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A novel long intergenic noncoding RNA indispensable for further cleavage of mouse two-cell embryos

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Transaction Report:

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

1st Editorial Decision 29 February 2016

Thank you for the submission of your manuscript to EMBO reports. I am sorry for the slight delay in the decision process; we have now received the full set of referee reports as well as referee cross-comments pasted below.

As you will see, all referees acknowledge that the findings are interesting and novel. However, they also all point out that the description of the experiments, data presentation, (over)interpretations and the language must be improved and that a number of controls, quantifications and statistical analyses are missing. These types of concerns must be addressed throughout the manuscript, and full gels should be shown for all data (the full gels can also be added as source data, see below). Referees 1 and 3 further think that complex formation of lincGET with hnRNP U and ILF2 and the interaction of lincGET with Carm1 should be confirmed and also further analyzed in terms of their functional relevance. Referee 1 suggests in her/his cross-comments that it should either be investigated whether the transcriptional and splicing events depend on any of the proteins identified as interactors (hn RNP U, ILF2, Srsf1 or Carm1) OR whether Carm1 has a role in the lincGET phenotype and whether lincGET has a role in Carm1-mediated lineage allocation (in case this can be investigated, see cross-comments by referee 2 below). It should also be determined whether lincGET depletion affects the expression of MAPK target genes and Carm1.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns, as indicated above and in their reports, must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete

point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

This manuscript by Wang and colleagues demonstrates for the first time a functional role for a long intergenic non-coding RNA in mouse preimplantation development. More particularly this lincRNA is MERV-associated and required for 2-cell onward development, a potentially significant insight considering the intimate involvement of MERV-associated transcription with the 2-cell stage and totipotency. For this reason the novelty and thus potential impact of this manuscript is high. It is anticipated that many more lncRNAs will be found to play important roles during developmental processes, including preimplantation development.
However, the manuscript suffers from poor communication of the results. My general points are as follows and should be corrected before publication:
1. The English is of a very poor standard. It is often difficult to understand the point the authors are trying to make. This should be corrected throughout.
2. The Figure legends are too brief. It is not always straightforward to understand the experimental design and thus a correct interpretation of the results is made more difficult and time-consuming. For example, often only the conclusion of the figure is mentioned, without any description of experimental approach or description of the figure itself. Furthermore N numbers (including experimental replicates as well as technical replicates) and statistics (where appropriate) should be included throughout. If the authors need more space, perhaps the materials and methods could be moved to the Supplementary Material?
3. The manuscript is made more difficult to follow by the inclusion of both 'EV' and 'S' additional

figures. Can these not be incorporated into a single file?
4. Is the word cognate used correctly here? Throughout the manuscript the authors refer to 'ERV cognate lincRNA'. What does this mean? I have not heard of this usage before. Cognate usually refers to the binding of a factor to its appropriate site, not just DNA or RNA sequences that are spatially associated. Do the authors have any evidence that the lincRNAs are binding to DNA? The term 'cognation' is even used at one point (page 5, paragraph 3), which I cannot say I have ever heard used before.

Specific comments
Figure 1.
The characterisation of LincGet is extensive and thorough, particularly the sequencing of the Northern blot result. However one point I don't understand. On page 6, the authors mention that 'we designed probes in the unique regions of lincGET andDyei and sequenced the TM-qPCR products. As a result there were no lincGET or Dyei-like sequences, suggesting the reliability of TM-qPCR.' Are these regions spliced out? Why would they not expect to see any product here? The Diagrams of the loci (in Figure 1A and EV1B) or text do not explain the reason sufficiently.
The authors mention that the other lincRNAs are expressed weakly or in random patterns (page 5 paragraph 2). This is not the case. Many, show interesting expression patterns. They merely need to say that lincGET was the most 2-cell and 4-cell specific. For example Loca1 shows an interesting 2-cell to 8-cell expression pattern that is neither weak not random. Moreover Dyei is barely expressed in the 2-cell stage. It is wrong to mention (same paragraph) that Dyei is 'highly expressed in 2- to 4-cell stage mouse embryos.

Figure 2.
The authors mention that knockdown with siRNA was not successful. Did they try dsRNA? If this is successful to induce knockdown of lincGET, a developmental experiment using this as an alternative method of knockdown should be included as LNAs can have other effects, such as preventing binding to DNA/proteins. This would significantly strengthen the manuscript.
Have they excluded that the LNA prevents reverse transcription/amplification of the lincGET RNA by RT-qPCR? If not, then knockdown cannot be concluded, the LNA could be just blocking binding of the RNA, which changes the interpretation of the results.
2A Is not referred to in the text.
2B What is the timing of this experiment?
2D. Embryos in LNA groups look identical to wild-type groups. Therefore this Figure only shows that embryos are the same as wild-type up to and including S phase. Only in conjunction with the phenotype this suggests G2 phase block (they cannot conclude a G2 block). From the BrdU staining it is not clear that the embryos have exited S phase. Can do a short pulse of EdU at say 46h to confirm this (with BrdU as before as positive control). Additionally, S10ph is not necessarily a specific marker of G2/M - this is currently controversial. They should use Cylcin B1 as a G2 marker in addition.
How do arrested embryos look - are they still replicating? Are they still S10ph positive?
Hoeschst staining is mentioned in the text, but PI is used in the Figure.
Figure 2H should be enlarged

Figure 3
3C - Statistics should be included.
3E - The images are not clear, unmerged panels should be included. The decreased level of pP38 in particular is not clear.

Figure 4
4A. Should mention in text or figure legend the lysate for RNA-pulldown was from 4-cell stage embryos.
4C. The authors mention that ILF2 is present in the nuclei of normal late 2-cell embryos but this is not apparent in the Figure. Unmerged images should be shown.
The authors mention in the text that ILF2 and hnRNP U are 'colocalising with lincGET' This is completely overstated. All they can say is that both are present in the nucleus at the 4-cell stage only.
4D. The, although hypothetical is not helpful here. It doesn't even explain why in LincGet knockdown (LNA) embryos there is an increase in hnRNP U and ILF2 protein.

Figure 5
5B. The statistics are insufficiently presented. What test was used? Which results are statistically significantly different?
5C and 5H there is no negative control here.
The Srsf1 and Carm1 data is weak, and almost included as an afterthought. Perhaps Srsf1 and Carm1 could be included in Figure 4 alongside the interaction with hnRNP U and ILF2, to strengthen the weak Figure 4.
Are the transcriptional and splicing events depicted in Figure 5 dependent on any of the protein identified as interactors (hn RNP U, ILF2, Srsf1 or Carm1). If so, this would substantially strengthen the end of the manuscript, which does let down the first half of the manuscript somewhat.

EV
Figure 5G full image looks different to the cropped image in the main text.

Discussion
page 13 The authors mention that LincGET could function as an inhibitor of transcription. There is no evidence for such a direct role - upregualted genes after knockdown (LNA) in Figure 3A could easily be just indirect effects.
page 13 they hypothesize that binding with LincGET may activate or improve the splicing activity of ILF2, hnRNP U and Carm1 because they increased at the protein level when LincGET was depleted. Firstly Carm1 has not been shown to increase. Even if they do increase upon knockdown, this does not suggest in any way that LincGET may activate or improve their splicing activity? Third, the reference here is Fig. 3G - it should be 4C.

Referee #2:

Interesting report describing novel and likely important phenomenon regarding control of early mammalian development. The authors should also be commended for the extraordinary amount of work involved and for the depth of the analysis. It would be a pity if the report is published in its present form since extremely poor writing will detract any reader from the importance of the message. I checked some published papers from this group and they are certainly capable of expressing themselves in acceptable scientific English. Either the authors or the journal will have to find an editor capable of making this paper legible.

Specific comments:

1. Page 3, line 4. There are many excellent reviews describing lineage allocation in preimplantation mouse embryos but Bischoff et al is not one of them.
2. Page 3, lines 5-6. Fist description of retrotransposons expression and discussion about their possible function in preimplantation embryos is by Peaston et al., Dev. Cell 7, 597, 2004. and Evsikov et al., Cytogen. Genome Res., 105, 240,2004.
3. Page 5, line 20, page 27, line 8 and Figure 1C. there is no * marking on the figure.
4. Page 7, line 10. Fig. 3A should be Fig. 2A
5. Page 8, line 8. Another inappropriate reference.
6. Page 9, lines 25-28. Data presented in Figure 4C (also in Figure 2D) cannot be interpreted as co-localization. IF demonstrate the presence of antigens in question in the nucleus and that is all. To establish co-localization, the authors must stain the same embryo with two different antibodies and provide much better resolution.
7. Page 12, line 19. References numbered 68 and 69 describe isolation of mouse ES cells and have nothing to do with "segregation of ICM and trophectoderm".
8. Page 12, line 22. See comment #2.
9. Page 13, line 3. See comment #2.
10. Page 13, line 26. There is no Fig. 3G
11. Pages 14-21. Methods are usually presented in passive voice as something that happened in the past and not in the command mode of a cookbook. Please rewrite.
12. Page 18, line 7. How long were the embryos cultured in the presence of BrdU? Or EU (line 23, same page)?
13. Reference 70, volume, pages?

Referee #3:

This is an interesting manuscript that identifies a long non coding RNA, lincGET, that has a key function in the early embryo in so much that its depletion leads to arrest at the 2-cell stage. Interestingly, loss of lincGET does not affect zygotic gene activation. Instead the authors present somewhat circumstantial evidence to suggest that lincGET might regulate the MAP kinase pathway, and a number of other genes by regulating alternative splicing pathways. These genes include regulators of cell cycle progression such as Cdk1. There is the essence of a very interesting story here. The difficulty with the paper is that none of the experiments described go far enough to really be totally confident of the interpretation given. Further work is required before the paper is ready for publication.

The data describing the complex formed by the lincGET is particularly scant. The authors claim to have identified hnRNP U and ILF2 as lincGET-associated proteins. However they should present all of their data - full gels including Pnceau stained gels and full lists of proteins identified by MS (Fig. 4A). This also applies to the proteins identified by IP (Fig. 4B) as this data is not convincing as shown. Moreover, few conclusions can be drawn form the immunostaining experiments presented in Figure 4C that show the nuclear localisation of these proteins and their apparent increase upon lincGET depletion.

The above comments are equally applicable to the pull down experiments that claim to show an association with SRSF1. The authors should provide the complete gels with a full set of control experiments. Little can be concluded from the data as presented - the reader has to take the authors' statement in good faith. The authors should also provide evidence that Srsf1 still interacts with hnRNP U or ILF2 upon lincGET depletion.

The relationship between lincGET and Carm1 is not demonstrated satisfactorily. One can conclude little from the data presented in Figure 5H. This is particularly important as the authors would like to make a great deal from this potential interaction given the role that CARM1 has been demonstrated to have in early development. The controls for the pull-down and IP experiments should also be provided to check the specificity of IgG or GST-beads binding to proteins. Ponceau staining (to give loading controls) should also be attached for all GST-Pulldown and co-IP experiments. This potential interaction should also be explored further. The authors should investigate the consequences of lincGET knockdown and CARM1 overexpression and determine whether CARM1 inhibition affects the lincGET-CARM1 interaction / function.

Other points
A better control for the RNA FISH experiment would be the sense strand rather than random sequence.

Fig. 3D and E show that MAPK pathway is less activated upon lincGET depletion. However there is also less of total forms of ERK1/2 and p38 explaining the lower levels of pERK1/2 and p38 and how MAPK activation might be affected.
The authors should determine whether lincGET depletion affects expression of MAPK regulated target genes.

Page 7 (LincGET depletion results in developmental arrest at late G2 phase of 2-cell stage) - figure number is 3A, should be 2A.

Fig. 4C - quantification of the fluorescence intensity is missing (hnRNP U and ILF2).

Cross-comments by referee 1:

I agree with referee 3 that the data on lincGET complex formation is weak, as I mention in my review. This part of the manuscript, is experimentally the weakest section and significantly brings down the impact of the manuscript as a whole. Thus, further details regarding the experiments the authors performed to identify this complex would be helpful as suggested by Referee 3. A better

characterization of the complex would enable a reorganisation of Figures 4 and 5 to bring in the data on Srsf1 and Carm1 into the complex characterisation. As I mention and as suggested by referee 3, the data on Carm1 currently is insufficiently presented to be able to draw any conclusions. The controls suggested by Referee 3 are essential.

Also functional relationships between the proteins identified in the complex would be helpful, such as my previous suggestions:

'Are the transcriptional and splicing events depicted in Figure 5 dependent on any of the protein identified as interactors (hn RNP U, ILF2, Srsf1 or Carm1)' At present the authors assume the functional roles of these proteins in the embryo, but do not demonstrate a role in transcription or splicing in the context of the lincGET phenotype.

Alternatively the authors could explore the interaction with Carm1 more extensively from a functional point of view, as suggested by Referee 3. The authors should check for any role of Carm1 in the lincGET phenotype they observe and in contrast, whether lincGET has any role in the ascribed role of Carm1 in lineage allocation (e.g. knockdown lincGET in 1 blastomere at the 2-cell stage and determine if this affects lineage allocation).

On balance I feel that exploring functionally either route; the hn RNP U and ILF2 or Carm1 interactions would be necessary. Controls for the interactions/complex formation as suggested by Referee 3 would also be essential.

Cross comments by referee 2 on referee 3's report:

Srsf interaction with hnRNP U or ILF2 can be examined though it is entirely possible and compatible with the model that the interaction is indirect and mediated by lincGET. If that is the case co-immunoprecipitation experiments will be negative

I am not quite sure what is the Reviewer 3 asking for. The authors could do the same as for the other genes, deplete lincGET and see what happens with CARM1. Overexpression of CARM1 supposedly influences the fate of the early blastomeres and it would be interesting to see if this is somehow mediated by lincGET, except for the fact that lincGET depleted embryos arrest before any fate determination takes place. The authors show that CARM1 is present in lincGET pulldown protein complex but they certainly did not show that any exon skipping takes place in early embryos and that this causes developmental arrest. They should tone down penultimate paragraph of the Results (page 11) and last paragraph of the Discussion.

1st Revision - authors' response 09 June 2016

We would like to thank you for your interest in our manuscript and for providing us an opportunity to revise it. We appreciate the reviewers' careful and thoughtful suggestions, which helped us improve our manuscript. As requested, we revised our manuscript and included additional content in response to the reviewers' comments. Our point-by-point response to the reviewers' comments can be found below.

POINT-BY-POINT RESPONSE

Editor's comment 1: As you will see, all referees acknowledge that the findings are interesting and novel. However, they also all point out that the description of the experiments, data presentation, (over)interpretations and the language must be improved and that a number of controls, quantifications and statistical analyses are missing. These types of concerns must be addressed throughout the manuscript, and full gels should be shown for all data (the full gels can also be added as source data, see below). Referees 1 and 3 further think that complex formation of *LincGET* with hnRNP U and ILF2 and the interaction of *LincGET* with Carm1 should be confirmed and also further analyzed in terms of their functional relevance. Referee 1 suggests in her/his cross-comments that it should either be investigated whether the transcriptional and splicing events depend on any of the proteins identified as interactors (hn RNP U, ILF2, Srsf1 or Carm1) OR whether Carm1 has a

role in the *LincGET* phenotype and whether
LincGET has a role in Carm1-mediated lineage allocation (in case this can be investigated, see cross-comments by referee 2 below). It should also be determined whether *LincGET* depletion affects the expression of MAPK target genes and Carm1.

Response: As requested, we revised our manuscript and included additional content based on the reviewers' comments.

Editor's comment 2: Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

Response: As suggested, the number "n" for how many independent experiments were performed, the bars, and error bars as well as the test used to perform the statistical analyses are now provided in the figure legends. Scale bars are also included for microscopy images.

Referee #1:

This manuscript by Wang and colleagues demonstrates for the first time a functional role for a long intergenic non-coding RNA in mouse preimplantation development. More particularly this lincRNA is MERV-associated and required for 2-cell onward development, a potentially significant insight considering the intimate involvement of MERV-associated transcription with the 2-cell stage and totipotency. For this reason the novelty and thus potential impact of this manuscript is high. It is anticipated that many more lncRNAs will be found to play important roles during developmental processes, including preimplantation development.
However, the manuscript suffers from poor communication of the results. My general points are as follows and should be corrected before publication:

Q1. The English is of a very poor standard. It is often difficult to understand the point the authors are trying to make. This should be corrected throughout.

Response: As suggested, we carefully edited our manuscript. In addition, the revised manuscript was edited by a professional scientific editor.

Q2. The Figure legends are too brief. It is not always straightforward to understand the experimental design and thus a correct interpretation of the results is made more difficult and time-consuming. For example, often only the conclusion of the figure is mentioned, without any description of experimental approach or description of the figure itself. Furthermore N numbers (including experimental replicates as well as technical replicates) and statistics (where appropriate) should be included throughout. If the authors need more space, perhaps the materials and methods could be moved to the Supplementary Material?
Response: As suggested, we modified the Figure legends to improve clarity by adding information regarding the experimental approach and a description of the figures **(lines 892-1234)**. In addition, the numbers of experimental replicates and embryos used in some assays have been included.

Q3. The manuscript is made more difficult to follow by the inclusion of both 'EV' and 'S' additional figures. Can these not be incorporated into a single file?
Response: While we understand the reviewer's concern, the terms "EV" and "S" are used as per the journal formatting guidelines. Unfortunately, EV and S figures cannot be incorporated in a single file.

Q4. Is the word cognate used correctly here? Throughout the manuscript the authors refer to 'ERV cognate lincRNA'. What does this mean? I have not heard of this usage before. Cognate usually refers to the binding of a factor to its appropriate site, not just DNA or RNA sequences that are spatially associated. Do the authors have any evidence that the lincRNAs are binding to DNA? The

term 'cognation' is even used at one point (page 5, paragraph 3), which I cannot say I have ever heard used before.
Response: As pointed out, the word cognate is not appropriate here. Thus, we have modified 'ERV cognate lincRNA' to 'ERV-associated lincRNA', and 'cognation' to 'association' throughout the revised manuscript.

Specific comments
Figure 1.
Q5: The characterization of *LincGET* is extensive and thorough, particularly the sequencing of the Northern blot result. However one point I don't understand. On page 6, the authors mention that 'we designed probes in the unique regions of *LincGET* and *Dyei* and sequenced the TM-qPCR products. As a result there were no *LincGET* or *Dyei*-like sequences, suggesting the reliability of TM-qPCR.' Are these regions spliced out? Why would they not expect to see any product here? The Diagrams of the loci (in Figure 1A and EV1B) or text do not explain the reason sufficiently.
Response: We thank the reviewer for his comment. We believe that the reviewer misunderstood our meaning owing to our language issues. The TM-qPCR means "real-time PCR with TaqMan probes" and not for Northern blot. We used TM-qPCR to measure the expression pattern of *LincGET* and *Dyei*. Because *LincGET* and *Dyei* are both ERV-associated, sequencing should be used to confirm the reliability of TM-qPCR results. As a result, there were no *LincGET* or *Dyei*-like sequences, suggesting the reliability of TM-qPCR' **(lines 135-139)**.

Q6: The authors mention that the other lincRNAs are expressed weakly or in random patterns (page 5 paragraph 2). This is not the case. Many, show interesting expression patterns. They merely need to say that *LincGET* was the most 2-cell and 4-cell specific. For example Loca1 shows an interesting 2-cell to 8-cell expression pattern that is neither weak not random. Moreover *Dyei* is barely expressed in the 2-cell stage. It is wrong to mention (same paragraph) that *Dyei* is 'highly expressed in 2- to 4-cell stage mouse embryos.
Response: As pointed out, the description of the qPCR results for the expression pattern of these novel transcripts was not accurate. In the revised manuscript, we followed your advice and modified the description as follows: "The result showed that *LincGET* and *Dyei* are the most 2-cell and 4-cell specific" (**lines 105–106**).

Figure 2.
Q7: The authors mention that knockdown with siRNA was not successful. Did they try dsRNA? If this is successful to induce knockdown of *LincGET*, a developmental experiment using this as an alternative method of knockdown should be included as LNAs can have other effects, such as preventing binding to DNA/proteins. This would significantly strengthen the manuscript.
Response: The reviewer raised a very good point. We tried dsRNAs (the fragment from 2490 to 2680 nt of *LincGET* and the fragment from 400 to 650 nt of *Dyei* were used). However, dsRNA cannot knockdown *LincGET* or *Dyei*. The results are presented in **Figure EV2A in the revised manuscript**. We speculate that dsRNA is processed into siRNAs when it is injected into the cytoplasm. Thus, dsRNA cannot knockdown *LincGET* or *Dyei* efficiently. This is also explained in our results section **(lines 165-167).**

Q8: Have they excluded that the LNA prevents reverse transcription/amplification of the *LincGET* RNA by RT-qPCR? If not, then knockdown cannot be concluded, the LNA could be just blocking binding of the RNA, which changes the interpretation of the results.
Response: We do not think that LNA would be extracted into the total RNA, because the column absorption method (RNeasy Mini Kit, QIAGEN, #74104) was used.

Q9: Figure 2A Is not referred to in the text.
Response: We apologize for this mistake. Figure 2A is now referred to in the revised manuscript **(lines 168-171)**.

Q10: Figure 2B What is the timing of this experiment?
Response: As suggested, we added the description of the timing of this experiment in the legend of **Figure 2B in the revised manuscript** as "LNA was injected at 25 h phCG and embryos were collected at 48 h phCG at the late 2-cell stage for TM-qPCR analysis" **(lines 939-941).**

Q11: Figure 2D. Embryos in LNA groups look identical to wild-type groups. Therefore this Figure only shows that embryos are the same as wild-type up to and including S phase. Only in conjunction with the phenotype this suggests G2 phase block (they cannot conclude a G2 block). From the BrdU staining it is not clear that the embryos have exited S phase. Can do a short pulse of EdU at say 46h to confirm this (with BrdU as before as positive control).
Response: We concluded that *LincGET* depletion leads to a G2 block from the results as follows: PI staining showing an interphase nucleus can exclude the M phase. CAF-1 negative staining in blocked embryos excludes the S phase. BrdU positive staining indicates G2 or S phase. Thus, we can conclude that *LincGET* depletion leads to a G2 block and we chose G2 phase 2-cell embryos at phCG 48 h as control. This is the reason why embryos in the LNA groups look identical to those in the wild-type groups. In addition, as suggested, we added BrdU at the late 2-cell stage (phCG 48 h) and tested the BrdU signal at the late 4-cell stage (phCG 62 h) to determine if the arrested embryos existed the S phase. The results showed that embryos injected with Control-LNA were BrdU positive and reached the 4-cell stage (the BrdU must be incorporated into the genome at the S phase of 4-cell stage), while the embryos injected with *LincGET*-LNA were BrdU negative and still arrested at the 2-cell stage (**Figure EV3B of the revised manuscript**). It indicated that the arrested embryos injected with *LincGET*-LNA were blocked at the G2 phase and no DNA replication happened. This experiment is discussed in the revised manuscript (**lines 178–194**).

Q12: Additionally, S10ph is not necessarily a specific marker of G2/M - this is currently controversial. They should use Cylcin B1 as a G2 marker in addition.
How do arrested embryos look - are they still replicating? Are they still S10ph positive?
Response: As suggested, we stained the embryos for cyclin B1 as the G2 marker. The results showed that the control embryos at phCG 48 h and the arrested embryos were both cyclin B1 positive, indicating the G2 block. As explained in the above response, we concluded that no DNA replication occurred in arrested embryos injected with *LincGET*-LNA. We moved the H3S10ph IF results to **Figure EV3A in the revised manuscript**, which shows that the arrested embryos were still H3S10ph positive at E3.5 days (**lines 185–187**).

Q13: Hoeschst staining is mentioned in the text, but PI is used in the Figure.
Response: We thank the reviewer for pointing out this error. PI was used in all nuclear staining experiments. We have revised our manuscript and changed “Hoechst staining” to “PI staining.”

Q14: Figure 2H should be enlarged
Response: As suggested, we have enlarged **Figure 2H in the revised manuscript**. More data are presented in **Figure EV3D in the revised manuscript** (**lines 227–229**).

Figure 3
Q15: Figure 3C - Statistics should be included.
Response: As suggested, we added p values for every gene expression change in **Figure 3C in the revised manuscript**.

Q16: Figure 3E - The images are not clear, unmerged panels should be included. The decreased level of pP38 in particular is not clear.
Response: As suggested, we replaced the merged images with unmerged images for **Figure 3E in the revised manuscript**. From the unmerged pictures, the decreased level of phospho-p38 is clear. To clearly quantify the change in phospho-p38, the signal intensity was quantified by using ImageJ software. The results are presented in **Figure 3F in the revised manuscript** (**lines 242–244**).

Figure 4
Q17: Figure 4A. Should mention in text or figure legend the lysate for RNA-pulldown was from 4-cell stage embryos.
Response: As suggested, this is now mentioned in the figure legend of **Figure 4A in the revised manuscript** (**lines 1008–1011**).

Q18: Figure 4C. The authors mention that ILF2 is present in the nuclei of normal late 2-cell embryos but this is not apparent in the Figure. Unmerged images should be shown.
Response: As suggested, we replaced the merged images with unmerged images for **Figure 6A and Figure S5 in the revised manuscript**.

Q19: The authors mention in the text that ILF2 and hnRNP U are 'colocalizing with *LincGET*' This is completely overstated. All they can say is that both are present in the nucleus at the 4-cell stage only.
Response: As suggested, we corrected the revised manuscript as follows: "The results showed that all of them are located in nuclei of normal or arrested late 2-cell and early 4-cell embryos". (**lines 344–345**)

Q20: Figure 4D. The, although hypothetical is not helpful here. It doesn't even explain why in *LincGET* knockdown (LNA) embryos there is an increase in hnRNP U and ILF2 protein.
Response: In *LincGET*-depleted 2-cell embryos, the protein levels of hnRNP U, FUBP1, and ILF2, which can bind with *LincGET*, increased while no significant change was observed at the mRNA level (**Figure 6A, 6B, 6C, and S5 in the revised manuscript**) (**lines 348–350**). We then overexpressed Egfp or *LincGET* in mouse ES cells and assessed changes in the protein levels of hnRNP U, FUBP1, and ILF2 by western blot. Results showed that *LincGET* overexpression led to a decrease of hnRNP U, FUBP1, and ILF2 protein levels. The qPCR results showed that *LincGET* overexpression had no effect on *Hnrnpu*, *Fubp1*, and *Ilf2* at the mRNA level, suggesting that *LincGET* can inhibit hnRNP U, FUBP1, and ILF2 at the post-transcriptional level (**lines 351–357**). We hope this answers the reviewer's concern.

Figure 5
Q21: Figure 5B. The statistics are insufficiently presented. What test was used? Which results are statistically significantly different?
Response: As suggested, the statistical tests used in this experiment are now described in the legend of **Figure 5B in the revised manuscript** as follows: "Student's t-test was used for the statistical analysis. Different letters (a, d, c, and d) mean $p < 0.01$." (**lines 1042–1044**)

Q22: Figure 5C and 5H there is no negative control here. The Srsf1 and Carm1 data is weak, and almost included as an afterthought. Perhaps Srsf1 and Carm1 could be included in Figure 4 alongside the interaction with hnRNP U and ILF2, to strengthen the weak Figure 4.
Response: As suggested, we repeated the co-IP with IgG antibody as the negative control in early 4-cell embryos and mouse ES cell lines overexpressing *LincGET*. The western blot results of pull-down or co-IP are shown in **Figure 4 in the revised manuscript** (**lines 256–275**). In addition, we removed results associated with Carm1. The role played by *LincGET* in Carm1-mediated lineage allocation will be discussed in a future paper.

Q23: Are the transcriptional and splicing events depicted in Figure 5 dependent on any of the protein identified as interactors (hnRNP U, ILF2, Srsf1 or Carm1). If so, this would substantially strengthen the end of the manuscript, which does let down the first half of the manuscript somewhat.
Response: Like the reviewer, we were also concerned regarding the role of hnRNP U, FUBP1, ILF2, and SRSF1 in *LincGET*-mediated transcription and RNA splicing regulation. Because *LincGET* depletion led to an increase of hnRNP U, FUBP1, and ILF2 protein levels, but not at the mRNA level, and had no effect on SRSF1 expression levels both at the RNA and protein levels (**Figure 6 and S5 in the revised manuscript**) (**lines 342–357**), we mainly studied hnRNP U, FUBP1, and ILF2.

First, a dual-luciferase reporter system was used to determine the role of these proteins in the effect of *LincGET* on transcription. 293T cells were used. Group 1, with GLKLTR-SV40pA-Luciferase-pA; group 2, with GLKLTR-SV40pA-Luciferase-pA and EF1a-*LincGET*-pA; group 3, with GLKLTR-SV40pA-Luciferase-pA, EF1a-*LincGET*-pA and CMV-EGFP-pA; group 4, with GLKLTR-SV40pA-Luciferase-pA, EF1a-*LincGET*-pA and CMV-Hnrnpu-pA; group 5, with GLKLTR-SV40pA-Luciferase-pA, EF1a-*LincGET*-pA and CMV-Fubp1-pA; group 6, with GLKLTR-SV40pA-Luciferase-pA, EF1a-*LincGET*-pA and CMV-Ilf2-pA. The results showed that, (1), overexpression of hnRNP U, FUBP1, and ILF2 had no effect on *LincGET* RNA levels; (2) group 2 is stronger than group 1, indicating that *LincGET* can mediate the activity of GLKLTR (this was also verified in **Figure 5B**) *in trans*, as the enhancer-like lincRNAs, which, however, function *in cis*; (3), group 3 was similar to group 2, indicating that EGFP cannot work as a transcription factor for *LincGET*; (4), group 4 and group 6 were similar to each other and both are stronger than group 2, indicating that hnRNP U and ILF2 can work as positive regulators for the enhancer-mediator activity of *LincGET*; (5), interestingly, group 5 is weaker than group 2, indicating that FUBP1 can work as a negative regulator for the enhancer-mediator activity of *LincGET*. (**Figure 5B in the revised manuscript**, **lines 291-294**)

Secondly, for RNA splicing regulation, we chose to assess the proportion of *CDK1-3ExS* as the measurement. Embryos were used. Group 1, injected with Control-LNA and siRNA for *Egfp*; Group 2, injected with Control-LNA and siRNA for *Hnrnpu*; Group 3, injected with Control-LNA and siRNA for *Fubp1*; Group 4, injected with Control-LNA and siRNA for *Ilf2*; Group 5, injected with *LincGET*-LNA2 and siRNA for *Egfp*; Group 6, injected with *LincGET*-LNA2 and siRNA for *Hnrnpu*; Group 7, injected with *LincGET*-LNA2 and siRNA for *Fubp1*; Group 8, injected with *LincGET*-LNA2 and siRNA for *Ilf2*; Group 9, injected with Control-LNA and *Egfp* mRNA; Group 10, injected with Control-LNA and *Hnrnpu* mRNA; Group 11, injected with Control-LNA and *Fubp1* mRNA; Group 12, injected with Control-LNA and *Ilf2* mRNA. LNA and siRNA were both used at 10 μM, and mRNA was used at 300 ng/μL. The results showed that: (1), knockdown of hnRNP U, FUBP1, and ILF2 in embryos injected with Control-LNA did not induce exon skipping of *Cdk1*; (2), knockdown of any of them in embryos injected with *LincGET*-LNA2 decreased *Cdk1*-3ExS level, especially *Fubp1* knockdown; (3), overexpression of hnRNP U, FUBP1, and ILF2 in embryos injected with Control-LNA increased *Cdk1*-3ExS level, especially *Fubp1* overexpression. These results indicated that hnRNP U, FUBP1, and ILF2, especially FUBP1, can promote exon skipping splicing, while *LincGET* is involved in the inhibition of exon skipping splicing, partially by decreasing the protein level of hnRNP U, FUBP1, and ILF2. (**Figure 7A in the revised manuscript, lines 358-372**)

EV
Q24: Figure 5G full image looks different to the cropped image in the main text.
Response: The cropped image in Figure 5G in the original manuscript was inverted and stressed, but truly derived from the full image in the appendix profile. In the revised manuscript, we changed the image as **Figure 5F in the revised manuscript**, showing that *LincGET* depletion induced dramatic exon skipping splicing of *Cdk1* exon 3, and that only the full length of *LincGET,* but not *LincGET* fragments can partially inhibit it (**lines 320–325**).

Discussion
Q25: page 13 The authors mention that *LincGET* could function as an inhibitor of transcription. There is no evidence for such a direct role - upregualted genes after knockdown (LNA) in Figure 3A could easily be just indirect effects.
Response: As pointed out, we have no evidence that *LincGET* could function as an inhibitor of transcription, even if we demonstrated that FUBP1 could work as a negative regulator for the enhancer-mediator activity of *LincGET* (**lines 292–294**). We have delete 'and can be either activator or inhibitor' in the revised manuscript.

Q26: page 13 they hypothesize that binding with *LincGET* may activate or improve the splicing activity of ILF2, hnRNP U and Carm1 because they increased at the protein level when *LincGET* was depleted. Firstly Carm1 has not been shown to increase. Even if they do increase upon knockdown, this does not suggest in any way that *LincGET* may activate or improve their splicing activity? Third, the reference here is Fig. 3G - it should be 4C.
Response: As pointed out, stating that *LincGET* may activate or improve the splicing activity of ILF2 and hnRNP U (we have removed the results about Carm1) in the original manuscript was an overstatement. In the revised manuscript, we demonstrated that (1) *LincGET* can inhibit the expression of hnRNP U, FUBP1, and ILF2 at the post-transcriptional level (**Figure 6 and S5 in the revised manuscript**); (2), knockdown of hnRNP U, FUBP1, and ILF2 in embryos injected with Control-LNA does not induce the exon skipping of *Cdk1*; (3), knockdown of hnRNP U, FUBP1, and ILF2 in embryos injected with *LincGET*-LNA2 decreased the *Cdk1*-3ExS level, especially *Fubp1* knockdown; (4), overexpression of hnRNP U, FUBP1, and ILF2 in embryos injected with Control-LNA increases *Cdk1*-3ExS levels, especially *Fubp1* overexpression. These results indicated that hnRNP U, FUBP1, and ILF2, especially FUBP1, can promote exon skipping splicing, while *LincGET* is involved in the inhibition of exon skipping splicing, partially through decreasing the protein levels of hnRNP U, FUBP1, and ILF2 (**Figure 7A in the revised manuscript**) (**lines 358–372**).

Referee #2:

Interesting report describing novel and likely important phenomenon regarding control of early mammalian development. The authors should also be commended for the extraordinary amount of work involved and for the depth of the analysis. It would be a pity if the report is published in its

present form since extremely poor writing will detract any reader from the importance of the message. I checked some published papers from this group and they are certainly capable of expressing themselves in acceptable scientific English. Either the authors or the journal will have to find an editor capable of making this paper legible.

Specific comments:

Q1. Page 3, line 4. There are many excellent reviews describing lineage allocation in preimplantation mouse embryos but Bischoff et al is not one of them.
Response: As suggested, we included the reference 'Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo' by Magdalena Zernicka-Goetz instead (**line 41**).

Q2. Page 3, lines 5-6. First description of retrotransposons expression and discussion about their possible function in preimplantation embryos is by Peaston et al., Dev. Cell 7, 597, 2004. and Evsikov et al., Cytogen. Genome Res., 105, 240,2004.
Response: As suggested, we added these 2 references (**lines 44–46**).

Q3. Page 5, line 20, page 27, line 8 and Figure 1C. there is no * marking on the figure.
Response: We added * in **Figure 1C in the revised manuscript**.

Q4. Page 7, line 10. Fig. 3A should be Fig. 2A
Response: We reviewed every figure citation carefully in the revised manuscript.

Q5. Page 8, line 8. Another inappropriate reference.
Response: As suggested, we changed the reference as 'Minami, N., T. Suzuki, and S. Tsukamoto, Zygotic gene activation and maternal factors in mammals. J Reprod Dev, 2007. 53(4): p. 707-15.' (**line 208**)

Q6. Page 9, lines 25-28. Data presented in Figure 4C (also in Figure 2D) cannot be interpreted as co-localization. IF demonstrate the presence of antigens in question in the nucleus and that is all. To establish co-localization, the authors must stain the same embryo with two different antibodies and provide much better resolution.
Response: As suggested, this is corrected in the revised manuscript as "The results showed that all of them are located in nuclei of normal or arrested late 2-cell and early 4-cell embryos". (**lines 344–345**)
.

Q7. Page 12, line 19. References numbered 68 and 69 describe isolation of mouse ES cells and have nothing to do with "segregation of ICM and trophectoderm".
Response: As suggested, we changed the reference to 'Papaioannou, V.E., Lineage analysis of inner cell mass and trophectoderm using microsurgically reconstituted mouse blastocysts. J Embryol Exp Morphol, 1982. 68: p. 199-209.' (**line 402**)

Q8. Page 12, line 22. See comment #2.
Response: As suggested, we changed the reference to 'Peaston, A.E., et al., Retrotransposons regulate host genes in mouse oocytes and preimplantation embryos. Dev Cell, 2004. 7(4): p. 597-606.' (**line 406**)

Q9. Page 13, line 3. See comment #2.
Response: As suggested, we added the references 'Peaston, A.E., et al., Retrotransposons regulate host genes in mouse oocytes and preimplantation embryos. Dev Cell, 2004. 7(4): p. 597-606.' and 'Evsikov, A.V., et al., Systems biology of the 2-cell mouse embryo. Cytogenet Genome Res, 2004. 105(2-4): p. 240-50.' (**line 415**)

Q10. Page 13, line 26. There is no Fig. 3G
Response: We reviewed every figure citation carefully in the revised manuscript.

Q11. Pages 14-21. Methods are usually presented in passive voice as something that happened in the past and not in the command mode of a cookbook. Please rewrite.

Response: As suggested, the materials and methods section was revised and written in the passive voice.

Q12. Page 18, line 7. How long were the embryos cultured in the presence of BrdU? Or EU (line 23, same page)?
Response: BrdU was added at 30 h phCG at the G2 phase of 1-cell stage and was detected at 48 h phCG at the G2 phase of 2-cell stage. For EU staining, EU was added at 40 h phCG at the early 2-cell stage and was detected at 48 h phCG at the late 2-cell stage. Thus, embryos were cultured for 18 h and 8 h in the presence of BrdU and EU, respectively. This information is now provided in the legend of **Figure 2A in the revised manuscript** (**lines 936–938**).

Q13. Reference 70, volume, pages?
Response: References have been updated by using EndNote.

Referee #3:

This is an interesting manuscript that identifies a long non coding RNA, *LincGET*, that has a key function in the early embryo in so much that its depletion leads to arrest at the 2-cell stage. Interestingly, loss of *LincGET* does not affect zygotic gene activation. Instead the authors present somewhat circumstantial evidence to suggest that *LincGET* might regulate the MAP kinase pathway, and a number of other genes by regulating alternative splicing pathways. These genes include regulators of cell cycle progression such as Cdk1. There is the essence of a very interesting story here. The difficulty with the paper is that none of the experiments described go far enough to really be totally confident of the interpretation given. Further work is required before the paper is ready for publication.

Q1: The data describing the complex formed by the *LincGET* is particularly scant. The authors claim to have identified hnRNP U and ILF2 as *LincGET*-associated proteins. However they should present all of their data - full gels including Pinceau stained gels and full lists of proteins identified by MS (Fig. 4A). This also applies to the proteins identified by IP (Fig. 4B) as this data is not convincing as shown.
Response: *LincGET* is expressed only in 2- to 4-cell mouse embryos; neither cell lines nor tissues express *LincGET* (**Fig. 1F, 1G, and S3B in the revised manuscript**). Thus, we can only use RNA-pulldown-MS to identify proteins that can interact with *LincGET in vitro* with biotin-labeled *LincGET* and early 4-cell embryos lysates (**Fig. 4A in the revised manuscript**) and then use western blot to verify the pulldown-MS results (**Fig. 4B in the revised manuscript**, which does not present IP results). One pull-down-MS experiment requires about 6,000 early 4-cell embryos, while one pulldown-WB experiment needs about 1,500 early 4-cell embryos. Thus, the pulldown-MS was only performed once and the pulldown-WB was performed 3 times to verify the pulldown-MS results (**lines 256–263**).

The full silver staining gel is shown in the last appendix profile (see below, left). From the silver staining gel, we found three specific bands in the *LincGET* lane compared to the *a-LincGET* lane (see below, right, or **Figure 4A in the revised manuscript**). We analyzed these three bands (not the direct pulldown results) by MS. Using MS, we identified these three bands as hnRNP-U, FUBP1, and ILF2 (see below, right, or **Figure 4A in the revised manuscript**). Data for FUBP1 have been added in the revised manuscript (**lines 256–263**).

[Figures have been removed per author's request]

The RNA Immunoprecipitation (RIP) is the perfect method to verify the binding between proteins and *LincGET in vivo* and this will make the data describing the complex formed by the *LincGET* more solid. However, to perform RIP, we would need a large amount of embryos that we cannot afford. Thus, instead, we used co-IP to verify the RNA-protein complex formed by *LincGET* with hnRNP U, FUBP1, ILF2, and SRSF1 both in embryos and mouse ES cells overexpressing MS2-labeled *LincGET* (we have removed the results associated with Carm1 for the revised manuscript and will study the role played by *LincGET* in Carm1-mediated lineage allocation in another paper). The co-IP results using anti-SRSF1 or anti-HA (HA-labeled MS2 coat protein) (with IgG as control, which was a weak point of the original manuscript) showed that *LincGET* truly

formed an RNA-protein complex with hnRNP U, FUBP1, ILF2, and SRSF1 (**Figure 4D in the revised manuscript**) (**lines 265–275**).

Q2: Moreover, few conclusions can be drawn form the immunostaining experiments presented in Figure 4C that show the nuclear localization of these proteins and their apparent increase upon *LincGET* depletion.
Response: We corrected this in the revised manuscript as follows: "The results showed that all of them are located in nuclei of normal or arrested late 2-cell and early 4-cell embryos". (**lines 344–345**)

In addition, in the revised manuscript, we demonstrated that (1) *LincGET* can inhibit the expression of hnRNP U, FUBP1, and ILF2 at the post-transcriptional level (**Figure 6 and S5 in the revised manuscript**) (**lines 342–357**); (2), knockdown of hnRNP U, FUBP1, and ILF2 in embryos injected with Control-LNA induced the exon skipping of *Cdk1*; (3), knockdown of hnRNP U, FUBP1, and ILF2 in embryos injected with *LincGET*-LNA2 decreased *Cdk1*-3ExS levels, especially *Fubp1* knockdown; (4), overexpression of hnRNP U, FUBP1, and ILF2 in embryos injected with Control-LNA increased *Cdk1*-3ExS level, especially *Fubp1* overexpression. These results indicated that hnRNP U, FUBP1, and ILF2, especially FUBP1, can promote the exon skipping splicing, while *LincGET* is involved in the inhibition of exon skipping splicing, partially by decreasing the protein levels of hnRNP U, FUBP1, and ILF2 (**Figure 7A in the revised manuscript**) (**lines 358–372**).

Q3: The above comments are equally applicable to the pull down experiments that claim to show an association with SRSF1. The authors should provide the complete gels with a full set of control experiments. Little can be concluded from the data as presented - the reader has to take the authors' statement in good faith.
Response: As explained above, we cut only the three *LincGET*-specific bands from the silver staining gel for MS analysis and no band for SRSF1 (34 KDa) was *LincGET*-specific. However, this cannot exclude that SRSF1 is present in the *LincGET*-protein complex. hnRNP U, FUBP1, and ILF2 were identified from the MS results. All of them are RNA alternative splicing regulators. Thus, we hypothesized that *LincGET* was involved in RNA alternative splicing regulation, which is also supported by the RNA-seq data. SRSF1 is a well-known alternative splicing factor and is highly expressed from 2- to 8-cell embryos (**Figure 4C in the revised manuscript**). Thus, we detected SRSF1 in *LincGET*-pulldown by western blot. As a result, SRSF1 was identified in the *LincGET*-pulldown (**lines 264–268**).

Furthermore, in the revised manuscript, we used co-IP to verify the RNA-protein complex that *LincGET* formed with hnRNP U, FUBP1, ILF2, and SRSF1 both in embryos and mouse ES cells overexpressing MS2-labeled *LincGET* (we removed the results associated with Carm1 and will study the role played by *LincGET* in Carm1-mediated lineage allocation in another paper). The co-IP results using anti-SRSF1 or anti-HA (HA-labeled MS2 coat protein) (with IgG as control, which was a weak point of the original manuscript) showed that *LincGET* truly formed an RNA-protein complex with hnRNP U, FUBP1, ILF2, and SRSF1 (**Figure 4D in the revised manuscript**) (**lines 269–273**).

Q4: The authors should also provide evidence that Srsf1 still interacts with hnRNP U or ILF2 upon *LincGET* depletion.
Response: In the revised manuscript, we performed co-IP with *LincGET*-depleted 2-cells and mouse ES cells that do not express *LincGET* using anti-SRSF1 or anti-HA. The results showed that SRSF1 interacts with hnRNP U, FUBP1, and ILF2 regardless of the presence or absence of *LincGET* (**Figure 4D in the revised manuscript**) (**lines 273–275**).

Q5: The relationship between *LincGET* and Carm1 is not demonstrated satisfactorily. One can conclude little from the data presented in Figure 5H. This is particularly important as the authors would like to make a great deal from this potential interaction given the role that CARM1 has been demonstrated to have in early development. The controls for the pull-down and IP experiments should also be provided to check the specificity of IgG or GST-beads binding to proteins. Pinceau staining (to give loading controls) should also be attached for all GST-Pulldown and co-IP experiments. This potential interaction should also be explored further. The authors should investigate the consequences of *LincGET* knockdown and CARM1 overexpression and determine whether CARM1 inhibition affects the *LincGET*-CARM1 interaction / function.
Response: We removed the results associated with Carm1 and will study the role played by *LincGET* in Carm1-mediated lineage allocation in another paper. In this paper, we want to focus on

the results indicating that *LincGET* is essential for mouse 2-cell embryonic development through transcription and exon skipping splicing regulation. Thus, the involvement of *LincGET* in lineage allocation is another story, though we obtained some promising results about this.

Other points

Q6: Fig. 3D and E show that MAPK pathway is less activated upon *LincGET* depletion. However there is also less of total forms of ERK1/2 and p38 explaining the lower levels of pERK1/2 and p38 and how MAPK activation might be affected. The authors should determine whether *LincGET* depletion affects expression of MAPK regulated target genes.
Response: As suggested, we determined the effect of *LincGET* on the expression of MAPK regulated genes by qPCR. The results are shown in **Figure 3G in the revised manuscript** and described in the results section (**lines 244-246**).

Q7: Page 7 (*LincGET* depletion results in developmental arrest at late G2 phase of 2-cell stage) - figure number is 3A, should be 2A.
Response: We reviewed every figure citation carefully in the revised manuscript.

Q8: Fig. 4C - quantification of the fluorescence intensity is missing (hnRNP U and ILF2).
Response: As suggested, we included the fluorescence intensity analysis performed by image J software in the revised manuscript (**Figure 3E and 6B in the revised manuscript**) (**lines 242–244**).

Cross-comments by referee 1:

Q1: I agree with referee 3 that the data on *LincGET* complex formation is weak, as I mention in my review. This part of the manuscript, is experimentally the weakest section and significantly brings down the impact of the manuscript as a whole. Thus, further details regarding the experiments the authors performed to identify this complex would be helpful as suggested by Referee 3. A better characterization of the complex would enable a reorganisation of Figures 4 and 5 to bring in the data on Srsf1 and Carm1 into the complex characterisation. As I mention and as suggested by referee 3, the data on Carm1 currently is insufficiently presented to be able to draw any conclusions. The controls suggested by Referee 3 are essential.
Response: Please refer to our response to **reviewer # 3's comment Q1**.

Q2: Also functional relationships between the proteins identified in the complex would be helpful, such as my previous suggestions: 'Are the transcriptional and splicing events depicted in Figure 5 dependent on any of the protein identified as interactors (hn RNP U, ILF2, Srsf1 or Carm1)' At present the authors assume the functional roles of these proteins in the embryo, but do not demonstrate a role in transcription or splicing in the context of the *LincGET* phenotype. Alternatively the authors could explore the interaction with Carm1 more extensively from a functional point of view, as suggested by Referee 3. The authors should check for any role of Carm1 in the *LincGET* phenotype they observe and in contrast, whether *LincGET* has any role in the ascribed role of Carm1 in lineage allocation (e.g. knockdown *LincGET* in 1 blastomere at the 2-cell stage and determine if this affects lineage allocation).
On balance I feel that exploring functionally either route; the hn RNP U and ILF2 or Carm1 interactions would be necessary. Controls for the interactions/complex formation as suggested by Referee 3 would also be essential.
Response: Please refer to our response to **reviewer # 1's comment Q23**.

Cross comments by referee 2 on referee 3's report:

Q1: Srsf interaction with hnRNP U or ILF2 can be examined though it is entirely possible and compatible with the model that the interaction is indirect and mediated by *LincGET*. If that is the case co-immunoprecipitation experiments will be negative
Response: As suggested, we performed co-IPs in the revised manuscript with *LincGET*-depleted 2-cells and mouse ES cells that do not express *LincGET* using anti-SRSF1 or anti-HA. The results showed that SRSF1 interacts with hnRNP U, FUBP1, and ILF2 regardless of the presence or absence of *LincGET* (**Figure 4D in the revised manuscript**) (**lines 264–275**).

Q2: I am not quite sure what is the Reviewer 3 asking for. The authors could do the same as for the other genes, deplete *LincGET* and see what happens with CARM1. Overexpression of CARM1 supposedly influences the fate of the early blastomeres and it would be interesting to see if this is somehow mediated by *LincGET*, except for the fact that *LincGET* depleted embryos arrest before any fate determination takes place. The authors show that CARM1 is present in *LincGET* pulldown protein complex but they certainly did not show that any exon skipping takes place in early embryos and that this causes developmental arrest. They should tone down penultimate paragraph of the Results (page 11) and last paragraph of the Discussion.
Response: We removed the results associated with Carm1 and will study the role played by *LincGET* in Carm1-mediated lineage allocation in another paper.

2nd Editorial Decision 23 June 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed report from referee 1 who was asked to assess it. Referee 1 is happy with the revised manuscript and only comments on the language, which should be improved and carefully checked throughout the manuscript before we can proceed with its official acceptance.

Please also answer all questions regarding statistis in the author checklist. The checklist will be part of the transparent peer-review file, and the questions about statistics need to be answered, given that statistics are calculated in several figures.

I would like to suggest to change the title slightly to:
"A novel long intergenic non-coding RNA indispensable for the cleavage of mouse 2-cell embryos"
Please let me know whether you agree with this change.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

I believe the comments have been sufficiently addressed to allow publication of the revised manuscript in EMBO Reports.

The authors of the manuscript have impressively addressed both the concerns of Reviewer 3 and my own concerns in the revised version. All 5 of Reviewer 3's comments have been well answered. The data on the interaction is sufficiently improved, which was the primary concern of Reviewer 3 (points Q1 and Q3), most noticeably in Figure 4D. Indeed it is quite impressive the amount of work involved to identify these interactions in mouse 4-cell stage embryos. As far as I can tell the full gels for the IP experiments are not shown (as requested by reviewer 3) although I do not think this is a major concern. Q2 has been well addressed and was also a concern of mine in the original manuscript. Qs 4 and 5 are also now clearly addressed in the revised version.
On the whole the standard of presentation of the results has significantly improved. However, on re-reading through the manuscript I have noticed some sentences that still are difficult to understand.
Examples:
lines 244-246, 255-256.
Thus, further work is still required on the text throughout.

2nd Revision - authors' response 04 July 2016

We would like to thank you for your interest in our manuscript and for providing us an opportunity to revise it again. We appreciate careful and thoughtful suggestions of you and the reviewer 1, which helped us improve our manuscript. As requested, we revised our manuscript and included additional content in response to the comments. Our point-by-point response to the comments can be found below.

POINT-BY-POINT RESPONSE

Editor's comment 1: Please also answer all questions regarding statistics in the author checklist. The checklist will be part of the transparent peer-review file, and the questions about statistics need to be answered, given that statistics are calculated in several figures.

Response: As requested, we have answered all questions regarding statistics in the author checklist.

Editor's comment 2: I would like to suggest to change the title slightly to:
"A novel long intergenic non-coding RNA indispensable for the cleavage of mouse 2-cell embryos"
Please let me know whether you agree with this change.

Response: We agree with this change, and we have changed the title to "A novel long intergenic non-coding RNA indispensable for the cleavage of mouse 2-cell embryos" in the revised manuscript.

Editor's comment 3: EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Response: We have added a short summary, highlights, and a synopsis image.

Referee #1:

I believe the comments have been sufficiently addressed to allow publication of the revised manuscript in EMBO Reports.

The authors of the manuscript have impressively addressed both the concerns of Reviewer 3 and my own concerns in the revised version. All 5 of Reviewer 3's comments have been well answered. The data on the interaction is sufficiently improved, which was the primary concern of Reviewer 3 (points Q1 and Q3), most noticeably in Figure 4D. Indeed it is quite impressive the amount of work involved to identify these interactions in mouse 4-cell stage embryos. As far as I can tell the full gels for the IP experiments are not shown (as requested by reviewer 3) although I do not think this is a major concern. Q2 has been well addressed and was also a concern of mine in the original manuscript. Qs 4 and 5 are also now clearly addressed in the revised version.
On the whole the standard of presentation of the results has significantly improved.

Comment 1: However, on re-reading through the manuscript I have noticed some sentences that still are difficult to understand. Examples:
lines 244-246, 255-256.
Thus, further work is still required on the text throughout.

Response: As suggested, we carefully edited our manuscript again with a professional scientific editor.

3rd Editorial Decision 07 July 2016

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Corresponding Author Name: Zhong-hua Liu
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For qPCR, about 50 embryos in each stage (13 different stages) were used for each experiment and three experimental replicates were performed (in total, about 2,000 embryos were used). For SSRT PCR, about 50 early 4-cell embryos were used for each experiment and three experimental replicates were performed (in total, about 150 early 4-cell embryos were used). For Subcellular localization analysis, about 500 early 4-cell embryos were used for each experiment and three experimental replicates were performed (in total, about 1,500 early 4-cell stage embryos were used). For RACE experiments, about 200 early 4-cell embryos were used for each experiment and three experimental replicates were performed (in total, about 1,200 early 4-cell stage embryos were used). For miRNA reverse northern blot, about 400 early 4-cell embryos were used for each experiment and three experimental replicates were performed (in total, about 1,200 early 4-cell stage embryos were used). For RNA-FISH, about 4-7 embryos for each stage (8 different stages) were used for each experiment and three experimental replicates were performed (in total, about 150 early 4-cell stage embryos were used). For immunofluorescence or DNA-FISH, about 15 embryos were used for each antibody for each experiment and three experimental replicates were performed (in total, about 300 Control-LNA injected embryos and 300 LincGET-LNA injected embryos were used). For RNA-seq, about 2,200 Control-LNA injected embryos and 2,000 LincGET-LNA injected embryos were used. For western blot, about 200 embryos were used for each lane for each experiment and three experimental replicates were performed (in total, about 2,000 Control-LNA injected embryos and 2,000 LincGET-LNA injected embryos were used). For pull-down-mass spectrometry analysis, 6,367 early 4-cell embryos were used and only one experiment were performed. For pull-down-WB analysis, about 1,500 early 4-cell embryos were used for each experiment and three experimental replicates were performed (in total, about 4,500 Control-LNA injected embryos and 4,500 LincGET-LNA injected embryos were used). For co-IP assays, about 2, 500 Control-LNA injected embryos and 2,500 LincGET-LNA injected embryos were used for each experiment and three experimental replicates were performed (in total, about 7,500 Control-LNA injected embryos and 7,500 LincGET-LNA injected embryos were used). In total, about 46,000 embryos were used. These embryos were collected from 1,854 female ICR mouse and 213 male ICR were used for mate.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	no
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	no
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A

5. For every figure, are statistical tests justified as appropriate?	YES, the number of experiments and statistics are described in the material and methods section as well as figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	The standard error of the mean were used to estimate the variation whitin group when three replicates were used.
Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All these information is included in the materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The mouse cell line used in this work were in-house produced and verified with commonly accepted immunofluorescent staining and/or functional assays.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The RNA-Seq data generated by this study have been deposited to the SRA database (SRX1478805;SRX1478806;SRR2990786;SRR2990793).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	The information and GenBank accession numbers of the newly identified 36 novel transcripts identifieid in this study is presented in Table EV2.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A