. We have verified that the level of Dcp1a was comparable between control and Nup358 siRNA treated cells (Fig 1*D*, revised manuscript). Dcp1 appears to be redistributed within the cell.

4. Generally, the authors should use at least two different siRNAs for functional experiments since siRNAs may also have off target effects and may therefore lead to miss interpretations.

Our Response: We had provided the data in the first version with three different siRNAs and had verified the authenticity of depletion as well as its effect on the miRNA pathway. Please refer to Fig S2*A*, current and previous versions.

5. Figure 2C: the authors should add the same controls that are used in Figure 2B.

Our Response: We thank the reviewer for the comment. We have included the same controls for Fig 3*C* as that were used in Fig 2*B* (please refer to Fig 3*C*, revised manuscript).

6. Figure 3B, western blots for Ago2 should be added to show that similar amounts of proteins have been immunoprecipitated. For the mRNA co-IP shown in Figure 3D, an IgG control could be added.

Our Response: From the same experiments (Fig 3*B* and Fig 3*C*, previous version), miRNA and target mRNA analysis were performed, but for ease of following, they were represented in two figures. In the current revised manuscript, we have combined the data and have been shown in Fig 3*B*. IgG-control was already added for mRNA co-IP in Fig. 3*D* (previous version). This is Fig. 3*C* in the revised manuscript.

7. Are miRNAs and miRNA target mRNAs co-immunoprecipitated with anti-Nup358 antibodies? This would strengthen the final model that is suggested by the authors.

Our Response: We thank the reviewer for suggesting this experiment. We have performed Nup358 immunoprecipitation and could detect significant amount of miRNA and target mRNA in the immunoprecipitates as assessed by q-PCR, and the data is included (Fig 3*F*, revised manuscript).

8. Generally, the authors should go through their text and tone down their statements (e.g. ...these results establish Nup358 as an essential player in the miRNA pathway...etc.). SIM as general Ago interaction motif needs to further functionally characterized in order to draw such strong conclusions.

Our Response: We have taken a note of the comments and have made changes appropriately.

9. Figure S3B attempts to analyze effects on global mRNA export. However not much can be seen in this figure and the authors should refrain from over-interpreting mild effects or low quality co-localization images.

Our Response: We agree with the reviewer that IF does not give a quantitative assessment of the effect of Nup358 depletion on mRNA export. As mentioned in the manuscript, we maintain that there was no discernible change in the nucleo-cytoplasmic distribution of mRNA between control and Nup358 siRNA treated cells.

10. Figure 3G: it is well possible that Ago and GW proteins interact with Nup358 via mRNA interactions. That would argue against a direct interaction with Ago2. Therefore, the authors should test whether the observed interaction is sensitive to RNase treatment.

Our Response: We thank the reviewer for this comment. We have performed the co-immunoprecipitation experiments with and without RNase A treatment, and found that Nup358 interacts with AGO2 and GW182 in an RNA-independent manner (Fig 3*E*, revised manuscript). Ago2-PABPC1 interaction was monitored under the same condition, which is RNA-dependent (Fig 3*E*), as previously reported [4].

11. Is Nup358 associated with components of the translation machinery, which could mediate binding to the miRNA pathway components?

Our Response: This is an interesting possibility and we thank the reviewer for the suggestion. We are pursuing this direction. However, we believe that at this point of time, these experiments will be beyond the scope of this manuscript.

12. miRNAs are also seen in polysomes and polysomes are not part of P bodies. Would that be consistent with the model presented in this study? Or are there different miRNA-guided regulatory pathways? This should be discussed.

Our Response: As the reviewer points out, there are indications that the miRNA-guided suppression could occur outside the structures called 'P bodies'. This is a distinct possibility and therefore we have removed the 'P body' component from the proposed model in the first version (Fig 7*E*, revised manuscript). We believe P bodies are microscopically distinct structures and at times, minute P body-like structures may be beyond the detection limit (resolution) of the microscope. Therefore we restrained from making a strong statement about this. However, what is amply clear is the requirement for many of the P components in miRNA function.

13. Abstract: the sentence starting with "AGO family of proteins ..." needs to be corrected.

Our Response: We thank the reviewer for the comments. We have reframed the sentence in the current manuscript (highlighted in red).

14. Annulate lamellae are not well introduced and characterized in this study. The authors state that these structures are 'pore-like structures'. However, Nup358 positive foci are found in the cytoplasm and are not associated with the nuclear envelope. So what 'pores' are formed? Furthermore, are there any other, more unrelated components of AL that could serve as control?

Our Response: *As per the reviewer's suggestion, we have included more information on AL in the 'introduction' section in order for the reader to understand the relevance of this paper. We thank the reviewer for pointing out this. Unfortunately, other than nucleoporins, no markers are found to represent AL.*

Referee #2:

In this manuscript, Sahoo et al. report that Nup358, a nucleoporin that localizes to the cytoplasmic side of the nuclear pore complex, is important for miRNA-mediated silencing. They show that Nup358 interacts with AGO subfamily proteins and propose that the interaction promotes the target recognition by miRISC. They further show that the SUMO-interacting motif of Nup358 acts as a binding site for Argonaute proteins, including AGO and PIWI subfamily proteins. Overall, the presented data themselves are generally sound and well performed, and this study may potentially provide an important insight in the RNA silencing field and. However, the data are sometimes over-interpreted toward a particular direction and the (direct or indirect) molecular mechanisms underlying the observed phenomena remains unclear. The following points should be carefully addressed before the manuscript is further considered.

Our Response: We thank the reviewer for the positive comments, raising valid concerns and providing valuable suggestions. We have attempted addressing all the concerns raised by the reviewer, which we believe has significantly strengthened and improved the manuscript.

Major points:

1. The major drawback of this study is the mechanistic ambiguity for how Nup358 promotes miRNA-mediated silencing. P-body was originally believed as a site for miRNA-mediated silencing, but it is now well accepted that P-body per se is dispensable for silencing (Chu and Rana, PLoS Biol. 2006; Eulalio et al., Mol. Cell. Biol. 2007). Thus, the proposed model for how Nup358 facilitates miRNA function appears somewhat superficial. The authors argue that Nup358 binds AGO2 and GW182 and mediates "the association of the target mRNA with miRISC." However, it is hard to imagine how, via such protein-protein interactions, Nup358 can exclusively facilitate the base pairing between the AGO-loaded miRNA and target RNA, but not the association between lambda-N-AGO2 and BoxB-RNA (Figure 3F), which is also in equilibrium and could theoretically be assisted by the protein interaction network.

Our Response: We thank the reviewer for the comments. We also agree with the reviewer that the relevance of 'P body' structure as such is debatable [please refer to our response to Reviewer 1 (#12)], as we found Nup358 positive AL and P bodies were physically and dynamically associating with each other, and Nup358 depletion had a profound effect on P body formation, we had included 'P bodies' in the model (first version). In the current manuscript, our additional data show that P body formation can be rescued by ectopic expression of AGO2 in Nup358 depleted cells (Fig 7*C*). However, miRNA reporter assays clearly suggest that restoring P body formation is not sufficient to rescue the miRNA defect in Nup358 deficient cells (Fig 7*C*, revised manuscript). Because of the lack of clarity with respect to the relevance of this structure in miRNA function, we preferred to remove 'P body' component from our model (Fig 7*E*, revised manuscript). However, we believe that the ambiguity is most likely due to the distinctness of the P body structures while visualizing through microscopes. As the components of P bodies are required for miRNA mediated suppression, we think the repression could occur outside the microscopically distinguishable structures (P bodies). Under physiological condition, the number and size of these structures could be an indication of the global miRNA activity in the cell.

2. In Figure 1C, the association of Nup358 with P-bodies should also be investigated in the normal condition. Also, the definition of "associated" and "free" should be clarified.

Our Response: We have quantitated the association between Nup358 and P bodies under normal condition, which is ~16% (highlighted in page 6 & 7). This is closer to what we found under oxidative stress condition (Fig 1*C*, revised manuscript). The association between AL and mRNP granules becomes more extensive and apparent when both GFP-Nup358 and RFP-Dcp1 are co-expressed (Fig S1D and Video S2, revised manuscript). Under endogenous conditions, the overall association is not that striking possibly due to the dynamic nature of interaction. Moreover, we found that the extent of association is similar to that of the well-characterized interaction between P bodies and SGs in HeLa cells (Fig 1*C*). The term "free" is replaced with "others", as per the suggestion by reviewer 3 (technical comments, #2). We have made changes in the text accordingly.

3. In Figure 1D, the expression level of Dcp1a should be quantified by Western blotting to clarify whether P-body formation per se or Dcp1a protein was destabilized by Nup358 depletion. If the level of Dcp1a (or other core P-body proteins) was in fact down-regulated by Nup358 depletion, the observed effects on miRNA-mediated silencing might all have to be attributed to Dcp1a (or others), not directly to Nup358.

Our Response: We thank the reviewer for the comments. As suggested, we have monitored the level of Dcp1a under control and Nup358 knockdown condition by western analysis and found it to be comparable (Fig 1*D*, revised manuscript). This indicates that the disruption of P bodies in Nup358 depleted condition is not a consequence of any change in the level of Dcp1a. Moreover, we could restore the formation of Dcp1a positive P bodies when AGO2 was ectopically expressed in Nup358 depleted condition (Fig 7*C*, revised version).

4. Throughout the manuscript, the data of reporter assays (currently shown only in fold de-repression) should be presented in the Rluc/Fluc ratio. Representation by fold de-repression hides all the important information of the efficiency of miRNA-mediated silencing itself.

Our Response: As per the suggestion, we have represented the reporter assays as RL/FL or FL/RL ratio in the revised manuscript (Fig 1*C*, 1*D*, 7*A*, 7*C*, EV2*A*, EV2*B*, EV2*C*, EV2*D*, EV4, EV5*B*).

5. To evaluate the effect of Nup358 on general miRNA biogenesis, the authors performed deep sequencing using HEK293T cells. However, it is clear that depletion of Nup358 in HEK293T is inefficient (Figure S3A) and it is unclear why the authors did not choose HeLa cells, in which Nup358 knockdown is quite efficient (Figure 2A), for this important analysis.

Our Response: We thank the reviewer for the comments. As mentioned by the reviewer, we had performed most of the miRNA functional assays in HeLa, where we had validated that the levels of specific miRNA (e.g. let-7a) remained comparable between control and Nup358 knockdown conditions using northern and qPCR (Fig 3*A*). We have used another cell line (HEK293T) for deep sequencing to test the generality of the finding. We have used shRNA-mediated knockdown to deplete Nup358 in HEK293T cells, which is still quite efficient (although may not be to the same extent as seen for HeLa using siRNA) (Fig EV3*A,* revised manuscript). We believe that the results do not undermine the conclusion.

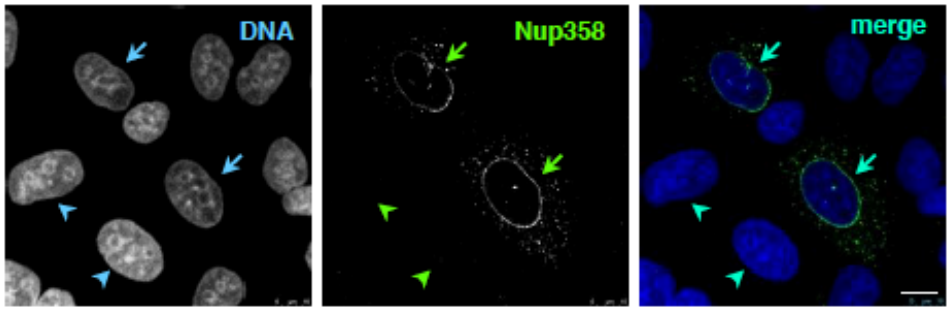
6. To conclude "AGO2 interaction is mainly dependent on SIM1" (p11, line 4), the authors should investigate whether or not IR1+2ΔSIM2 interacts with Ago2.

Our Response: As suggested by the reviewer, we have included IR1+2ΔSIM2 and AGO2 interaction data (Fig 5*E*, revised manuscript). The results clearly indicate that SIM1, and not SIM2, is important for interaction with AGO2 in the IR1+2 context.

Minor points:

1. The specificity of immunostaining of Nup358 should be validated using the siRNA against Nup358.

Our Response: The Nup358 antibody has been used in many other studies and had been validated [5-7]. In this study, this Nup358 antibody has been used for immunostaining, western and immunoprecipitation. Specific to the reviewer's comment, the immunostaining in Fig 1D showed almost complete removal of Nup358 positive signals in the depleted cells. We are providing an additional immunostaining data showing the specificity of Nup358 antibodies (Reviewers' Fig 2). The data shows that Nup358 is depleted from both NE and AL.



Reviewers' Fig 2. Specificity of Nup358 antibodies used in the study. *HeLa cells were treated with Nup358 specific siRNA and were immunostained using affinity-purified rabbit Nup358 antibodies (green). DNA was stained with Hoechst 33342 (blue). Arrow heads indicate Nup358 depleted cells and arrows indicate cells where siRNA-mediated depletion is not seen. Note that the Nup358 positive signals both on the NE as well as in the cytoplasmic AL are specific. Scale bars, 10* mm.

2. Page 3, line 22: The statement "Although many GW/WG sequences are present in GW182, two tryptophan residues (W623 and W634 in human TNRC6B isoform) that are interspersed by a minimum length of 10 amino acids majorly contribute to the AGO- GW182 interaction" is misleading. Although Pfaff et al. [9] reported that W623 and W634 are crucial for the interaction between AGO2 and a 83-aa short peptide derived from TNRC6B (positions 599-683), they clearly

showed that full-length GW182 has many other Ago-binding tryptophan residues in the GW-rich repeat (positions 162-996).

Our Response: We thank the reviewer for pointing out this. We have removed the sentence to avoid confusion (page 3, first para, last sentence).

3. Page 3, line 28: In general, siRNA triggers cleavage of mRNA, but not suppression of translation.

Our Response: As per the suggestion, we have made appropriate changes (page 3, second para, highlighted in red).

4. Several IP data are too faint to follow the authors' conclusion, e.g., Figures 4B, 4D, 5E. Western blotting data of long exposure should be added.

Our Response: We thank the reviewer for the concerns raised. We have repeated the experiments and provided new blots. Please refer to Fig 4*B*, 4*D* and 6*B* (5*E* in the previous version) in the revised manuscript.

5. The coomassie staining gel in the direct binding assay (Figure 5H) should be presented without cropping in order to validate that the interaction is not mediated by other components.

Our Response: As mentioned in the 'materials and methods' and 'results' sections, GST pull down assay was performed after mixing soluble lysates made from bacteria expressing GST-control or GST-SIM1 with soluble lysates from bacteria expressing MBP-control or MBP-AGO2. As we used both GST and MBP controls, and pull downs were performed under identical conditions, other components mediating the interactions are not relevant. To monitor the comparative GST-pull downs we had coomassie stained the blot that was used for western analysis with anti-MBP antibodies to detect MBP-AGO2 (Fig 6D, revised version).

Referee #3:

In this manuscript, Sahoo et al establish a relation between annular lamellae (AL), a so far relatively poorly understood subcellular structure deriving from the perinuclear ER, and miRNA silencing. They reveal that the nuclear pore complex protein Nup358 localizes to AL and plays a role in miRISC binding to target mRNA by direct interaction with Ago2, and that this interaction is required for target mRNA silencing. Finally the authors dissect the Nup358 domains relevant for the interaction and derive that Ago2 interacts with the SUMO binding domain of Nup358, whereas the interaction is conserved across species and maintained for different family members of Ago and Piwi proteins. This is an interesting study which is presented in a systematic manner that is easy to follow, and the main conclusions seem well supported by the data. The characterization of annular lamellae and the elucidation of their functional role in RNA silencing is an important contribution of relevance for a large audience not only in the RNA field but cell biology in general. Prior to publication I have only a few major and some technical comments:

Our Response: We thank the reviewer for the positive comments, raising valid concerns and providing valuable suggestions. We have attempted addressing all the concerns raised by the reviewer, which we believe has significantly strengthened and improved the manuscript.

1. The paper is lacking a conclusion in light of the physiological relevance of this newly found interaction, ie whether the Nup358-Ago2 interaction is required to increase quantitative efficiency of miRISC to encounter target mRNA molecules by compartmentalization, or whether the interaction is affecting miRISC - mRNA interaction at the molecular level (such as by eg altering Ago conformation, recruiting further factors etc).

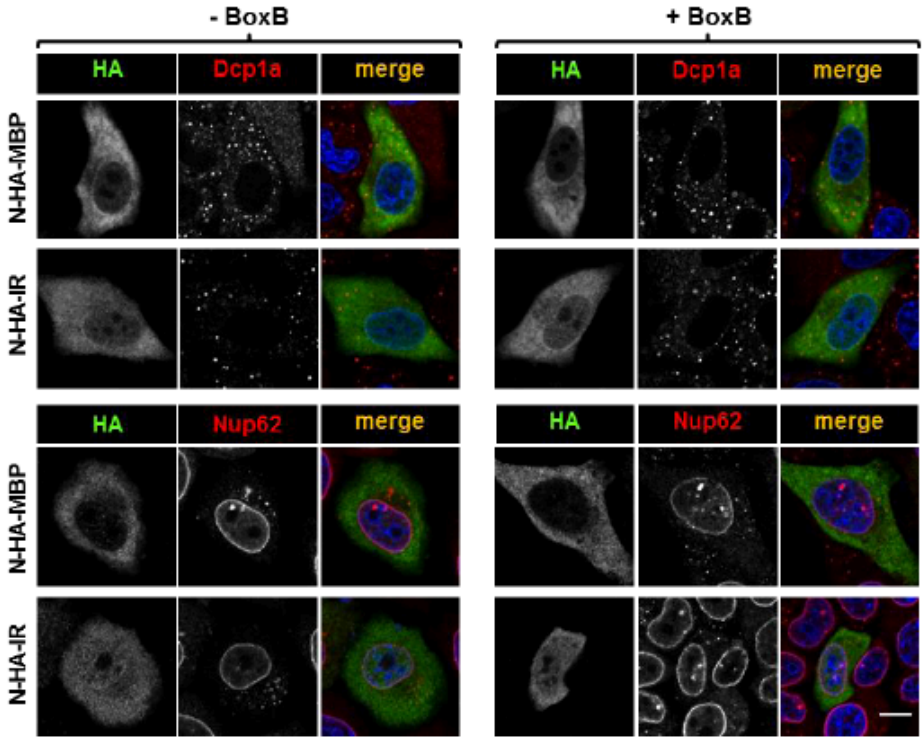
Our Response: We thank the reviewer for the insightful suggestions and comments. We have performed some experiments to address the questions raised by the reviewer. The question as to whether Nup358-AGO2 interaction is required for increasing the efficiency of miRISC with target mRNAs by compartmentalization or through affecting miRISC-mRNA interactions at the molecular level requires further investigation. Based on the results obtained (from previous and revised versions), it appears that Nup358 and AGO2 could possibly function outside AL and P bodies,

respectively. However, under endogenous conditions, the compartmentalization could help in increasing the efficiency of miRNA function. One major problem in interpreting the data in the context of compartmentalization is that the compartments are defined based on the visual distinctness of them under the microscope. For example, P bodies are considered as microscopically distinguishable structures with the presence of marker proteins. However, there can be minute particles beyond the detection limit (or resolution) of the microscope, which could be functional as well. Therefore, as much as possible, we refrained from making specific comments on the compartmentalization.

Some experimental results in this context are discussed below.

Related to this question the data in Figure 6B are curious: if loss of RNA silencing upon Nup358 knockdown can be rescued by tethering of N-HA-IR domains - which likely lack the AL localization moiety - to the target reporter, wouldn't this suggest that localization to AL is not the main relevance of Nup358 - Ago interaction? Where does this Nup358 truncation mutant (N-HA-IR1+2) localize?

Fig 6B (old version) is represented as Fig 7A in the revised version. This experiment was not performed in Nup358 depleted condition as pointed out by the reviewer. This was performed under normal conditions to check if tethering of IR would be sufficient to suppress the target RNA, possibly by recruiting AGO proteins. The results supported this notion (Fig 7A, revised manuscript). Even if this experiment was not performed under siRNA treated condition, the reviewer's point is valid, that is, whether IR functions by localizing to AL or not. We have monitored the localization of N-HA-IR localization when RL reporter having (+) or not having (-) the Nl-peptide binding BoxB elements in relation to AL and P bodies. Under both the conditions, we failed to detect N-HA-IR in AL (Reviewers' Fig 3). N-HA-IR was mostly diffused throughout the cell, indicating that localization of IR to AL may not be absolutely required for the suppression. However, under endogenous situation, localization to AL could enhance the efficiency of suppression, a possibility which needs to be tested by further experiments. This, particularly, requires understanding of how Nup358 gets targeted to AL. It is also possible that under overexpressed condition, whether the localization of N-HA-IR to AL occurs or not is difficult to monitor, as the cytoplasmic localization of N-HA-IR makes it difficult distinctly see the AL localization.

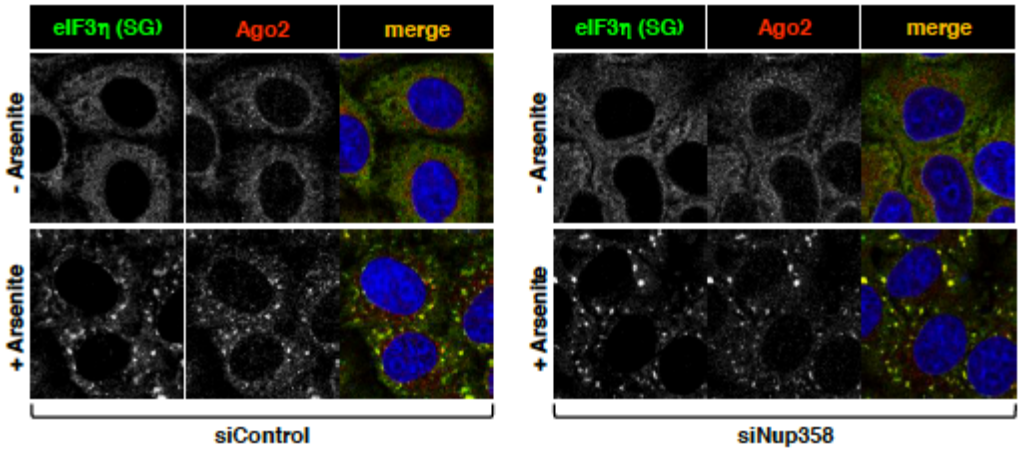


Reviewers' Fig 3. Relative localization of N-HA-IR with P bodies and AL. *HeLa cells were transfected with N-HA-MBP (control) or N-HA-IR with (+) or without (-) RL-5BoxB construct*

(BoxB). Cells were immunostained for HA (green) and Dcp1a (red, P body marker) or Nup62 (red, AL marker) as indicated. DNA was stained with Hoechst 33342 (blue, merge). Scale bar, 10 mm.

How does Ago localization change upon Nup358 knockdown?

We lacked good antibodies that showed appreciable and reproducible localization of endogenous AGO2 in P bodies. However, ectopic expression of AGO2 to monitor the localization of AGO2 restored the P body formation in Nup358 depleted cells. Due to these technical limitations, we could not conclusively assess the localization of endogenous AGO2 under Nup358 depleted condition. Studies using rat anti-AGO2 antibody (monoclonal anti-AGO2 antibody – 11A9 clone, Sigma, SAB4200085) [8], indicated that in Nup358 depleted cells, AGO2's SG localization does not get affected (Reviewers' Fig 4). This antibody failed to detect AGO2 localization to P bodies in HeLa cells reproducibly [8].



Reviewers' Fig 4. Nup358 is not required for localization of AGO2 to SGs. *HeLa cells were transfected with control (siControl) or Nup358 (siNup358) specific siRNA. Cells were treated with (+) or without (-) 0.5 mM sodium arsenite for 30 min, fixed and stained for eIF3*h *(green, SG marker) and AGO2 (red) using specific antibodies. DNA was stained with Hoechst 33342 (blue, merge).*

Does Nup358 depletion affect quantitative efficiency or result in a complete loss of RNA silencing?(which is impossible to assess since the data in Figure 2B for example show only a single condition/time point and dose which results in complete loss of silencing).

One of the experiments we performed based on the reviewer's comments (for point 2 below), showed that ectopic expression of AGO2 restored P body formation under Nup358 depleted condition (Fig 7C, revised version). Interestingly, AGO2 expression was not sufficient to rescue the miRNA function (Fig 7C, revised version). These results are in support of the conclusion that Nup358 is an important component of miRNA pathway involved in bridging AGO2 (miRISC) to target the mRNA. If Nup358 would be involved in enhancing the quantitative efficiency of miRISC and mRNA interaction, then overexpression of AGO2 would have been able to rescue (at least partially) the miRNA defect in Nup358 depleted cells.

Is the Ago2-Nup358 interaction RNA dependent? These are important questions that need to be addressed in order to understand the relevance of the presented findings.

We have performed experiments to test if interaction of Nup358 with AGO2 and GW182 is RNA-dependent. Based on the co-immunoprecipitation assays performed in the presence and absence of RNase A, we conclude that Nup358 interacts with AGO2 and GW182 in an RNA-independent manner. (Fig 3*E*, revised manuscript). Under the same conditions, as previously reported [4], AGO2 interaction with PABPC1 was found to be RNA-dependent.

2. Since the data in Figure 1B show that Nup358 is required for P-body formation, the rescue of silencing upon Nup358 knockdown with tethering Ago2 to the target mRNA reporter (Figure 3F)

may suggest that P-body formation is not required for RNA silencing. This is an important point since there is still an unresolved controversy in the field about whether P-bodies are required for RNA silencing, or build up in consequence to RNAs being targeted for degradation. The authors should test whether P-body formation is rescued together with the reporter knockdown upon Ago tethering and should comment on the implications of these data in light of the role of P bodies in RNA silencing in the discussion.

Our Response: We thank the reviewer for the comments. While trying to address the question raised by the reviewer, we found that AGO2 expression (tethered or untethered conditions), almost completely restored the P body formation in Nup358 depleted cells (Fig 7*C*, revised version). Interestingly, this was not sufficient to rescue the miRNA defect (Fig 7*C*, revised version). This experiment clearly indicates that impairment in miRNA pathway under Nup358 depletion is not due to the inability of cells to form P bodies. Moreover, mere expression of AGO2 (HA- or NHA-AGO2 in the absence or presence of tethering reporter) restored P body formation. Therefore, from these data it is difficult to arrive at any answer to the question the reviewer has raised.

3. The authors mention in the introduction that Annular Lamellae are deriving from perinuclear ER but this information somehow gets lost throughout the elaboration of the conclusions and the discussion. However, this would be relevant information in order to relate this study to the emerging roles of the ER in RNA silencing, translation of non-secreted proteins as well as vesicular trafficking. Too often is there a disconnect in the literature due to a lack of emphasis of such relations - one prominent example being that Ago was initially discovered as Golgi/ER protein (GERP95, Cihaluk 1999) but it still took over ten years to recognize the role of these compartments in RNA silencing. Since many readers may be unfamiliar with AL biology, I would therefore encourage the authors to reiterate the relation of AL to the ER in more prominent passages of the manuscript, ie the abstract as well as comment on the findings of this study in light of emerging ER biology in the discussion.

Our Response: We thank the reviewer for the comments. In principle we agree with the reviewer. Our results support such a role for ER, particularly for AL, in this process. However, to unequivocally state this we might need more evidence. The results documented in this manuscript certainly provide a framework for further exploration of the role of ER in miRNA-mediated suppression. We had discussed about this in the previous version, and, we have again highlighted this in the revised manuscript as well (Discussion session, para 2).

Technical comments:

• *The co-localization in Figure 1A and Figure S1A, C, F, G should be quantified*

Our Response: We have quantified the data and included in the revised manuscript.

• *Figure 1C and Figure S1D and E: The fraction of P-bodies or stress granules that do neither localize with NE nor AL should not be called 'free' (perhaps rather 'other'), since the authors do only look at those two compartments and cannot exclude that these granules associate with other structures*

Our Response: We agree with the reviewer and as suggested, have relabelled this pool as ‘others’.

• *Figure 1B, S1D and S1E lack scaling bars*

Our Response: We have provided the scale bars for all the microscopic images in the revised manuscript.

• *Figure 1C: the markers rather than the compartments should be specified in the image.*

Our Response: The markers are specified as suggested.

• *For all co-localization studies, a positive control for alignment / chromatic aberration between the two channels (such as by TetraSpeck beads) should be shown as supplementary information.*

Our Response: The microscopes are routinely calibrated by the company engineers with TetraSpeck beads for colocalisation/ aberration check. We have provided the same in supplementary information (Appendix Fig S1).

• *The experiment in Figure 1D lacks supplemental information on the efficiency of the knockdown.*

Our Response: We have included a western blot showing the extent of knockdown in the revised version (Fig 1*D*).

• *Page 7, line 7: '...HeLa cells have higher levels of endogenous let7a...' - this is unclear, higher compared to?*

Our Response: This sentence has been reframed (highlighted in red).

• *The relatively modest effect of Dicer knockdown on the de-repression of the reporter in Figure 2B is curious - how are mature let7 levels affected?*

Our Response: Under Dicer knockdown condition, the mature levels of miRNA have been substantially reduced (Fig 3*A*). We are not sure if we can compare the extent of decrease miRNA levels directly to the extent of de-repression. Although we do not have a reason, we presume that this could depend on the kinetics of other processes. The extent of depletion, miRNA levels and miRNA activity may have to be monitored at different time points for making any meaningful interpretation. We, however, consistently see a significant de-repression upon Dicer knockdown.

• *Figure S2A: which of the three Nup358 siRNAs are being used in all other experiments with Nup358 knockdown?*

Our Response: We had provided the information about the siRNA being used in all the experiments in the 'materials and methods' section (kindly refer to page 18, revised manuscript). We have included it in the figure legends as well (Fig S2*A*).

• *The rescue data in Figure S2C are not entirely conclusive - also for control siRNA an increased level of repression is observed. Perhaps this indicates that overexpression of Nup358 in general enhances RNA silencing but the authors should perform experiments to address this issue (this may relate to my first point in the major comments: How is the quantitative RNA silencing efficiency and Ago2 subcellular distribution affected upon Nup358 overexpression?)*

Our Response: We thank the reviewer for the comments. We have overexpressed Nup358 and found it to enhance miRNA-mediated repression (Fig EV2*C*, revised manuscript). This indicates that Nup358 could be a rate limiting factor for the miRNA pathway in cells. However, endogenous AGO2 localization to P bodies or other compartments could not be monitored by immunofluorescence microscopy due to the technical problem mentioned in response to reviewers' question #1.

• *How are the IP data in Figure 3B normalized - should be normalized to Ago2 levels which are pulled down, but this is unclear from the experimental description given.*

Our Response: The IP data has been normalized to the amount of GAPDH RNA co-immunoprecipitated with AGO2 in each condition. If normalized to the extent of AGO2 pull down, then in AGO2 siRNA condition, the decrease in pull down would not be apparent, as it was used as a control for the specificity of AGO2 pull down. The amount of AGO2 remained similar under different conditions (except for siAGO2 condition) and therefore we believe that this does not undermine the conclusion.

• *Page 9, line 1: there is an unspecified reference given*

Our Response: We are sorry for overlooking this. The correct reference has been included (highlighted in red, Page 9, middle para)

• The co-fractionation of Ago2 with Nup358 in Figure S4C is not convincing: since the two proteins both fractionate broadly and don't peak with each other, the fact that both proteins are found in some fractions together does not allow to make any conclusions about whether they are in the same complexes - as the authors suggest by the boxes in the figure and the wording in the legend and text. Unless the authors perform additional IPs in all fractions to assess interaction in each of these 'complexes' I would suggest to remove this data altogether.

Our Response: We have removed this data from the revised manuscript as per the reviewer's suggestion.

• Which Ago2 structure is being used for the data in Figure 5J?

Our Response: We had provided the information and reference in the 'materials and methods' (page 27, previous version, and page 29, current version, highlighted). We have now included this information in the figure legends a well (Fig 6*F*, revised version).

• The discussion is rather long and has several passages which appear redundant with the Results section - this should be shortened

Our Response: We have rewritten the discussion taking away redundant passages and discussing the miRNA pathway in the context of ER or AL.

• The model in Figure 6C is not instructive and in fact rather confusing (eg why are there two export paths shown for mRNAs? Why are there two alternate arrows from mRNAs to miRNA repression? Etc) - this should be improved. Also, again the relation of AL to the ER should be indicated.

Our Response: We thank the reviewer for the concerns raised. The original reason for putting up two locations was that we are not sure if the exported RNA could be coupled to miRISC at the NPC or at the AL or at both the sites. As this part is not clear from our work, in the model, we proposed that the coupling could occur at both the places. We do not have the evidence to rule out one or the other possibility. Therefore, we have preferred to depict both the sites/possibilities in the model. We have considerably revised the model and based on the suggestions, we have included AL and ER connection in our model (Fig 7*D*, revised manuscript).

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1. Cordes VC, Reidenbach S, Rackwitz HR, Franke WW (1997) Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments. *J Cell Biol* 136: 515-529.
2. Wu X, Kasper LH, Mantcheva RT, Mantchev GT, Springett MJ, van Deursen JM (2001) Disruption of the FG nucleoporin NUP98 causes selective changes in nuclear pore complex stoichiometry and function. *Proc Natl Acad Sci U S A* 98: 3191-3196.
3. Tirard M, Hsiao HH, Nikolov M, Urlaub H, Melchior F, Brose N (2012) In vivo localization and identification of SUMOylated proteins in the brain of His6-HA-SUMO1 knock-in mice. *Proc Natl Acad Sci U S A* 109: 21122-21127.
4. Weinmann L, Hock J, Ivacevic T, Ohrt T, Mutze J, Schwille P, Kremmer E, Benes V, Urlaub H, Meister G (2009) Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. *Cell* 136: 496-507.
5. Joseph J, Liu ST, Jablonski SA, Yen TJ, Dasso M (2004) The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions in vivo. *Curr Biol* 14: 611-617.
6. Joseph J, Dasso M (2008) The nucleoporin Nup358 associates with and regulates interphase microtubules. *FEBS Lett* 582: 190-196.
7. Murawala P, Tripathi MM, Vyas P, Salunke A, Joseph J (2009) Nup358 interacts with APC and plays a role in cell polarization. *J Cell Sci* 122: 3113-3122.
8. Rudel S, Flatley A, Weinmann L, Kremmer E, Meister G (2008) A multifunctional human Argonaute2-specific monoclonal antibody. *RNA* 14: 1244-1253.

2nd Editorial Decision 14 September 2016

Thank you for the submission of your revised manuscript to our journal. I apologize for the delay, which is due to the recent summer break and many referees being out of the office. We have now received the full set of referee reports that is copied below.

As you will see, while the referees acknowledge that the manuscript has been improved, all of them have remaining concerns and point out that the original reports have not been addressed completely. I think that all points raised are important (especially the comments from referee 3) and have been mentioned in the first round of review, and they should therefore all be addressed. We usually only allow one round of revision, so please perform the experiments and send us a newly revised manuscript as soon as possible.

If you can successfully address all remaining concerns please note that at the moment we can only offer a maximum of 5 EV (expanded view) figures. You can either combine the smaller EV figures in order to reduce the total number or move some EV figures to the Appendix. I look forward to seeing a new, final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

In the revised version of their manuscript, Sahoo et al. have addressed all points that I had raised on their previous version. New experiments have been added and low quality images were replaced with better ones. For a direct proof of the model presented, I had asked whether miRNAs and mRNAs could be co-immunoprecipitated with NUP358. This experiment has been provided. However, a good control for this qPCR experiments would have been a luc mRNA that lacks miRNA binding sites. This is only a minor point and I am generally satisfied with the revised manuscript.

Referee #2:

The authors have addressed most of my previous concerns and the additional data have strengthened the manuscript. However, the authors have missed to respond to the latter half of my original Major Point #1. Please provide reasonable explanation as to why Nup358 can exclusively strengthen the base pairing.

1. The authors argue that Nup358 binds AGO2 and GW182 and mediates "the association of the target mRNA with miRISC." However, it is hard to imagine how, via such protein-protein interactions, Nup358 can exclusively facilitate the base pairing between the AGO-loaded miRNA and target RNA, but not the association between lambda-N-AGO2 and BoxB-RNA (Figure EV4), which is also in equilibrium and could theoretically be assisted by the protein interaction network. Please discuss why or how Nup358 facilitates the association between miRNA-loaded Ago and mRNA, but not between lambda-N-Ago and BoxB-mRNA.

Referee #3:

The authors have performed a number of experiments that have significantly improved the manuscript and most minor points have been addressed. The major issue I had raised however remains still unaddressed, ie what the functional and mechanistic relevance of the newly identified interaciton is. There are two main and conceptionally very different possiilities - ie a relevance of the interaction to make RNA silencing more efficient (thermodynamically and/or kinetically) through compartmentalization or a requirement of the Nup358 complex formation with Ago2 to interact with mRNA at a molecular level through eg conformational rearrangements.

I disagree with the authors arguments that answering this question is technically not feasible due to inavailability of suitable Ago2 anitbodies. (1) The 11A9 Ago2 antibody has been used successfully in immunofluorescence detection of endogenous Ago2 in several previous studies. Ectopic expression of Ago2 is well established to result in ectopic P-bopdy localization which is not

recapitulaing endogenous Ago2 localization, and therefore the absence of 11A9 to detect Ago in P bodies is not an indication of poor antibody quality.(2) Independent of this, immunofluorescence would in fact not be the ideal approach to address this question since Ago2 localizes in many compartments. Instead, biochemical fractionation would be a straightforward approach to assess whether Nup358 knockdown changes the localization of miRNA-loaded Ago2, since the complex associates almost exclusively to rough ER membrane. (3) A second experiment which would be straightforward to do, and as indicated in my previous review, would be to perform dose response and time course data for RNA silencing by transfection with exogenous miRNA / siRNA in absence and presence of Nup358 knockdown, which would allow to assess changes in quantitative RNAi efficiency in a straightforward manner.

Both of these experiments would allow to support either one or the other models and should be performed in order to provide any insight into the functional role of the newly found interaction within this manuscript.

2nd Revision - authors' response 13 November 2016

We are submitting the revised manuscript entitled “Elucidation of Nup358’s role in miRNA pathway reveals a new motif for interaction with AGO family of proteins” by Sahoo et al., for consideration for publication in the EMBO Reports as ‘Research Article.’

In the revised manuscript, we have attempted to address all the concerns raised by the reviewers. We have provided a point-by-point response to the reviewers’ comments. As suggested by you, we have restricted the number of EVs to 5, and moved the remaining Figures to Appendix.

We believe that the new data added has improved the manuscript, and will now be suitable for publication in EMBO reports as a full article.

Referee #1:
In the revised version of their manuscript, Sahoo et al. have addressed all points that I had raised on their previous version. New experiments have been added and low quality images were replaced with better ones. For a direct proof of the model presented, I had asked whether miRNAs and mRNAs could be co-immunoprecipitated with NUP358. This experiment has been provided. However, a good control for this qPCR experiments would have been a luc mRNA that lacks miRNA binding sites. This is only a minor point and I am generally satisfied with the revised manuscript.

Our Response: We thank the reviewer for the positive comments. As suggested, we could use *luc* mRNA that lacks miRNA binding site as a control. However, our experiments clearly suggest that Nup358, as compared to IgG control, specifically associates with target mRNA not only with the exogenously expressed artificial reporter, but also with an authenticated endogenous mRNA, DnaJb11 (Fig 3*F*).

Referee #2:
The authors have addressed most of my previous concerns and the additional data have strengthened the manuscript. However, the authors have missed to respond to the latter half of my original Major Point #1. Please provide reasonable explanation as to why Nup358 can exclusively strengthen the base pairing. 1. The authors argue that Nup358 binds AGO2 and GW182 and mediates "the association of the target mRNA with miRISC." However, it is hard to imagine how, via such protein-protein interactions, Nup358 can exclusively facilitate the base pairing between the AGO-loaded miRNA and target RNA, but not the association between lambda-N-AGO2 and BoxB-RNA (Figure EV4), which is also in equilibrium and could theoretically be assisted by the protein interaction network. Please discuss why or how Nup358 facilitates the association between miRNA-loaded Ago and mRNA, but not between lambda-N-Ago and BoxB-mRNA.

Our Response: We thank the reviewer for the positive comments. As suggested by the reviewer, we have discussed the possible reason why Nup358 facilitates the association of miRNA-loaded AGO2 with target RNA, and not lambda-N-AGO2 with BoxB-RNA (Discussion, Page 15 and 16, highlighted in red font).

Referee #3:
The authors have performed a number of experiments that have significantly improved the manuscript and most minor points have been addressed. The major 2 issue I had raised however remains still unaddressed, ie what the functional and mechanistic relevance of the newly identified interaction is. There are two main and conceptionally very different possibilities - ie a relevance of the interaction to make RNA silencing more efficient (thermodynamically and/or kinetically) through compartmentalization or a requirement of the Nup358 complex formation with Ago2 to interact with mRNA at a molecular level through eg conformational rearrangements.

I disagree with the authors arguments that answering this question is technically not feasible due to inavailability of suitable Ago2 antibodies. (1) The 11A9 Ago2 antibody has been used successfully in immunofluorescence detection of endogenous Ago2 in several previous studies. Ectopic expression of Ago2 is well established to result in ectopic P-body localization which is not recapitulating endogenous Ago2 localization, and therefore the absence of 11A9 to detect Ago in P bodies is not an indication of poor antibody quality. (2) Independent of this, immunofluorescence would in fact not be the ideal approach to address this question since Ago2 localizes in many compartments. Instead, biochemical fractionation would be a straightforward approach to assess whether Nup358 knockdown changes the localization of miRNA-loaded Ago2, since the complex associates almost exclusively to rough ER membrane. (3) A second experiment which would be straightforward to do, and as indicated in my previous review, would be to perform dose response and time course data for RNA silencing by transfection with exogenous miRNA / siRNA in absence and presence of Nup358 knockdown, which would allow to assess changes in quantitative RNAi efficiency in a straightforward manner.

Both of these experiments would allow to support either one or the other models and should be performed in order to provide any insight into the functional role of the newly found interaction within this manuscript.

Our Response: We thank the reviewer for the comments. As suggested by the reviewer, we have analysed the localization of AGO2 to the rER using membrane flotation assay under control and Nup358 siRNA conditions. The results showed that Nup358 depletion does not affect the targeting of AGO2 to the rough ER (Fig 7*D*, revised manuscript). However, we believe that the second experiment suggested by the reviewer would not provide a conclusive answer to whether Nup358 is required for increasing the miRNA efficiency or has an essential role in miRNA pathway under siRNA-mediated knockdown condition (due to partial depletion). However, even if Nup358 is absolutely required for miRNA pathway, under knockdown condition (where some amount of Nup358 still remains), the miRNA efficiency would reach a level comparable to that of control at a later time point. The same results would be expected even if Nup358 is involved in increasing miRNA efficiency. Therefore, we preferred adding the data showing localization of AGO2 to ER in the revised manuscript, and discussed the possible functions of Nup358-AGO2 interaction in miRNA pathway (Discussion, Page 15 and 16, highlighted in red font).

3rd Editorial Decision 24 November 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Corresponding Author Name:	Jomon Joseph
Journal Submitted to:	EMBO reports
Manuscript Number:	EMBOR-2016-42386V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Genrally considered sample size for the experiments documented in this study has been n = 3 or 4, which is routinely by our group and other groups. The details have been provided in the figure legends as well as in the methods section. No power calculation was performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization procedure was followed. However, as mentioned above critical experiments were performed by different individuals in the group to minimize the subject bias.
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Most of the critical experiments were performed by different individuals in the group to test the reproducibility and to avoid biased interpretation. Randomization as such was not performed.
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Details of antibodies used in the study have been provided in the methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Mycoplasma contamination was routinely tested by Hoechst staining.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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