Non-canonical argonaute loading of extracellular vesicle-derived exogenous single-stranded miRNA in recipient cells

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MS TITLE: Non-Canonical Ago Loading of EV-Derived Exogenous Single Stranded miRNA in Recipient Cells

AUTHORS: Bartika Ghoshal, Edouard Bertrand, and Suvendra N Bhattacharyya
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I have now received comments on your manuscript from three experts. As you will see from their reports, the recommendations regarding publication are somewhat mixed. While referee #3 considers that your experimental data does not support the ambitious conclusions, the other referee thought that the work was potentially quite interesting and significant but all also raised a number of concerns that must be dealt with. Please address the issues raised by the three reviewers as thoroughly as possible. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. I would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the paper “Non-Canonical Ago Loading of EV-Derived Exogenous Single Stranded miRNA in Recipient Cells” from Ghoshal and colleagues the authors describe the interesting finding that miRNA are transferred from secreting cells to receiving cells by non-canonical Ago integration to downregulated target genes. The findings would be interesting and relevant for cell biologists in the area of miRNA function and EV biology.

Comments for the author

The data of the paper looks promising, but the text is not well written and it is not easy to understand what authors did to reach to their conclusions. Figure legends and text should have a bit more redundancy, for example the text is not comprehensive on itself, the figures not well enough labeled. The authors should take care to explain better their reasoning and motivation for each step of the manuscript. However, with extensive text adjustments, the paper can be improved a lot to maybe make it a good fit for JCS. It is hard to judge in the current form.

Major comment:
As miRNA were discussed to be attached to the outside of EV and not packaged inside, an important control experiment would be to test RNAse treatment with and without Tritonx100, in addition to the shown DNAse treatment. If miRNA are inside EVs, they should be protected in the absence of TX100, while the biological function should be gone if the miRNA is outside or just co-purified with EVs irrespective of TX100.

Minor comments:
Labeling is not sufficient, all graphs should have one letter to them, not several in one subfigure.
The authors used mir122 and 146 a miRNA foreign to Hela cells to understand general transfer, but in the abstract and introduction it is mentioned just miRNA in general.

- The miR122* was also not detected in recipient HeLa and was in the non-reliable detection limit in EVs derived from miR-122 expressing HeLa cells: Please explain why you analyze miR112* in the text.
- The transferred miR-122 was found to cause downregulation the target messages (Figure 1B): This sentence is incomplete.
- we detected no increase of EV-delivered miRNA content in presence of its target messages in recipient cells, rather a decrease in total miRNA content was noted in addition to no change in Ago2-associated miRNA content was detected (Figure S2F-G): The sentence is too long and confusing.
- …..can be hypothesised that upon lowering the pH of endocytic vesicles, fusion between the exosomal membrane and internalized EV-membrane should occur: This phrase reads wrong. Fusion between endosomal membrane and internalized EV-membrane?

Figure legends:

- Level of internalization of exsomal miR-146a when expressed in donor HeLa cells via EVs to recipient HeLa: This sentence could be improved

- Transfer of miR-122 repressive activity between HeLa cells and functional transfer of mature miR-122 in recipient cell Ago2 protein: Reads confusing

Reviewer 2

Advance summary and potential significance to field

This manuscript presents some interesting concepts and makes several major claims: (1) miRNA are present in EVs as a single stranded mature form not bound to any proteins, (2) EVs these miRNA containing EVs are endocytosed in a dynamin dependent fashion and the miRNA content is delivered after acidification of the endosomal compartment, (3) EV fusion is mediated by pH and Ago2 present on the cytosolic leaflet of the endosome binds to miRNA where it is then available for binding to mRNA on the rER. Unfortunately, while a description of such a pathway is of great interest to the EV field and extremely important in cell biology generally, I do not believe the data presented here conclusively supports all these claims and I have some major concerns that need to be addressed prior to publication.

Comments for the author

Non-Canonical Ago Loading of EV-Derived Exogenous Single Stranded miRNA in Recipient Cells

Bartika Ghoshal , Edouard Bertrand and Suvendra N. Bhattacharyya *

General: This manuscript presents some interesting concepts and makes several major claims: (1) miRNA are present in EVs as a single stranded mature form not bound to any proteins, (2) EVs these miRNA containing EVs are endocytosed in a dynamin dependent fashion and the miRNA content is delivered after acidification of the endosomal compartment, (3) EV fusion is mediated by pH and Ago2 present on the cytosolic leaflet of the endosome binds to miRNA where it is then available for binding to mRNA on the rER. Unfortunately, while a description of such a pathway is of great interest to the EV field and extremely important in cell biology generally, I do not believe the data presented here supports all these claims and I have some major concerns that need to be addressed prior to publication.

Generally, the manuscript is not well written. There are many instances of data presented in figures not being referred to in the results section. There are also instances where the conclusions of the data are incorrect or overstated. Data is not clearly presented, there are multiple pieces of data displayed in the same subfigure that are not clearly related and important information is in the supplementary figures. The paper lacks clarity for these reasons.

Major concerns regarding data: EV characterisation:
The extracellular vesicles used here are not well characterised. **Figure S1** Characterisation of EVs is provided in the supplementary information in figure S1. Here the authors compare the EVs from HeLa cells +/- miR-122 over-expression. Unfortunately, the EVs isolated are not well characterised and it remains unclear whether the material they have isolated contains contaminants that could also carry miR-122. Authors need to address the following points relating to this:

- As per the MISEV2018 guidelines, the authors need to show that their preparation does not contain protein or apoptotic contaminants by probing for a range of proteins in the EV pellet by Western blotting. The WB data presented only shows that the material they are using is positive for ALIX and CD63, both of which should be present, but no negative controls are provided. They should also probe for an ER/Golgi protein that is not expected to be present at high levels in EVs. Additionally, the presence of lipoproteins in EV preparations is a major problem for the EV field, particularly when discussing miRNA as they are known to carry miRNA. While it is unlikely in this system, it remains important to also formally show that the isolated EV preparation does not contain lipoproteins. All EV lysates should be run alongside cell lysates as a positive control which will also show the relative abundance of enrichment in EVs.

- The size of the EV population has been characterised by NTA and AFM which is good, however only the NTA is represented graphically. The AFM data should also be represented graphically displaying the numbers of EVs analysed (figure states an average but from how many?).

- The characterisation of EVs is from HeLa cells when HeLa cells expressing CD63-GFP are used for imaging experiments. The over-expression of CD63-GFP is known to change the size distribution of EVs and is thought to increase the concentration of EVs released. These EVs should also be characterised with and without miR-122 over-expression.

- It is important to show that miR-122 is inside EVs. This can be achieved by treating intact EVs with RNase, in activating the RNase with an inhibitor, and checking for levels of miR-122. This is commonly done in the EV field and is needed to show that any miRNA of interest is inside the lumen of EVs and not just associated or non-specifically co-isolated.

EV uptake studies using microscopy:

- Several figures show GFP-CD63 (or GFP-CD63?) EVs associated with recipient cells, however it is very difficult to tell whether the EVs are inside the cell as the authors claim. The imaging is not presented in a way that clearly shows the EVs are inside the cells, they could simply be associated with the cell surface. It is also unclear from the figure legend and methods whether this is a 3D stack or a slice. In the results it states that this is 3D imaging, if this is the case, the images presented do not show that the EVs are inside the cells as surface associated EVs will also be present.

- In the methods section under “Microscopic analysis of EV entry into recipient cells” it states that EVs were isolated using “Exosome Isolation Reagent (Thermo Scientific) from cell culture medium” however this is not the method used previously for characterisation of EVs or what is described under “Exosomes isolation and characterisation” methods. Why is this? If EVs are isolated using a different method they also need to be characterised. Clearly the isolated material from this kit has GFP-CD63 positive material but kits are also thought to contain more contaminants than UC material. Additionally, the methods do not state how the EVs were quantified prior to incubation with recipient cells – was NTA used every time to ensure the same number of EVs were used in each assay?

- Figure 2 A/B – uptake studies using GFP-CD63 show very few EVs associated with cells after 4 h and only 10 on average at 16 h. Again, it is unclear whether we are looking at a stack or a slice. The graphical representation suggests whole cells were counted. If this is the case, there is a very big difference in the estimated number of miR-122 molecules delivered based on miR-122 in recipient cells as estimated in the first section on page 7 “The average copy number of mature miR-122 per EVs was 1.95 while the total amount of miR- 122 transferred to recipient HeLa after treatment for 16h was just above 10,000 molecules per cells.” Based on this estimated number of molecules delivered (10,000/cell) with a copy number of the miR-122 in EVs (about 2), 5000 EVs would need to be taken up per cell to produce these numbers. However, only 10 EVs/cell are detected at 16h. Even taking into consideration that EVs would eventually be degraded, one would expect to see more EVs taken up after 16h on average. Therefore, unless the copy number is significantly higher than 2/EV, the number of molecules delivered is probably markedly over estimated due to EVs that are either loosely associated with cells or the well the cells are growing in rather than being internalised. The authors should comment on this. Only 19 cells per time point counted, this is not enough cells to get a good average, especially given the variability.
Additionally, as mentioned above, the methods state that the EVs used for microscopy have been purified using an Exosomal Isolation Reagent not UC as depicted in the schematic in Figure 2A.

- Figure S2 A/B – The numbers of GFP positive EVs in the representative image are much greater than what is plotted in the graph. Are these cells not represented in the graphical data? Are these cells outliers? Data seems inconsistent in this figure and is also different from the uptake studies presented in figure 2 where the average EV/cell count was about 10 with a large spread (up to 20), now it is about 4 with max 6 EVs/cell. Only 10 cells per condition measured. Given this variability it is hard to draw solid conclusions with siDynamin2 as the average clearly fluctuates. Additionally, data detecting miR-122 levels decreasing in the recipient cells should be accompanied by the luciferase activity assay.

- **Figure 1A** does not demonstrate an increase of miR-122 in recipient cells as stated:

"Increase of miR-122 in recipient HeLa cells, is the measure of the miRNA- transferred after the pre-treatment of HeLa cells with EVs derived from miR-122 expressing HeLa cells (Figure 1A)." The image in Figure 1A shows the association of CD63-GFP containing EVs with recipient cells. If this sentence referrers to the workflow diagram only, please label (I) and (ii) and specify in the text to avoid confusion. This sentence should also be reworded for clarity.

Cell fractionation and endosome imaging experiments:

- Figure 3 B – Cell fractionation is an interesting way of investigating where EVs are after internalisation. The WB shows that HRS isolates in factions 1-3 and calnexin is predominantly fractions 6-8. However, LAMP-1 is predominantly in fractions 1-2 and 5-8 which overlaps heavily with HRS and calnexin. No late endosome marker was used. Given that LAMP1 isolated with many fractions and there is much overlap it is hard to draw conclusions about late endosomes/lysosomes. Furthermore, the summary of this data in the results section is somewhat overstated "Fractions 2, 3 were enriched for early endosomes while fractions 4-6 represented late endosomes and lysosomes and fractions 7-9 had enrichment for ER marker protein." Finally, the WB is not aligned properly and there appears to be 9 lanes in for LAMP1 on the right-hand side with lane 10 cropped out. This makes is hard to see which fractions LAMP1 is in. Later in the manuscript the authors KO Rab5a, Rab7 and RILP as makers for different endosomal compartments, why have these not been used here as well?

- Page 11 and Figure 3F– "Measuring the effect of knockdown of these factors, we found a stronger effect of RILP depletion on internalized miRNA content." This refers to the uptake of miR-122 EVs, again this is measured with the relative level of miRNA associated with cells and not specifically taken up. If this did measure uptake, would you not expect to see decreased miR-122 in recipient cells? Why is there an increase in most cases? This is contradictory to what would be expected. Why was uptake was not measured by imaging to confirm?

- Page 11 – "In a steady state, endosome numbers were not found to significantly alter on knockdown of the endosomal proteins individually (Figure S3A-C)." The number of cells used to see if there is a difference in the total numbers of endosomes is only 15. There is a large spread of endosomal numbers detected in the control cells and therefore this result is not surprising, clearly more cells need to be analysed to conclude if there is a change in the numbers of endosomes in Rab5a, Rab7 and RILP knockout cells. As these proteins are required for endosomal trafficking it is surprising that there is no decrease in the total endosomes, looking at the data there is a decreasing trend in the Rab7 and RILP knockdown cells. Furthermore, the representative images also look as though there is a decrease in the knockdown cells.

- Page 13 – "Therefore a large fraction of endosome localized Ago2 is present on the outer side of the endosomes and thus sensitive to Proteinase K (Figure 4H)." How have the authors controlled for lysis of endosomes in the presence of proteinase K? By also probing with a cell surface protein or receptor which is primarily on the outer leaflet of the PM and that should be present within endosomes, the authors would control for lysis and this would clearly show that Argo2 is associated with the outer membrane of endosomes and not the inside.

- Page 13/14 – "It was found that the exosomal miRNA content in the supernatant was increased in presence of FCCP and the increase has happened in a time dependent manner (Figure 4K)." Again, how did the authors control for FCCP induced damage to endosomes? To ensure this was specific to release of EV contents other endosomal contents should also be measured in the supernatant after FCCP treatment.

- Page 14 – "Western blot before and after the assay and this showed that a similar amount of endosomes was recovered with FCCP, ruling out that a change in endosomes themselves cause the changes (Figure 4I, K)." Figure 4I does not compare this and actually shows that the endosomes isolated do not contain calnexin and LAMP1 (as per figure legend. Not mentioned in results). This is very surprising when comparing with the cell fractionation data in figure 3B, while the calnexin data is consistent, LAMP1 now appears to be absent when it was strongly detected in fractions 2-3 previously. Can the authors explain this difference?
Also, why are the levels of HRS and ALIX in the supernatant the same as the pellet? Figure 4K does

not show that there is no difference in the numbers of endosomes before and after treatment with FCC, the levels of ALIX and HRS appear to be variable (ALIX decreases and HRS increases?).

No data referred to in results/data included but not referred to:

Page 6 “We then further confirmed the presence of mature miR-122 in the EVs isolated from miR-122 expressing donor HeLa cell.” None of the data presented so far supports this statement nor does the data that follows. The authors need to directly show data demonstrating that it is the mature miR-122 in EVs not pre-miR-122.

Page 7 Authors state “The miR- 122* was also not detected in recipient HeLa and was in the non-reliable detection limit in EVs derived from miR-122 expressing HeLa cells.” And later in the same paragraph “No pre-miR-122 was detected in recipient HeLa cells treated with mature miR-122 positive EVs.” Again, no data is referred to for these statements. The authors suggest they have data showing that EVs contain mature miR-122 not in complex with dicer/TRPB but it is not presented. Figure 1C is the closest to this but does not directly show this as recipient cells are analysed not the EVs directly and they do not look at miR- 122*. Please clarify/add relevant data.

Figure 1C - “The mature miR-122 content was found to increase in the recipient cells treated with miR-122 containing EVs (Figure 1C)” – this data does not directly demonstrate that the EVs are inside the recipient cells because to measure the miRNA content the cells (and associated EVs) must be lysed to extract miRNA for measurements. Therefore, all EVs, including those internalised and cell surface associated will be measured. Furthermore, the word ‘endogenous’ appears underneath the miR-122 column in the right panel, this is misleading as miR-122 is not endogenous and is exogenously overexpressed. miR-146 is endogenous. Please clarify for this figure and 2F far RHS.

Page 9 – “Upon knockdown of Dynamin2 in the recipient cell, the internalization of miRNA was significantly reduced.” What data is this in reference to?

Figure S2 (E) is not referred to in the results

Other more minor concerns/comments:

Abstract:

Use EV after the initial abbreviated definition – change Extracellular vesicles-derived to EV-derived

“Following endocytosis of miRNA-containing extracellular vesicles, loading of host cell Ago occurs on the endosomal membrane where pH-dependent membrane fusion triggers the release of internalized miRNAs to form exogenous miRNP pool.” This statement is very strong unless fusion is shown release or other should be used.

Figure of key findings:

Why are only the endosomal membranes depicted as double membranes? This is inconsistent. All membranes should be depicted the same way unless it is actually a double membrane as is the case for the autophagosome. No figure legend.

Introduction

The English throughout needs editing to improve grammatical correctness and readability, examples:

The first sentence would benefit from two commas – one after “cells” and the other after “tissues”.

“cells and thus considered as a very important” should read ‘cells and are thus considered a very

important'

"Exosomes the 40-100nm sized vesicles, positive for the marker protein CD63, and are formed during membrane invagination of late endosomes to generate multivesicular bodies (MVBs) followed by MVB fusion to cell membrane" should read 'Exosomes are vesicles 40- 100 nm in size, positive for the marker protein CD63, and are formed during membrane invagination of late endosomes to generate multivesicular bodies (MVBs). Following MVB fusion to cell membrane exosomes are released into the extracellular space." Or similar.

Extracellular vesicles defined multiple times. Use abbreviate after the first instance in the following sentences:

"Extracellular vesicles or exosomes, in particular, have"

"Transfer of functional microRNAs by exosomes or extracellular vesicles (EVs) to"

Same as above for MVB – change the following: "selectively for packaging into Multivesicular bodies(MVBs)"

"MicroRNAs are sorted selectively for packaging into Multivesicular bodies(MVBs) by specific sets of proteins before the MVBs can fuse with cell membrane to release the EVs, packed with specific miRNAs in the intercellular space in a context dependent manner" This sentence makes it sound like the sorting and packaging is required for MVB fusion with the cell membrane which it is not or at least this has not been shown. Please change to read "MicroRNAs are sorted selectively for packaging into MVBs by specific sets of proteins before MVBs fuse with cell membrane to release the intraluminal EVs, packed with specific miRNAs in the intercellular space in a context dependent manner" This sentence is also very long and grammatically incorrect.

Methods:

More information in this section would benefit the reader. Specifically, the luciferase activity assay used to measure the transfer of miR-122 is not fully explained and authors refer to the manufactures instructions.

In results the plasmid used to measure endosome numbers is called "YFP-Endo" and in the supplementary information described as "YFP-tagged early endosome expression plasmid" rather than saying what the protein that is YFP tagged actually is. This makes it difficult to replicate.

Results:

Page 7 "The transferred miR-122 was found to cause downregulation the target messages (Figure 1B)" This is very general and not descriptive of the results. Figure 1B shows a decrease in mRNA expression of 2 genes not mentioned in the results section and the use of a luciferase activity assay. Furthermore, there is no mention of what CAT1 is in the results or figure legend (the reader would have to look this up – I assume it is cationic amino acid transporter 1 (CAT1). Have the authors checked what the impact of overexpressing miR-122 in HeLa cells is? Are the miR-122 donor cells compromised in any way? Are they healthy? Have they been characterised?

Figure legend 1D EVs are referred to as "exosomes"

Figure 1B – The data for the luciferase activity assay is presented as a ratio however it would also be good to know what percentage repression this is, still with reference to the FL activity. I imagine this is quite a small decrease in RL activity?

Page 7 "In order to revalidate the exosomal uptake of miRNA in recipient HeLa cells," the term 'exosomal' is used here instead of the more accurate term 'EV'

Figure 1F – authors do not discuss all the data presented in this figure in the results when

discussing the rest of the data presented in figure 1F. This is discussed later after moving to the next figure. For this reason, presenting the data here is complicating the issue and authors should consider moving the coculture Ago2 and CAT1 data to a separate section (figure 1J).

Page 8 – "The recipient cells were incubated with CD63-GFP positive EVs at different time points of 0, 4 and 16 hours and internalization of GFP-CD63 was quantified microscopically. The internalization of CD63-GFP increased with time till 16h of observation (Figure 2A,B)." Is the GFP tagged CD63 construct N- or C-terminal? CD63 contains a lysosomal targeting signal on the c-terminus so would usually be on the N-terminus – please clarify as both are written in the results.

Page 9 – "the involvement of specific proteins known to control endocytic processes were targeted." Authors only look at dynamin2 as far as I can see. Many studies have looked at the effect of blocking endocytosis (of different types) on the uptake of EVs. A range of effectors have been found to be involved, but few are required for internalisation suggesting many routes are used. Did the authors look at any other regulators of endocytosis as suggested as dynamin2 independent pathways exist?

Page 10 – "Therefore fitness of ER controls the internalization and functioning of the EV-derived miR-122 in human cells." This is novel and interesting finding, however the data for this is buried in the supplementary data and this has only been measured by looking at the levels of miR-122 in recipient cells. The authors have not looked at the EV uptake by microscopy nor have they assessed the functional miR-122 delivery using their luciferase activity assay. ER stress induction is shown by blotting for eIF2alpha phosphorylation which has increased slightly, they could have also assessed BIP to further demonstrate stress induction.

Figure 3F – miR-122 referred to as endogenous where should be exogenously expressed

Figure 4 – siRNA cell fractionation shows an increase of miR-122 in the early endosomal fractions. It did the authors also do the same cell fractionation after Bafilomycin treatment? It would have further strengthened this data if the result was similar.

Reviewer 3

Advance summary and potential significance to field

In this study Goshal et al investigated Extracellular Vesicle-mediated exchange of miRNA between donor and acceptor cells. They propose that miRNA loaded within EV are delivered within acceptor cells after 1) endocytosis 2) pH-dependent fusion with the endosomal membrane. This seems to be a pre-requisite for a DICER-independent miRNA loading with Ago at the cytosolic face of the endosomal membrane, possibly associated with endoplasmic reticulum membranes. Importantly, delivered miRNAs are functional.

Comments for the author

The study is ambitious and tries to address several key questions related to EV-content, including miRNA delivery within the acceptor cells. This is interesting, however the initial enthusiasm was severely tempered by several concerns:

First, results demonstrating EV-mediated delivery of functional miRNAs are strong and it seems convincing that delivery requires endocytosis and pH-acidification. This is consistent with recent papers that should be cited 1) Joshi BS, de Beer MA, Giepmans BNG, Zuhorn IS. Endocytosis of Extracellular Vesicles and Release of Their Cargo from Endosomes. ACS Nano. 2020 Apr 28;14(4):4444-4455. doi: 10.1021/acsnano.9b10033.
Epub 2020 Apr 16. PMID: 32282185; PMCID: PMC7199215. 2) Bonsergent E, Lavieu G. Content release of extracellular vesicles in a cell-free extract. FEBS Lett. 2019 Aug;593(15):1983-1992. doi: 10.1002/1873-3468.13472. Epub 2019 Jun 17. PMID: 31175663°.

However, results dealing with the main location of 1) delivered miRNA and 2) ARGO association are less convincing.
How can the authors rule out that miRNAs are not delivered within the cytosol of the acceptor cells and then immediately processed for ARGO association.
The Authors decided to start with complex organelle separation, that are not always very convincing (for instance in fig3 B lysosomal and ER markers are within the same fractions (6-9) which prevents clear interpretation).
It seems more logical to first separate membrane from cytosolic fractions (using mechanical disruption of the acceptor cells after EV feeding) and test whereas miRNA 122 is more predominantly found in cytosolic or in membrane fractions. Then they could assess if BAf treatment prevents cytosolic release. Similarly, they should questioned where miRNA/ARNO association is predominantly found.
If they rule out that miRNA is never found in cytosol (which would be very surprising) then it will make sense to identify with which organelles miRNAs are associated and processed with ARGO.

-Another general comment is that many controls are missing.
Some examples:
a) FigS1 C, they show only two markers within the EVs; Cell lysate should be tested in parallel. The authors did not test for usual negative markers for EVs (ER or Golgi markers for instance); in addition, CD63 shows a very unusual profile. In all established publications, CD63 runs as a smear due to the presence of heterogeneous glycan chains.
b) FIgS1 E the authors tested the resistance to DNase but omit to test resistance to RNAse. Topology of EV-associated miRNAs is still controversial and the authors should demonstrate that miRNAs are indeed within the EVs (protected from RNAse except in the presence of detergent)
c) Figure 4 H: the authors only tested proteinase K resistance of Ago2, they should add markers that are known to be on the cytosolic face of endosome (positive control) and the luminal face of the endosomes (negative control)that should be proteinase K resistant. In addition, the samples should be tested for other organelles markers to establish lack of contaminations. Without all thess controls, experiments are not validated and it is impossible to rigorously conclude.
-Presentation of the data are not always linear and the logic of the order is not clear. In several occasions there is a back-and- forth reference of data throughout the manuscript. This greatly perturb the reading and the comprehension of the paper. For example, internalization of GFP-CD63 is showed in Fig1 but the again in Fig2B where it is also quantified. This make the paper hard to follow.
-Some results are not mentioned in the core manuscript and it is therefore not clear why they were performed. One example: Figure 1C, why did they use mi146. If this is a control, why is it only a one-shot control and not a systematic one.

Overall the message is interesting but this reviewer feels that the execution of the experiments and the results do not fully support the claims.

First revision

Author response to reviewers' comments

Response to Reviewers' Comments
Reviewer 1 Advance Summary and Potential Significance to Field:
In the paper "Non-Canonical Ago Loading of EV-Derived Exogenous Single Stranded miRNA in Recipient Cells" from Ghoshal and colleagues the authors describe the interesting finding that miRNA are transferred from secreting cells to receiving cells by non-canonical Ago integration to downregulated target genes. The findings would be interesting and relevant for cell biologists in the area of miRNA function and EV biology.

We thank the reviewer for his/her appreciation of the concept of the work. We have tried our best to address all the queries and concern raised by the reviewers by doing additional experiments and also by providing explanations as appropriate.

Reviewer 1 Comments for the Author:
The data of the paper looks promising, but the text is not well written and it is not easy to understand what authors did to reach to their conclusions. Figure legends and text should have a bit more redundancy, for example the text is not comprehensive on itself, the figures not well enough labeled. The authors should take care to explain better their reasoning and motivation for each step of the manuscript. However, with extensive text adjustments, the paper can be improved a lot to maybe make it a good fit for JCS. It is hard to judge in the current form.
We addressed this concern in the revised version and have changed the text and figure legends appropriately to make the manuscript an easy read and communicative.

Major comment:
As miRNA were discussed to be attached to the outside of EV and not packaged inside, an important control experiment would be to test RNAse treatment with and without Tritonx100, in addition to the shown DNAse treatment. If miRNAs are inside EVs, they should be protected in the absence of TX100, while the biological function should be gone if the miRNA is outside or just co-purified with EVs irrespective of TX100.
We would like to clarify that the localization of RNAs has been usually observed to be inside the EVs (Del Pozo-Acebo et al., 2021; Fabbiano et al., 2020; Shurtleff et al., 2017; Valadi et al., 2007). It has been observed that upon treating EVs with RNase and Triton X-100 together, the EV associated RNAs undergo degradation due to disruption of the EV membranes. Likewise, we have also demonstrated that the miRNAs localized inside the EVs are protected from RNase treatment. However upon sonication, that is known to disrupt the membranous structures; (Alvarez-Erviti et al., 2011; Kojima et al., 2018) the EV-associated RNAs become sensitive to RNase (**New Figure S1H-I**). When the EVs isolated from miR-122 expressing HeLa cells were subjected to sonication followed by RNase A treatment, we found that the levels of miR-122 decreased compared to the EVs which were not sonicated but treated with RNase A alone (**New Figure S1I**). Sonication, like Triton X-100, ruptured the EVs leading to the miRNAs being exposed to RNase A leading to their degradation. This data suggest majority of the EV associated miRNAs are present inside the EVs and not outside.

Minor comments:
Labeling is not sufficient; all graphs should have one letter to them, not several in one subfigure.
We have modified the panels as suggested.

The authors used mir122 and 146 a miRNA foreign to Hela cells to understand general transfer, but in the abstract and introduction it is mentioned just miRNA in general.
Yes, apart from detecting miR-122 transfer to recipient cells, we have also performed the miRNA transfer experiment with another miRNA, miR-146a, which like miR-122, is also not endogenously expressed in recipient HeLa cells. We have observed that similar to miR-122 being transferred by EVs, miR-146a is also transferred and detected in the recipient cells. Thus, we have concluded that this mode of transfer is not limited to particularly miR-122 but it can occur for other miRNAs as well. But we agree with the reviewer to mention specific miRNAs in the abstract and introduction.

•The miR122 was also not detected in recipient HeLa and was in the non-reliable detection limit in EVs derived from miR-122 expressing HeLa cells: Please explain why you analyze miR112* in the text.*
The miR-122* is the anti-sense strand of miR-122 which was analysed. The reason for this strand of miRNA being analysed is to see whether only the mature strand or both the sense and anti-sense strands of miR-122 could be transferred via EVs to the recipient cells. That has now been explained in the text.

•The transferred miR-122 was found to cause downregulation the target messages (Figure 1B): This sentence is incomplete.
We have changed the sentence to make it understandable.

•we detected no increase of EV-delivered miRNA content in presence of its target messages in recipient cells, rather a decrease in total miRNA content was noted in addition to no change in Ago2-associated miRNA content was detected (Figure S2F-G): The sentence is too long and confusing.
We have changed the long sentence and have broken it into two to convey the message more clearly. Thanks for the suggestion.

•…..can be hypothesised that upon lowering the pH of endocytic vesicles, fusion between the exosomal membrane and internalized EV-membrane should occur: This phrase reads wrong. Fusion between endosomal membrane and internalized EV-membrane?
We have taken all the review comments into consideration and have provided the explanation and have changed the language as appropriate in the main text.

Figure legends:
•Level of internalization of exsomal miR-146a when expressed in donor HeLa cells via EVs to recipient HeLa: This sentence could be improved
We have changed the text.

•Transfer of miR-122 repressive activity between HeLa cells and functional transfer of mature miR-122 in recipient cell Ago2 protein: Reads confusing
We have made the changes as mentioned and suggested by the reviewer.

Reviewer 2:
General: This manuscript presents some interesting concepts and makes several major claims: (1) miRNA are present in EVs as a single stranded mature form not bound to any proteins, (2) EVs these miRNA containing EVs are endocytosed in a dynamin dependent fashion and the miRNA content is delivered after acidification of the endosomal compartment, (3) EV fusion is mediated by pH and Ago2 present on the cytosolic leaflet of the endosome binds to miRNA where it is then available for binding to mRNA on the rER. Unfortunately, while a description of such a pathway is of great interest to the EV field and extremely important in cell biology generally, I do not believe the data presented here supports all these claims and I have some major concerns that need to be addressed prior to publication.
We would like to thank the reviewer for pointing out the importance of the findings and also appreciate his/her suggestions to improve the experiments to make the manuscript a better read and strong with its claims. We have done additional experiments to address the concerns raised by the reviewer and have tried to answer them all. Our response is listed below.

Generally, the manuscript is not well written. There are many instances of data presented in figures not being referred to in the results section. There are also instances where the conclusions of the data are incorrect or overstated. Data is not clearly presented, there are multiple pieces of data displayed in the same subfigure that are not clearly related and important information is in the supplementary figures. The paper lacks clarity for these reasons.
This is an important concern and we have tried to revise the text and figures as appropriate. We have also taken precaution to present the data in a more meaningful manner and also avoided the overstatement and claims.

Major concerns regarding data:
EV characterisation:
The extracellular vesicles used here are not well characterised. Figure S1 Characterisation of EVs is provided in the supplementary information in figure S1. Here the authors compare the EVs from HeLa cells +/- miR-122 over-expression. Unfortunately, the EVs isolated are not well characterised and it remains unclear whether the material they have isolated contains contaminants that could also carry miR-122. Authors need to address the following points relating to this:
- As per the MISEV2018 guidelines, the authors need to show that their preparation does not contain protein or apoptotic contaminants by probing for a range of proteins in the EV pellet by Western blotting. The WB data presented only shows that the material they are using is positive for ALIX and CD63, both of which should be present, but no negative controls are provided. They should also probe for an ER/Golgi protein that is not expected to be present at high levels in EVs. Additionally, the presence of lipoproteins in EV preparations is a major problem for the EV field, particularly when discussing miRNA as they are known to carry miRNA. While it is unlikely in this system, it remains important to also formally show that the isolated EV preparation does not contain lipoproteins. All EV lysates should be run alongside cell lysates as a positive control which will also show the relative abundance of enrichment in EVs.
We have now EV-characterization as the new **Figure S1**. We have provided Western Blots for the different EV markers in cellular extract as well as in EVs. The EVs, isolated from control and miR-122 transfected HeLa cells, are positive for Alix, HRS, Flotilin and negative for ER marker Calnexin,

lysosomal marker Lamp1. Cytochrome C as well as GAPDH was also found to be absent in the EVs (**New Figure S1E**) to rule out presence of apoptotic structures/bodies in EV preparation. We could not detect CD63 in cell lysate of HeLa cells. The EV preparation is also free from ApoE, a lipoprotein.

- The size of the EV population has been characterised by NTA and AFM which is good, however only the NTA is represented graphically. The AFM data should also be represented graphically displaying the numbers of EVs analysed (figure states an average but from how many?).
Unfortunately, the software plugin required to analyse the number of EVs by AFM was unavailable in the system we have used. We apologise for our inability to produce the data. However, we have shown a larger field view of the same samples to supplement the existing data (**New Figure S1D right panel**).

- The characterisation of EVs is from HeLa cells when HeLa cells expressing CD63-GFPareused for imaging experiments. The over-expression of CD63-GFP is known to change the size distribution of EVs and is thought to increase the concentration of EVs released. These EVs should also be characterised with and without miR-122 over-expression.
We have characterised the EVs isolated using Exosome Isolation Reagent by NTA and Western Blot. We have found that the EVs isolated from untransfected control and CD63-GFP transfected HeLa cells have shown no significant difference in size and number. We also compared the EVs isolated by this commercial reagent aided method with traditional ultracentrifugation based method to note no change in size and concentration (**New Figure S2A, B, D, E**). This has been reported previously also (Cheng et al., 2019). However, upon Western Blot analysis, the Alix protein levels were found to be higher in the CD63-GFP positive EVs than control set suggesting an increase in the specific protein components in the CD63-GFP positive EVs (**New Figure S2C**).
The purpose of using CD63-GFP EVs was to track the internalization of EVs in recipient cells and not to follow miRNA transfer with CD63-GFP positive EVs. The tagging of EVs by using expression plasmid for tagged marker protein has been reported earlier (Heusermann et al., 2016; Ren et al., 2019). Thus, using a similar approach, we wanted to measure the kinetics of the internalization of EVs in recipient cells. As they were not used for measuring the miRNA trafficking as such, hence the effect of miR-122 cargo co-expression on CD63-GFP expressing cells' secreted EVs were not used for analysis.

- It is important to show that miR-122 is inside EVs. This can be achieved by treating intact EVs with RNase, in activating the RNase with an inhibitor, and checking for levels of miR-122. This is commonly done in the EV field and is needed to show that any miRNA of interest is inside the lumen of EVs and not just associated or non-specifically co-isolated.

We appreciate the concern raised by the reviewer. The localization of RNAs have been usually observed to be inside the EVs (Del Pozo-Acebo et al., 2021; Fabbiano et al., 2020; Shurtleff et al., 2017; Valadi et al., 2007). It has been observed that upon treating EVs with RNase and TritonX together, the EV associated RNAs undergo degradation due to disruption of the EV membranes. Likewise, we have also demonstrated that the miRNAs localize inside the EVs as they are protected from RNase treatment unless the EVs are disrupted by sonication (**Figure S1H-I**). When the EVs isolated from miR-122 expressing HeLa cells were subjected to sonication, we found that the levels of miR-122 get decreased upon RNase A treatment (**New Figure S1I**). This suggests that EV associated miRNAs are primarily inside the EVs.

EV uptake studies using microscopy:
- Several figures show GFP-CD63 (or GFP-CD63?) EVs associated with recipient cells, however it is very difficult to tell whether the EVs are inside the cell as the authors claim. The imaging is not presented in a way that clearly shows the EVs are inside the cells, they could simply be associated with the cell surface. It is also unclear from the figure legend and methods whether this is a 3D stack or a slice. In the results it states that this is 3D imaging, if this is the case, the images presented do not show that the EVs are inside the cells as surface associated EVs will also be present.

Firstly, we would like to apologise for the confusion regarding CD63-GFP or GFP-CD63. The plasmid used was CD63-GFP. Secondly, we have done 3D imaging of CD63-GFP EVs inside the recipient cells to visualize the internalization process. We isolated EVs from CD63-GFP transfected donor HeLa cells. This was followed by incubation of recipient HeLa cells with or without GFP tagged EVs for 16 hours. After incubation of the recipient cells with the CD63-GFP positive EVs, the cells were washed extensively with 1X PBS to minimise the chance of any surface bound EVs being present. These cells

were visualised and 3D imaging was done (**New Figure 1A lower right panel**). The grey colour depicts tubulin filaments with EVs (in green) being present on the cell surface as well as inside the cell. Thus, apart from taking images in slices we also captured 3D images to ensure that the EVs were localised inside the cells rather than merely on the surface. However other images presented in other Figures (including the colour images of **New Figure 1A top right panel**) are the confocal image slice that clearly show the internalized CD63-GFP positive EVs there.

- In the methods section under "Microscopic analysis of EV entry into recipient cells" it states that EVs were isolated using "Exosome Isolation Reagent (Thermo Scientific) from cell culture medium" however this is not the method used previously for characterisation of EVs or what is described under "Exosomes isolation and characterisation" methods. Why is this? If EVs are isolated using a different method they also need to be characterised. Clearly the isolated material from this kit has GFPCD63 positive material but kits are also thought to contain more contaminants than UC material. Additionally, the methods do not state how the EVs were quantified prior to incubation with recipient cells – was NTA used every time to ensure the same number of EVs were used in each assay?

We have characterised the kit based EVs (**New Figure S2**). Also, as mentioned, the sole purpose was to visualise the EV internalization, we used crude EVs isolated by the kit. Furthermore, upon comparing the size and concentration of the EVs released by control and CD63-GFP transfected cells, we found that there was no difference in size and numbers observed between the isolated EVs obtained either from the kit or ultracentrifugation (**New Figure S2A, B, D, E**).
To address the amount of EVs added to recipient cells for each assay, we used the approach used by Valadi et al in their paper (Valadi et al., 2007). We used a definite cell ratio of 10:1 for donor: recipient. This means that EVs were isolated from donor cells 10 times the number of recipient cells for detection of miRNA internalization as well CD63-GFP internalization. The amount of EVs released by 10 times the donor cells were quantified by NTA and since, the number of EVs produced from cells grown in identical condition did not alter significantly; this cell ratio was fixed for all the experiments described here.

- Figure 2 A/B – uptake studies using GFP-CD63 show very few EVs associated with cells after 4 h and only 10 on average at 16 h. Again, it is unclear whether we are looking at a stack or a slice. The graphical representation suggests whole cells were counted. If this is the case, there is a very big difference in the estimated number of miR-122 molecules delivered based on miR-122 in recipient cells as estimated in the first section on page 7 "The average copy number of mature miR-122 per EVs was 1.95 while the total amount of miR-122 transferred to recipient HeLa after treatment for 16h was just above 10,000 molecules per cells." Based on this estimated number of molecules delivered (10,000/cell) with a copy number of the miR-122 in EVs (about 2), 5000 EVs would need to be taken up per cell to produce these numbers. However, only 10 EVs/cell are detected at 16h. Even taking into consideration that EVs would eventually be degraded, one would expect to see more EVs taken up after 16h on average. Therefore, unless the copy number is significantly higher than 2/EV, the number of molecules delivered is probably markedly over estimated due to EVs that are either loosely associated with cells or the well the cells are growing in rather than being internalised. The authors should comment on this. Only 19 cells per time point counted, this is not enough cells to get a good average, especially given the variability. Additionally, as mentioned above, the methods state that the EVs used for microscopy have been purified using an Exosomal Isolation Reagent not UC as depicted in the schematic in Figure 2A.

We have recounted the number of EVs internalized using a bigger data set. We have replaced the old data with new data that shows relatively higher number of internalized EVs (**New Figure 1B, C left panel**). We have utilised the *Imaris* software plug-in to generate the number of green surfaces present inside the cell using confocal slices.
Secondly, to address the concern whether the copy number of miRNA per EV was an accurate estimation or not, we have repeated the experiments and have redone the calculation taking into account a bigger data set to consider. We have found that the miRNA copies per cell remains to be approximately 2 per EV while the number of transferred miRNAs in the recipient cell is about 2,800 copies (**New Figure S1J right panel and 1D right panel**). Earlier studies concerning miRNA copies in EVs have shown a similar number (Alexander et al., 2015). We have utilised a method as described previously (Simmonds, 2019). Our imaging data shows approximately 70 EVs on average being internalised and detected at 16h time-point inside the cells. However, our microscopy results do not take into consideration all the EVs that have already been internalized over the 16-hour period as

they may have already undergone recycling or degradation of CD63-GFP signals after internalization. The results do not account for the EVs that already have released the contents into the recipient cells. Sensitivity of the detection of GFP is another issue that may account for the apparent anomaly in EV-uptake and miRNA content because we may be losing the signals of many EVs presents in other focal plane. Also, some fraction of unlabelled EVs might also be internalised contributing to the EV derived miRNA pool in the recipient cells. These could answer to the apparent paradox as to why we observe a smaller number of EVs compared to the miRNA copy number to account for. Furthermore, it has been reported that EVs carry the entire miRNA processing machinery that can process precursor miRNAs into mature ones before being released into the recipient cells (Melo et al., 2014). There is always a chance that despite detecting only 2 copies of miRNA in the EVs, for instance, extra copies may be processed at the time of delivery into the cells. Another report suggests that over a period of time, the internalized EVs tend to aggregate together making it difficult in counting the exact number of EVs (Cheng et al., 2019) further reaffirming that despite visualising a smaller number of EVs, it might not be the exact representation of the number of EVs actually entering the cells with miRNAs. However, previously we had analysed the cells in 19 fields but we have increased the number of cells visualised now. The data of uptake of EVs at different time points was analysed from approximately 170 cells in different fields. The EV-entry for miRNA has been done with EVs isolated with ultracentrifugation. Therefore, we have corrected the work flow diagram in panels **New Figure1A and S1A**.

- Figure S2 A/B – The numbers of GFP positive EVs in the representative image are much greater than what is plotted in the graph. Are these cells not represented in the graphical data? Are these cells outliers? Data seems inconsistent in this figure and is also different from the uptake studies presented in figure 2 where the average EV/cell count was about 10with a large spread (up to 20), now it is about 4 with max 6 EVs/cell. Only 10 cells per condition measured. Given this variability it is hard to draw solid conclusions with siDynamin2 as the average clearly fluctuates. Additionally, data detecting miR-122 levels decreasing in the recipient cells should be accompanied by the luciferase activity assay.

We apologise for the apparent variability in our data. We had used *Imaris* software to perform the analysis of the number of EVs internalized in the recipient cells. We had previously counted the EVs by counting the number of green surfaces imaged on HeLa cells. However, upon reanalysing and repeating the experiment for larger number of cells, we found that even if the numbers increased, the result remained that the same. That is, upon siDynamin2 transfection, the uptake of CD63-GFP positive EVs decreased (**New Figure S3A and B left panel**).
We have also performed luciferase assay in recipient cells transfected with siCon or siDynamin2 and consistent to our finding of decrease in miR-122 internalization in Dynamin2 knock-down cells, we found a decrease in the activity of the miR-122 as they were not being internalized sufficiently for repression of reporter plasmid RL-perfect-122 (**New Figure S3C**).

- Figure 1A does not demonstrate an increase of miR-122 in recipient cells as stated:
"Increase of miR-122 in recipient HeLa cells, is the measure of the miRNA- transferred after the pre-treatment of HeLa cells with EVs derived from miR-122 expressing HeLa cells (Figure 1A)." The image in Figure 1A shows the association of CD63-GFP containing EVs with recipient cells. If this sentence referrers to the workflow diagram only, please label (I)and (ii) and specify in the text to avoid confusion. This sentence should also be reworded for clarity.

We thank the reviewer for this correction. We have done the needful to explain it better.

Cell fractionation and endosome imaging experiments:
- Figure 3 B – Cell fractionation is an interesting way of investigating where EVs are after internalisation. The WB shows that HRS isolates in factions 1-3 and calnexin is predominantly fractions 6-8. However, LAMP-1 is predominantly in fractions 1-2 and 58 which overlaps heavily with HRS and calnexin. No late endosome marker was used. Given that LAMP1 isolated with many fractions and there is much overlap it is hard to draw conclusions about late endosomes/lysosomes. Furthermore, the summary of this data in the results section is somewhat overstated "Fractions 2, 3 were enriched for early endosomes while fractions 4-6 represented late endosomes and lysosomes and fractions 7-9 had enrichment for ER marker protein." Finally, the WB is not aligned properly and there appears to be 9 lanes in for LAMP1 on the right-hand side with lane10 cropped out. This makes is hard to see which fractions LAMP1 is in. Later in the manuscript the authors KO Rab5a,

Rab7 and RILP as makers for different endosomal compartments, why have these not been used here as well?

We have redone the gradient analysis. This method of subcellular fractionation depicts that a particular fraction/fractions will show a higher protein level for an organelle compared to other fractions. This has been described in several of our previous publications and is very standardized method in the lab (Ghosh et al., 2015; Mukherjee et al., 2016). Thus, in this case, Lamp1 is predominantly present in fractions 4, 5 while Calnexin is found in fractions 6, 7, 8, 9. We found the early endosome markers like Rab5a, EEA1 (early endosome antigen) in the regions 2, 3 mainly while Rab7a was found almost in the fractions similar to Lamp1 (not in figure) (**New Figure 3B and C**).
As we have used pooled fractions for our experiments, to show localization of transferred miR-122, we have done Western Blot analysis for the different marker proteins in the pooled fractions as well. We have seen that EEA1, HRS are found in the fractions 2,3 indicating them to be the endosomal fractions. Lamp1 was found predominantly in the fractions 4,5,6 while Calnexin was found in both the 4,5,6 and 7,8,9 pools. Hence, as Lamp1 is detected mainly in the 4,5,6 fractions we can safely comment that this is the late endosomal/lysosomal pool while the 7,8,9 fractions which do not have Lamp1 predominantly is the ER fraction (**New Figure 3C**). It has been pointed out earlier, that the late endosomes and lysosomes make contact with the Endoplasmic Reticulum (Elbaz-Alon et al., 2020; Friedman et al., 2013; Raiborg et al., 2015a; Raiborg et al., 2015b) thus explaining the reason for presence of some Calnexin protein in the 4, 5, 6 fractions.
Thus, we can state that upon Real Time PCR between fractions (2,3), (4,5,6) and (7,8,9) we have provided a comparison between the early/late endosomes, lysosomes and ER.

- Page 11 and Figure 3F- "Measuring the effect of knockdown of these factors, we found a stronger effect of RILP depletion on internalized miRNA content." This refers to the uptake ofmiR-122 EVs, again this is measured with the relative level of miRNAassociated with cellsand not specifically taken up. If this did measure uptake, would you not expect to see decreased miR-122 in recipient cells? Why is there an increase in most cases? This is contradictory to what would be expected. Why was uptake was not measured by imaging to confirm?

As we have already mentioned in the text, the knockdown of RILP, leads to an increase in the internalized miRNA levels which is actually contradictory to the fact that the levels of miRNA internalized should decrease down the endocytic pathway. The reason for this anomaly is that, the internalized miRNA is compartmentalised and stored such that it is unable to reach the Endoplasmic Reticulum, the site of nucleation for the miRNA machinery, to repress target genes (as observed by the luciferase assay). Hence, the miRNA remains trapped in the endosomal compartment without being utilised and degraded leading to their increased levels being detected by Real Time.
We had performed microscopic analysis of the number of CD63-GFP positive EVs internalized into recipient cells either knocked down for Rab5a, Rab7a or RILP. Contrary to our finding that the levels of miRNA increase upon knockdown of RILP, we found that the internalization of EVs had a reverse data. That is, upon knockdown of the endocytic pathway proteins, there was observed an overall decrease in the number of EVs internalised in the recipient cells whether it was knocked down for Rab5a or RILP (**New Figure S4D, E**). The probable reason for the observed decrease in the EV number was an effect of transfection which could have altered the uptake process. More importantly, as we knockdown the early endosomal protein Rab5a, the endocytic pathway is hampered, blocking the uptake of the EVs. This is the similar observation for knockdown of Rab7a. However, for RILP knockdown, also a decrease in the EV internalization is observed. It has been reported earlier that knockdown of RILP delayed the trafficking of EGFR from early to late endosomes as well as prevented their degradation in lysosomes (Progida et al., 2007). Consistent with this, we have observed that due to disruption of morphology of late endosomes, the miRNAs are not degraded and hence stored away in the early endosomes. This shows that despite the faulty endocytic pathway hindering the uptake process, the internalized miRNA gets blocked such that it is not degraded in the lysosomes nor used for repression of target genes. In a recent manuscript from the lab posted in BioRxIV (**doi:** https://doi.org/10.1101/2020.12.24.424324), it has been shown the lysosomal targeting of endosome associated miRNPs is important for its repressive function although a fraction of miRNA and Ago2 in the process may get degraded in lysosomes. This may be a quality control step but exploration of that pathway is beyond the scope of the current manuscript. Similarly, here the EV-internalized miRNA after getting loaded with Ago2 possibly requires a lysosomal targeting for its target RNA interaction. This can explain why the problem with lysosomal targeting resulted in higher miRNAs/miRNPs with endosomal compartment. However, as the dynamic integration of the

internalized EV content get arrested in early to late endosomal states. it is not surprising that the total EV internalization would be affected. However, even considering a 2-fold decrease in EV internalization, the miRNA levels would increase even higher if normalized with total EV internalized! This signifies endosome maturation as the key step for functional miRNA machinery in mammalian cells. We have discussed the issue in the discussion part. We have also discussed why siRNA transfection in general could have an inhibitory effect on EV-uptake process.

- Page 11 – "In a steady state, endosome numbers were not found to significantly alter on knockdown of the endosomal proteins individually (Figure S3A-C)." The number of cells used to see if there is a difference in the total numbers of endosomes is only 15. There is a large spread of endosomal numbers detected in the control cells and therefore this result is not surprising, clearly more cells need to be analysed to conclude if there is a change in the numbers of endosomes in Rab5a, Rab7 and RILP knockout cells. As these proteins are required for endosomal trafficking it is surprising that there is no decrease in the total endosomes, looking at the data there is a decreasing trend in the Rab7 and RILP knockdown cells. Furthermore, the representative images also look as though there is a decrease in the knockdown cells.

We have increased the number to 30 cells per analysis however; the variation in the number of endosomes is statistically insignificant. New confocal images are provided (**New Figure S4A-C**). Please also see our reply to the previous comments.

- Page 13 – "Therefore a large fraction of endosome localized Ago2 is present on the outerside of the endosomes and thus sensitive to Proteinase K (Figure 4H)." How have the authors controlled for lysis of endosomes in the presence of proteinase K? By also probing with a cell surface protein or receptor which is primarily on the outer leaflet of the PM and that should be present within endosomes, the authors would control for lysis and this would clearly show that Argo2 is associated with the outer membrane of endosomes and not the inside.

This is an important concern. We have repeated the experiment using HEK293 cells and observed that apart from Ago2 protein, endosomal membrane protein HRS and EEA1also showed a decline in the protein levels when the proteinase K levels were increased to 5ng/ul (**New Figure 5H**). Furthermore, we have previously reported that these isolated vesicles are free from any organelle markers like Calnexin. However, we tried to check for the cell surface receptor, CD44, but unfortunately it was not observed to be enriched in the endosomal fractions. The ApoE protein is a lipoprotein which is endocytosed by low density lipoprotein receptors and hence should be present in the endosomes (Dekroon and Armati, 2002). We observed that this protein is protected from PK suggesting the intactness of the endosomes during the treatment to conclude on Ago2 being present on outer surface of the endosomes analysed.

- Page 13/14 – "It was found that the exosomal miRNA content in the supernatant was increased in presence of FCCP and the increase has happened in a time dependent manner (Figure 4K)." Again, how did the authors control for FCCP induced damage to endosomes? To ensure this was specific to release of EV contents other endosomal contents should also be measured in the supernatant after FCCP treatment.

We have observed by Western Blotting that after FCCP treatment, the endosomal membrane proteins are found in the pellet and not in the supernatant suggesting that the FCCP does not degrade or disrupt the membranes (**New Figure 5G**). Furthermore, Atomic Force Microscopy of these endosomes was done and we found the endosomal vesicles to have the similar appearance with pH5+/- FCCP treatment. This should suggest that the endosomal integrity is not compromised during the assay (**New Figure S5C**).

***-** Page 14 – "Western blot before and after the assay and this showed that a similar amount of endosomes was recovered with FCCP, ruling out that a change in endosomes themselves cause the changes (Figure 4I, K)." Figure 4I does not compare this and actually shows that the endosomes isolated do not contain calnexin and LAMP1 (as per figure legend. Not mentioned in results). This is very surprising when comparing with the cell fractionation data in figure 3B, while the calnexin data is consistent, LAMP1 now appears to be absent when it was strongly detected in fractions2-3*

previously. Can the authors explain this difference? Also, why are the levels of HRS and ALIX in the supernatant the same as the pellet? Figure4K does not show that there is no difference in the numbers of endosomes before and aftertreatment with FCC, the levels of ALIX and HRS appear to be variable (ALIX decreases and HRS increases?).

We thank the reviewer for bringing this issue. We have now repeated the experiments with isolated endosomes as we did Optiprep gradient analysis for Lamp1 (**New Figure 3B**). The new blots for fractionation for Lamp1 showed no enrichments in fraction 2, 3 (**New Figure 3B**). Also, we had previously shown that HRS and Alix was present both in supernatant and pellet but in blots for repeat experiments, only the pellet contains proteins specific for the endosomes (**New Figure 5B**). Therefore, we have redone the experiments and the current data is more clear and consistent. We have done additional analysis of proteins and NTA to confirm the data. We have discussed this in the relevant section of the result part. We have replaced the blots for Alix and HRS (**New Figure 5G**) for endosomes treated with or without FCCP after doing new experiment and the bands, now as they appear, are comparable. We have also performed NTA to show that the concentration and size of the endosomes does not change before and after FCCP treatment (**New Figure S5A, B**).

No data referred to in results/data included but not referred to:
Page 6 "We then further confirmed the presence of mature miR-122 in the EVs isolatedfrommiR-122 expressing donor HeLa cell." None of the data presented so far supports this statement nor does the data that follows. The authors need to directly show data demonstrating that it is the mature miR-122 in EVs not pre-miR-122.

We had performed Real Time PCR for the amount of mature miR-122 in the EVs derived from miR-122 transfected or untransfected HeLa cells. Subsequently, we also quantified the relative levels of precursor miR-122 and miR-122* in the EVs using primers specific for the precursor, miR-122 and the miR-122*. The Applied biosystem miRNA assays don't detect the pre-miR-122 as it has been shown clearly in the previous literature and company data sheet. We have used Taqman MicroRNA Assay kit to perform the Real Time PCR, these kits are sensitive only to the mature miRNAs and not the precursor forms. These kits have been known to incorporate a target-specific stem-loop reverse transcription primer which provides specificity for mature miRNAs as well as eliminates the problem of short length of mature miRNAs which inhibit the design of a random-primer followed by RT-PCR. Although some pre-miR-122 was detected in EVs, it was not found to get enriched in recipient cells, suggesting an en-route processing of pre-miR-122 before being delivered, as it has been reported elsewhere (Melo et al., 2014). This is consistent with identification of trace amount of miR-122* in EV from miR-122 expressing HeLa cells but no miR-122* in the recipient cells (**New Figure 1D-E, S1F, S1G, S1J**).

Page 7 Authors state "The miR- 122 was also not detected in recipient HeLa and was in the non-reliable detection limit in EVs derived from miR-122 expressing HeLa cells." And later in the same paragraph "No pre-miR-122 was detected in recipient HeLa cells treated with mature miR-122 positive EVs." Again, no data is referred to for these statements. The authors suggest they have data showing that EVs contain mature miR122 not in complex with dicer/TRPB but it is not presented. Figure 1C is the closest to this but does not directly show this as recipient cells are analysed not the EVs directly and they do not look at miR-122*. Please clarify/add relevant data.*

Firstly, upon Real Time PCR analysis of the recipient cells, we found no change in the precursor levels of miR-122 in cells incubated with or without miR-122 EVs (**New Figure 1E**). It may be noted that the expression level of pre-miR-122 is negligible compared to hepatic Huh7 cells as the miR-122 is a liver specific miRNA that allowed us to investigate the mechanism of miRNA transfer in a miR-122 minimal background (Lagos-Quintana et al., 2002; Landgraf et al., 2007). Furthermore, as mentioned we quantified the levels of miR-122* both in the EVs and the recipient cells. We found that this was not in the detectable region in the recipient cells while in the miR-122 positive EVs, the values were too high for reliability (**New Figure 1D left panel and S1J left panel**).
We would like to clarify that we have found recipient cell Dicer is not involved in the internalization of EV derived miR-122. We have data in the main text showing that knockdown of Dicer in the recipient cells does not affect the internalization of the miRNAs. The reason for this being, that mainly the mature form is transferred to the recipient cell while the anti-sense strand is absent and the precursor levels remain unchanged between cells treated or untreated with miR-122 positive EVs.

We can't comment on the mature miR-122 association with Ago2 in the EVs during the transport. However, recent literature question Dicer1 and Ago2 presence in the EVs (Jeppesen et al., 2019). Additionally, the mature miRNAs in recipeint cells were found to get associated with the HA-Ago2 expressed exclusively in the recipeint cells.

Figure 1C - "The mature miR-122 content was found to increase in the recipient cells treated with miR-122 containing EVs (Figure 1C)" - this data does not directly demonstrate that the EVs are inside the recipient cells because to measure the miRNA content the cells (and associated EVs) must be lysed to extract miRNA for measurements. Therefore, all EVs, including those internalised and cell surface associated will be measured. Furthermore, the word 'endogenous' appears underneath the miR-122 column in the right panel, this is misleading as miR-122 is not endogenous and is exogenously overexpressed. miR-146 is endogenous. Please clarify for this figure and 2F far RHS.

The cells were washed thrice to remove all loosely bound EVs before proceeding for RNA extraction. Even arguably a fraction of EVs may remain with the cells but that should not be the major contributor as we have a low miR-122 "internalized" value with siDynamin2 treated cells. If cell membrane adsorbed or loosely bound miRNAs are major contributors in miRNA increase detected in recipient cells, we would have seen no change in the value with siDynamin2. Further, the cell lysate analysed for Optiprep would show enrichment of miRNAs in the top fractions (1-3) (**New Figure 3D**). However, we never detected a significant amount of miR-122 in those fractions in recipient cells after EV treatment. Therefore, the contamination from non-internalized EV is minimal.
If the signal that we observe for the transferred miRNAs was from the surface bound EVs, then these external EV associated miRNAs would not be responsible for the repression of target genes like CAT-1 or RL-perfect-122 reporter as seen by Real Time PCR and luciferase respectively. Previous studies have also isolated RNA from recipient cells after sufficient washing (Valadi et al., 2007) and thus we conclude that the miRNA content increase that we found was inside the recipient cells only.

Page 9 - "Upon knockdown of Dynamin2 in the recipient cell, the internalization of miRNA was significantly reduced." What data is this in reference to?
Figure S2 (E) is not referred to in the results
We have clarified the same in the main text.

Other more minor concerns/comments:
Abstract:
Use EV after the initial abbreviated definition - change Extracellular vesicles-derived to EV derived
We have done the needful.

"Following endocytosis of miRNA-containing extracellular vesicles, loading of host cell Ago occurs on the endosomal membrane where pH-dependent membrane fusion triggers the release of internalized miRNAs to form exogenous miRNP pool." This statement is very strong unless fusion is shown release or other should be used.
We thank the reviewer for the concern, we have rectified the statement.

Figure of key findings:
Why are only the endosomal membranes depicted as double membranes? This is inconsistent. All membranes should be depicted the same way unless it is actually a double membrane as is the case for the autophagosome. No figure legend.

We have corrected the same and figure legends provided

Introduction
The English throughout needs editing to improve grammatical correctness and readability, examples: The first sentence would benefit from two commas - one after "cells" and the other after "tissues". "cells and thus considered as a very important" should read 'cells and are thus considered a very important'
"Exosomes the 40-100nm sized vesicles, positive for the marker protein CD63, and are formed during membrane invagination of late endosomes to generate multivesicular bodies(MVBs) followed by MVB fusion to cell membrane" should read 'Exosomes are vesicles 40-100 nm in size, positive for the marker protein CD63, and are formed during membrane invagination of late endosomes to generate

multivesicular bodies (MVBs).Following MVB fusion to cell membrane exosomes are released into the extracellular space." Or similar.
Extracellular vesicles defined multiple times. Use abbreviate after the first instance in the following sentences:
"Extracellular vesicles or exosomes, in particular, have"
"Transfer of functional microRNAs by exosomes or extracellular vesicles (EVs) to"
Same as above for MVB - change the following: "selectively for packaging into Multivesicular bodies (MVBs)"
"MicroRNAs are sorted selectively for packaging into Multivesicular bodies (MVBs) by
specific sets of proteins before the MVBs can fuse with cell membrane to release the EVs, packed with specific miRNAs in the intercellular space in a context dependent manner" This sentence makes it sound like the sorting and packaging is required for MVB fusion with the cell membrane which it is not or at least this has not been shown. Please change to read
"MicroRNAs are sorted selectively for packaging into MVBs by specific sets of proteins before MVBs fuse with cell membrane to release the intraluminal EVs, packed with specific miRNAs in the intercellular space in a context dependent manner" This sentence is also very long and grammatically incorrect.

We have done the changes in the main text and we thank the reviewer for these detailed insights.

Methods:
More information in this section would benefit the reader. Specifically, the luciferase activity assay used to measure the transfer of miR-122 is not fully explained and authors refer to the manufactures instructions.
In results the plasmid used to measure endosome numbers is called "YFP-Endo" and in the supplementary information described as "YFP-tagged early endosome expression plasmid" rather than saying what the protein that is YFP tagged actually is. This makes it difficult to replicate.

We have made the necessary changes.

Results:
Page 7 "The transferred miR-122 was found to cause downregulation the target messages (Figure 1B)" This is very general and not descriptive of the results. Figure1B shows a decrease in mRNA expression of 2 genes not mentioned in the results section and the use of a luciferase activity assay. Furthermore, there is no mention of what CAT1 is in the results or figure legend (the reader would have to look this up - I assume it is cationic amino acid transporter 1 (CAT1). Have the authors checked what the impact of overexpressing miR-122in HeLa cells is? Are the miR-122 donor cells compromised in any way? Are they healthy?Have they been characterised?

We have described the results as mentioned by the reviewer. Yes, we have performed TUNEL assay to show the health of miR-122 transfected HeLa cells and seen that compared to the DNase treated positive control cells, the percentage of dead cells in transfected and untransfected population was very low (**New Figure S1K**). This indicated that transfected cells are healthy.

Figure legend 1D EVs are referred to as "exosomes"
We have made the change

Figure 1B - The data for the luciferase activity assay is presented as a ratio however it
would also be good to know what percentage repression this is, still with reference to the FL activity. I imagine this is quite a small decrease in RL activity?

This has been referred in the legends. We have compared the firefly normalized RL-reporter values for recipient cells treated with control or miR-122 containing EVs (**New Figure 1G left panel**) and found that the expression of RL-perfect-miR-122 substrate decreases in presence of miR-122, compared to that of control recipient cells without miR-122, by 90%.

Page 7 "In order to revalidate the exosomal uptake of miRNA in recipient HeLa cells," the term 'exosomal' is used here instead of the more accurate term 'EV'

This has been corrected

Figure 1F - authors do not discuss all the data presented in this figure in the results when discussing the rest of the data presented in figure 1F. This is discussed later after moving to the next figure. For this reason, presenting the data here is complicating the issue and authors should consider moving the coculture Ago2 and CAT1 data to a separate section (figure 1J).

We thank the reviewer for the suggestion and have done the necessary changes to have separate figures for EV characterization, entry and mechanism now.

Page 8 - "The recipient cells were incubated with CD63-GFP positive EVs at different timepoints of 0, 4 and 16 hours and internalization of GFP-CD63 was quantified microscopically. The internalization of CD63-GFP increased with time till 16h of observation (Figure 2A,B)." Is the GFP tagged CD63 construct N- or C-terminal? CD63 contains a lysosomal targeting signal on the c-terminus so would usually be on the N-terminus - please clarify as both are written in the results.

We once again would like to apologise for this confusion. The plasmid we have used is CD63-GFP and not otherwise and hence the GFP is N-terminally located in the fusion construct.

Page 9 - "the involvement of specific proteins known to control endocytic processes were targeted." Authors only look at dynamin2 as far as I can see. Many studies have looked at the effect of blocking endocytosis (of different types) on the uptake of EVs. A range of effectors have been found to be involved, but few are required for internalisation suggesting many routes are used. Did the authors look at any other regulators of endocytosis as suggested as dynamin2 independent pathways exist?

The macropinocytic pathway utilises macropinosome to culminate into the endocytic pathway (Recouvreux and Commisso, 2017). Further evidences show that macropincytosis, clathrin dependent and independent endocytic pathway deliver contents to the early endosomes (Mayor and Pagano, 2007; Mayor et al., 2014). However, some clathrin independent pathways (like CDC42 and ARF6-regulated pathways) and the macropinocytosis pathways do not use Dynamin2 for the pinching of the internal vesicles (Doherty and McMahon, 2009; Mayor and Pagano, 2007). Thus, it is possible that the EV-associated miRNAs are either internalized by clathrin dependent pathway or Dynamin dependent but clathrin independent pathways (for instance caveolar dependent pathways). We discussed this in the main text.
We have used inhibitors for endocytosis to see the uptake of miR-122 via EVs. We have used some inhibitors that have been previously used by some reports (Costa Verdera et al., 2017). We have seen that on treatment of recipient cells with EIPA, a macropinocytosis inhibitor, there was a decrease in the internalization of miRNA content. Similarly, upon treatment with Genistein (a clathrin independent pathway inhibitor) as well as Chlorpromazine (a clathrin dependent pathway inhibitor), we found a decrease in the uptake of miR-122 suggesting that macropinocytosis, clathrin dependent and clathrin independent pathways can facilitate the EV internalization process for the HeLa cells (**New Figure S3D**).

Page 10 - "Therefore fitness of ER controls the internalization and functioning of the EV derived miR-122 in human cells." This is novel and interesting finding, however the data for this is buried in the supplementary data and this has only been measured by looking at the levels of miR-122 in recipient cells. The authors have not looked at the EV uptake by microscopy nor have they assessed the functional miR-122 delivery using their luciferase activity assay. ER stress induction is shown by blotting for eIF2alpha phosphorylation which has increased slightly, they could have also assessed BIP to further demonstrate stress induction.

We have performed luciferase assay to show the repression of reporters in the presence of Thapsigargin and observed that consistent with the uptake result, the level of repression decreased as the uptake of EV derived miRNA is low in Thapsigargin treated cells. We have also performed Western Blot for BiP protein for the ER stressed cells (**New Figure S3F, G**).

Figure 3F - miR-122 referred to as endogenous where should be exogenously expressed

Corrected

Figure 4 – siRNA cell fractionation shows an increase of miR-122 in the early endosomal fractions. It did the authors also do the same cell fractionation after Bafilomycin treatment? It would have further strengthened this data if the result was similar.

We have performed similar fractionation studies for recipient cells treated with Bafilomycin and observed that the transferred miR-122 levels do increase in the fractions 2,3 compared to the DMSO treated cells (**New Figure 4H**). This data does revalidate our findings of differential localization of miR-122 upon RILP knockdown in recipient cells.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this study Ghoshal et al investigated Extracellular Vesicle-mediated exchange of miRNA between donor and acceptor cells. They propose that miRNA loaded within EV are delivered within acceptor cells after 1) endocytosis 2) pH-dependent fusion with the endosomal membrane. This seems to be a pre-requisite for a DICER-independent miRNA loading with Ago at the cytosolic face of the endosomal membrane, possibly associated with endoplasmic reticulum membranes. Importantly, delivered miRNAs are functional.

We thank the referee for his thoughtful comments and insight. We have tried to address the concerns as below.

Reviewer 3 Comments for the Author:
The study is ambitious and tries to address several key questions related to EV-content, including miRNA, delivery within the acceptor cells. This is interesting; however, the initial enthusiasm was severely tempered by several concerns:
First, results demonstrating EV-mediated delivery of functional miRNAs are strong and it seems convincing that delivery requires endocytosis and pH-acidification. This is consistent with recent papers that should be cited 1) Joshi BS, de Beer MA, Giepmans BNG, Zuhorn IS. Endocytosis of Extracellular Vesicles and Release of Their Cargo from Endosomes. ACS Nano. 2020 Apr 28;14(4):4444-4455. doi: 10.1021/acsnano.9b10033. Epub 2020 Apr 16. PMID: 32282185; PMCID: PMC7199215. 2) Bonsergent E, Lavieu G. Content release of extracellular vesicles in a cell-free extract. FEBS Lett. 2019 Aug;593(15):1983-1992. doi: 10.1002/1873-3468.13472. Epub 2019 Jun 17. PMID: 31175663°.
We thank the reviewer for the insightful information. We have done the needful in the main text to incorporate these references.
However, results dealing with the main location of 1) delivered miRNA and 2) ARGO association are less convincing. How can the authors rule out that miRNAs are not delivered within the cytosol of the acceptor cells and then immediately processed for ARGO association? The Authors decided to start with complex organelle separation, that are not always very convincing (for instance in fig3 B lysosomal and ER markers are within the same fractions (6-9) which prevents clear interpretation). It seems more logical to first separate membrane from cytosolic fractions (using mechanical disruption of the acceptor cells after EV feeding) and test whereas miRNA 122 is more predominantly found in cytosolic or in membrane fractions. Then they could assess if BAf treatment prevents cytosolic release. Similarly, they should question where miRNA/ARNO association is predominantly found. If they rule out that miRNA is never found in cytosol (which would be very surprising) then it will make sense to identify with which organelles miRNAs are associated and processed with ARGO.
This is a very interesting question raised by the reviewer. We have provided evidences where the miRNA does not seem to be delivered directly into the cytosol. Firstly, we have shown that the internalization of miRNAs requires Dynamin2. The dependence on Dynamin2 indirectly shows that the EVs are internalized into vesicles and not into the cytosol by fusion (**New Figure S3A-C**). Secondly, the involvement of the endocytic pathway protein like RILP in the internalization and activity of EV derived miRNA, also hints upon the fact that the miRNAs are not simply delivered into the cytosol but rather the endocytic pathway is instrumental in the internalization process.
However, to address this interesting question directly, we have performed a digitonin fractionation which helps to separate the cytosolic and membrane fractions of cells. The recipient cells treated with EV containing miR-122 were subjected to digitonin fractionation to see the localization of miRNA in cytosol and membranes. We have used liposome mediated delivery of synthetic single stranded miR-122 as a control. Upon either EV treatment or liposomal delivery of miR-122, we found that majority of the miRNA localized in the membrane fraction than in the cytosolic fraction (**New Figure S3K**). Western blot analysis revealed that the membrane fractions were enriched in Calnexin (a membrane marker) and low on GAPDH levels while the cytosolic fractions had no Calnexin protein

but high levels of GAPDH (**New Figure S3L**). This experiment further strengthens our data of localization of miRNA as well its Ago2 association on the Endoplasmic Reticular membranes.
We went on to perform a digitonin fractionation on recipient cells pre-treated with DMSO or Bafilomycin and found that while there was no change in the cytosolic levels of miR-122 between DMSO and Bafilomycin, the membrane levels of miR-122 increased in Bafilomycin treated cells (**New Figure 4G**). This is consistent with other data and strengthens the data that like knockdown of RILP, Bafilomycin acts in a similar manner to compartmentalise the miRNA in the membranous part of the recipient cells.

Another general comment is that many controls are missing.
We have worked hard to address this important concern. Please see the revised panels.

Some examples:
a) FigS1 C, they show only two markers within the EVs; Cell lysate should be tested in parallel. The authors did not test for usual negative markers for EVs (ER or Golgi markers for instance); in addition, CD63 shows a very unusual profile. In all established publications, CD63 runs as a smear due to the presence of heterogeneous glycan chains.
We have provided Western Blots for the different EV markers in cells as well as EVs. The EVs, isolated from control and miR-122 transfected HeLa cells, are positive for Alix, HRS, Flotilin and negative for ER marker Calnexin, lysosomal marker Lamp1. Cytochrome C as well as GAPDH was also found to be absent in the EVs (**New Figure S1E**). We have used the same antibody used in two established papers (Basu and Bhattacharyya, 2014; Mukherjee et al., 2016) and hence observed same single thick bands for CD63 that were observed there also. This is not surprising as the CD63 should be in matured state of glycosylation for it to be localized to EV membrane rather the heterogeneously glycosylated forms that are present in the cell. This may be reason why we have no detection of CD63 in the cell lysate due to lower concentration of matured glycosylated form in cell extract.

b) FIgS1 E the authors tested the resistance to DNase but omit to test resistance to RNAse. Topology of EV-associated miRNAs is still controversial and the authors should demonstrate that miRNAs are indeed within the EVs (protected from RNAse except in the presence of detergent).
We appreciate the concern raised by the reviewer. The localization of RNAs have been usually observed to be inside the EVs (Del Pozo-Acebo et al., 2021; Fabbiano et al., 2020; Shurtleff et al., 2017; Valadi et al., 2007). It has been observed that upon treating EVs with RNase and Triton X-100 together, the EV associated RNAs undergo degradation due to disruption of the EV membranes. Likewise, we have also demonstrated that the miRNAs localize inside the EVs and are protected from RNase treatment, unless the EVs isolated from miR-122 expressing HeLa cells were subjected to sonication followed by RNase A treatment. This caused a decrease in miR-122 compared to the EVs which were not sonicated but treated with RNase A alone (**New Figure S1I**). This suggests miRNAs are within EVs and that sonication, like detergents, ruptured the EVs leading to the miRNAs being exposed to RNase A leading to their degradation.

c) Figure 4 H: the authors only tested proteinase K resistance of Ago2, they should add markers that are known to be on the cytosolic face of endosome (positive control) and the luminal face of the endosomes (negative control) that should be proteinase K resistant. In addition, the samples should be tested for other organelles markers to establish lack of contaminations. Without all these controls, experiments are not validated and it is impossible to rigorously conclude.
We have repeated the experiment using HEK293 cells and observed that apart from Ago2 protein, endosomal membrane protein EEA1, HRS also showed a decline in the protein levels when the proteinase K levels were used as low as 5ng/ul (**New Figure 5H**). We have done Western Blot to detect PK resistance of the ApoE lipoprotein which is supposed to be in the matrix of endosomes.

-Presentation of the data are not always linear and the logic of the order is not clear. In several occasions, there is a back-and- forth reference of data throughout the manuscript. This greatly perturbs the reading and the comprehension of the paper. For example, internalization of GFP-CD63 is showed in Fig1 but the again in Fig2B where it is also quantified. This make the paper hard to follow.
We apologise for the confusion; we have tried to rectify the error and having the justified logical flow of the manuscript as appropriate by reorganizing the figures.

-Some results are not mentioned in the core manuscript and it is therefore not clear why they were performed. One example: Figure 1C, why did they use miR146. If this is a control, why is it only a one-shot control and not a systematic one.

We would again like to apologise for our inability to communicate the reason for using miR-146a in the aforementioned experiment. The miR-146a was used to show that apart from miR-122 being transferred by EVs, other miRNAs can be transferred in the same manner. For this we isolated EVs from donor cells transfected with miR-122 or miR-146a mimic and added them to recipient cells. Consistent with our data of miR-122 uptake, we found that miR-146a also was internalised by the recipient HeLa cells. We had to pick miR-146a that like miR-122 is not expressed in recipient cells, in this case HeLa cells, in order to enable us to monitor the trafficking process. This was required to show the common way in which different miRNAs can be transferred. In later experiments we concentrated on use of miR-122 to explore the mechanistic detail of this transfer process.

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Second decision letter

MS ID#: JOCES/2020/253914

MS TITLE: Non-Canonical Ago Loading of EV-Derived Exogenous Single Stranded miRNA in Recipient Cells

AUTHORS: Bartika Ghoshal, Edouard Bertrand, and Suvendra N Bhattacharyya
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers recognize that most of their initial criticisms have been addressed in your revised manuscript. However, reviewer #2 still raised issues that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have successfully improved the writing of the manuscript to make it clear. The additional experiments are in line with the presented hypothesis and conclusions.

Comments for the author

I don't have additional open points other than some more proof-reading and correcting wording.

Reviewer 2

Advance summary and potential significance to field

This manuscript presents novel and interesting insights into the delivery of miRNA from extracellular vesicles (EV) to recipient cells. This is of great interest to the wider cell biology community, including EV researchers. This manuscript, in my opinion, is now suitable for publication in JCS.

Comments for the author

The authors have provided a substantial amount of data to address the concerns I, and other reviewers, had in the previous version. The manuscript has also been edited to make it easier to read and understand. These changes have improved the manuscript significantly and I believe that this is now suitable for publication in JCS. I thank the authors for their extensive changes and numerous additional experiments, I appreciate the amount of work that was required.

I have a few minor comments that should be addressed before final publication if accepted:
The western blotting characterisation of EVs:
- CD63 usually appears as a smear between 30-60 kDa due to the various glycoforms. This is not the case here and the blot is cropped with the bands on an angle. I believe that this was also commented on by other reviewers. The authors suggest that this is because CD63 is in a mature form, however there are numerous publications showing that CD63 is a smear when detected in EVs from HeLa cells. I would suggest that CD63 presenting here as a single band is more likely due to the specific antibody used.

- GAPDH appears 3 times. I assume this is because there are several blots used to probe for all these markers – this should be stated in the figure legend.

Page 6. “Transfection don’t induce apoptosis” should read Transfection didn’t induce apoptosis

Page 8. There is a question in the text that should not be there or needs to be re-written as a statement.

Page 13 and Fig 3/S4: The siRNA studies on Rab5A, Rab7 and RILP are somewhat contradictory, and this is discussed briefly in the results, but this could use further clarification. The authors state that there are less EVs detected per cell due to uptake being blocked but is it more complex than this. If uptake is blocked, then there should be less detectable miR-122 in the KD cells as it is not taken up as efficiently – this is the opposite to their findings. Interestingly the number of EVs per cell are similar in all siRNA conditions but this is not reflected in the relative levels or repressive activity of miR-122. Therefore, I agree that due to perturbations in the endosomal pathway the progression of miR-122 delivery has changed and perhaps in siRILP (and siRab5A&7) the EV miR-122 is sequestered in an altered endosomal compartment. This would explain the increased level of miR-122 and decreased repression activity. However, I’m unclear about how there can be an increase of Argo2 associated miR-122 in under these conditions. Please comment.
Also, in S4 the quantification of EVs per cell and the representative images seem contradictory also and there appears to be more EVs in the siRILP cells than siCon or siRab5A/7.

Reviewer 3

Advance summary and potential significance to field

the authors satisfyingly answered reviewer's previous comments

Comments for the author

the authors satisfyingly answered reviewer's previous comments

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Second revision

Author response to reviewers' comments

Response to Reviewers
Reviewer 1 Advance summary and potential significance to field
The authors have successfully improved the writing of the manuscript to make it clear. The additional experiments are in line with the presented hypothesis and conclusions.

Reviewer 1 Comments for the author
I don't have additional open points other than some more proof-reading and correcting wording. We thank the reviewer for the appreciation and suggestion that are taken into consideration.

Reviewer 2 Advance summary and potential significance to field
This manuscript presents novel and interesting insights into the delivery of miRNA from extracellular vesicles (EV) to recipient cells. This is of great interest to the wider cell biology community, including EV researchers. This manuscript, in my opinion, is now suitable for publication in JCS.

Reviewer 2 Comments for the author
The authors have provided a substantial amount of data to address the concerns I, and other reviewers, had in the previous version. The manuscript has also been edited to make it easier to read and understand. These changes have improved the manuscript significantly and I believe that

this is now suitable for publication in JCS. I thank the authors for their extensive changes and numerous additional experiments, I appreciate the amount of work that was required.
We thank the reviewer for the kind words and appreciation.

I have a few minor comments that should be addressed before final publication if accepted:
The western blotting characterisation of EVs:
-CD63 usually appears as a smear between 30-60 kDa due to the various glycoforms. This is not the case here and the blot is cropped with the bands on an angle. I believe that this was also commented on by other reviewers. The authors suggest that this is because CD63 is in a mature form, however there are numerous publications showing that CD63 is a smear when detected in EVs from HeLa cells. I would suggest that CD63 presenting here as a single band is more likely due to the specific antibody used.
The CD63 antibody we have used detected a single band of the protein. There are many reports which show a smeary appearance of CD63 in western blots. However, earlier reports from our lab have also shown similar single bands (Basu and Bhattacharyya, 2014; Mukherjee et al., 2016) for CD63. Other independent studies have also detected single bands for CD63 (Cossetti et al., 2014; Kosaka et al., 2010; Sento et al., 2016; Zhu et al., 2014). We agree with the reviewer that this occurs presumably due to the antibody used in these studies. We mentioned that in western blot description in the Material and Method section.

-GAPDH appears 3 times. I assume this is because there are several blots used to probe for all these markers – this should be stated in the figure legend.
We have mentioned this in the figure legends.

Page 6. "Transfection don't induce apoptosis" should read Transfection didn't induce apoptosis
We have changed this in the main text

Page 8. There is a question in the text that should not be there or needs to be re-written as a statement.
We have changed this in the main text

Page 13 and Fig 3/S4: The siRNA studies on Rab5A, Rab7 and RILP are somewhat contradictory, and this is discussed briefly in the results, but this could use further clarification. The authors state that there are less EVs detected per cell due to uptake being blocked but is it more complex than this. If uptake is blocked, then there should be less detectable miR-122 in the KD cells as it is not taken up as efficiently – this is the opposite to their findings. Interestingly the number of EVs per cell are similar in all siRNA conditions but this is not reflected in the relative levels or repressive activity of miR-122. Therefore, I agree that due to perturbations in the endosomal pathway the progression of miR-122 delivery has changed and perhaps in siRILP (and siRab5A&7) the EV miR-122 is sequestered in an altered endosomal compartment. This would explain the increased level of miR-122 and decreased repression activity. However, I'm unclear about how there can be an increase of Argo2 associated miR-122 in under these conditions. Please comment.
Despite the number of internalized EVs being lower than the control, the levels of EV-miR-122 inside the cells were found to be increased. One reason for this apparent anomaly is the faulty endosomal pathway for which the miRNA is trapped with early/late endomes, away from its target due to the damage in early and late endosmal sorting. TargetmRNA-miRNA interaction can lead to degradation of the respective miRNAs (Bitetti et al., 2018; de la Mata et al., 2015; Ghini et al., 2018). Thus, with no interaction between the miRNA-mRNA, more miRNAs should accumulate in the cells transfected with siRNAs against Rab5, Rab7 or RILP compared to control. However, this accumulation appears to be highest in case of siRILP treated cells than the others. Recently it has been observed that RILP is essential for lysomal targeting of endosome associated Ago2 miRNPs for its re-targeting to mRNAs on ER and thus mRNA-miRNA interaction and miRNA turnover (bioRxiv 2021.02.11.430878). Therefore the effect with siRILP is even robust. We have included a short discussion on that in the main text on that.
Also, as the endosomes contain Ago2 on their membranes, the internalized miRNA upon release from the EV membranes binds to these Ago2 before finally reach the ER at a steady state for target RNA repression. Thus, upon blocking of the endosomal pathway, the released miRNA could bind to Ago2 but cannot get relocalized any further in cells treated with siRNAs specific to Rab5, Rab7 or RILP.

Also, in S4 the quantification of EVs per cell and the representative images seem contradictory also and there appears to be more EVs in the siRILP cells than siCon or siRab5A/7.
We have replaced the image with an more representative image to justify the statistical results.

Reviewer 3 Advance summary and potential significance to field
the authors satisfyingly answered reviewer's previous comments
Reviewer 3 Comments for the author
the authors satisfyingly answered reviewer's previous comments
We thank the reviewer for the kind comments.

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Third decision letter

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MS TITLE: Non-Canonical Ago Loading of EV-Derived Exogenous Single Stranded miRNA in Recipient Cells

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.