Hbxip is essential for murine embryogenesis and regulates embryonic stem cell differentiation through activating mTORC1

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Reviewer 1

Evidence, reproducibility and clarity

In this manuscript the authors study the role of Hbxip in early development, by performing a KO of this protein and using ESCs of these embryos, in a mouse model.
They conclude with classical loss of function approaches that Hbxip KO is embryonic lethal, and affects differentiation possibly acting via the mTOR signalling pathway, notably MTORC1. This is interesting work, although several aspects should be considered as there may be simpler explanations to the results than what the authors propose:

1- While the authors show some evidence for limited self renewal in the ESC KO line, cell cycle analyses and apoptosis should also be monitored, as should the stability of the KO line in term of karyotype. This is especially important as Hbxip also seems to interact with microtubules/centrosomes and other biological functions in cells (notably in cancer) that have little to do with differentiation. The authors should control for these aspects, as they may be skewing the data and actually lead to different interpretations of the data. The fact that the authors perform other loss of function experiments later on (related to MTORC1) function does not resolve this issue. They actually could form the core of a totally different paper not focused on Hbxip at all.

2- The authors should show EB differentiation and also differentiation markers at the protein level (WB or ICC). as it currently stands this data is not convincing at this level. THe same is true for pulipotency markers as localization (nuclear or not) is equally important, besides levels of expression. Besides the existing WB these ICC images must be shown.
3-To fully validate the recovery data the WB (3G) should be quantified, and the authors show that differentiation is now normal.4- Localization of Hbxip should be shown by ICC in Fig 6.

Significance

This would be interesting to both people in the pluripotency field and mammalian embryo development, as well as in mTOR signalling and therefore metabolism. This are things I have some expertise in.

Although some aspects need improvement and more experiments this is interesting, if unspectacular, work that will be of interest to a specialized audience.

Reviewer 2

Evidence, reproducibility and clarity

Qin et al dissect the function of Hbxip in early mouse development and embryonic stem cells. But using a variety of loss-of-function (LOF) models, the authors present an involvement of Hbxip in differentiation and exit from pluripotency in post-implantation embryos. In ES cells, Hbxip KO leads to self-renewal defects and skewed differentiation. The authors show that Hbxip regulates mTORC1 activity in ES cells and argue that this could be the mechanism for embryonic lethality of Hbxip KO embryos. In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function. The results are insightful, however relevant controls and rigorous statistical testing are often missing. Embryo and ES phenotypes contradict each other, and the claim for perturbed differentiation is not sufficiently supported. Overall, the paper could be more streamlined. If the authors focus on key findings and provide definitive supportive evidence, the study would be more informative.

Major comments

o Hbxip is not essential for ES differentiation, since the Hbxip KO ES cells do differentiate. Therefore the title is not correct.

o For all KOs, the evidence for the loss of protein is missing. In Fig.1, only genotyping results are shown, but a staining or western blot showing loss of the protein is not presented. In Fig. 2, the shown western blot is not convincing. The authors themselves show higher expression of Hbxip in these cells (Fig 6A), therefore a better western blot is required. For the KO cell lines presented in Figs. 6 and 7, no evidence of functional KO at the protein level.

o Similarly, for the overexpression/rescue shown in Fig. 3G as well as for the siRNA-KD shown in Fig.4 no evidence is provided to show that these methods work. Also in Fig. 4 it is unclear which control is used. In general, the authors need to provide more detailed explanations in the methods section and direct evidence supporting their KO/KD/OE models.

o qPCRs: Applied statistical tests seem to be T-test, which is not appropriate for the data presented. The authors need to apply two-way ANOVA because of the testing of multiple genes in multiple samples.

o The biggest discrepancy in the paper is the different outcome of Hbxip KO in the embryo and in ES cells. In the embryo, the KO leads to the inability to differentiate and persistence of pluripotency at E8.5. However, the KO ES cells lose self-renewal potential and have to blockage of differentiation. What is the author's take on this? ES data clearly shows that reduced proliferation due to Hbxip LOF is not an obstacle against exit from pluripotency or differentiation. Therefore the authors' argument of a proliferation defect causing persistent pluripotency at E8.5 does not hold. The embryo data points to gastrulation defects. One can envision a scenario where the KO epiblast does not initiate gastrulation and as a result does not exit pluripotency. Staining E7.5 embryos for gastrulation-associated genes would provide a clearer picture.

o Fig. 2/3: Mouse ES cells do not spontaneously differentiate to TE, unless driven by a major TE TF. The shown genes categorized as TE in Fig. 2/3 are not only TE-specific. Therefore the authors should decategorize these genes as TE markers and reinterpret the data as such.

o Fig. 4 is only loosely connected to the rest of the story. The shown si-injected embryos are not blastocysts, it is not indicated which control is used, no evidence of actual KD is presented, no evidence of maternal transcripts in the KO embryos are presented, and as such the whole argument is not convincing. If the authors want to keep this figure, then supporting evidence is necessary. Alternatively, the figure may be removed.

o Almost all differentiation analysis relies on RNA expression analysis. Supporting evidence at the protein level is necessary to substantiate the claims. The authors can stain for markers of different lineages and quantify.

o If mTORC1 activation is mediated by Hbxip function, but there is still mTORC1 activity in Hbxip KO cells, then Hbxip is probably required to promote mTORC1 activity above a certain threshold. mTOR heterozygous KO is not lethal, similar to Hbxip heterozygous KO. So mTOR dosage does not seem to be very critical for embryonic development. Since the authors' argument is that Hbxip functions by regulating TORC1 activity, it would be helpful to see the levels of mTORC1 activity in Hbxip homozygous vs heterozygous KOs vs wt embryos. The authors need to at least discuss how Hbxip/mTOR dosage regulates development in the discussion.

Minor comments

o For westerns please provide size markers and uncropped images (at least as a reviewer figure).

o In general, the differences in the expression levels of lineage markers are quite small and hard to read as presented in the figures. The data could be better understood if the authors plot log2FC to show fold change in expression.

o In Fig. 3a, b, it is better to plot expression of each gene relative to its levels in ES cells. This way the reader can see that lineage markers are actually upregulated during differentiation of wt cells, but perturbed in KO cells if that is the case.

o Please show individual data points for qPCR graphs.

o What is the staining in Fig. 1D? No explanation is provided.

o Please explain the reason for using EIIa-Cre in the text. This is crucial information to understand the nature of the KO in the embryo.

o Fig. 2H shows GO terms for downregulated genes. Please also show the same analysis for upregulated genes, as this subset comprises a large portion of the differentially expressed genes.

o Fig. 6D is a bit misleading because in 6E and F we see substantial pS6K1 in Hbxip KO, although it may be reduced compared to wt. But definitely not as reduced as in 6A.

Significance

In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function.

Role of different aspects of mTOR biology in early development is not well understood. Therefore the advance here could be useful.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

Qin, Ni, et al. investigate the role of Hepatitis B X-interacting protein (HBXIP, also known as LAMTOR5) during development. To do so, they use a conditional Hbxip mouse model and delete exon 2 of the gene upon recombination with a constitutive Cre. They find that Hbxip mutant embryos are delayed and die ~E7.5. They also make use of Hbxip knock out mouse embryonic stem cells to investigate the potential differentiation defects, and through co-immunoprecipitation coupled to proteomics and epistatic experiments, they conclude that Hbxip plays a role in epiblast

differentiation, interacts with ragulator complex proteins, and activates mTORC1 (pS6K1) signalling pathway.

Their findings suggest that Hbixp impacts differentiation at early stages of development and deletion of Hbixp diminishes mTOR signaling activity. I particularly like their proteomic approach to identifying HBXIP interacting partners. This allows them to show clearly and convincingly that HBXIP defects can be recapitulated by mutating other ragulator complex proteins. However, it is not clear what specific effects are primary or secondary to the phenotype, and how does mTOR regulate stem-cell renewal or differentiation.

Major concerns:

- The phenotype of the mouse post-implantation mutants is not clear and the pattern of expression of Hbxip at E7.5 and E8.5 in wild-type embryos is not well described (Figure 5). The authors could co-stain HBIXP at the onset of the phenotype (E7.5) together with markers of ectoderm and endoderm to demonstrate that HBIXP is expressed in the epiblast and whether there is a downregulation of ectoderm and endoderm markers in the mutant embryos. In addition, it would be good that the authors perform statistics on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios.

- The role of HBIXP in the regulation of OCT4 seems contradictory in the mouse model in comparison with their claims/findings in mouse stem cells. Mutant embryos seem to arrest/delay their development from ~E7.5 and they observe that embryos at E8.5 retain OCT4 expression (Figure 5). In mouse stem cells the authors state that "The mRNA levels of pluripotency genes, Nanog, Oct4, and Sox2, as well as the protein levels of Nanog and Oct4, are declined in Hbxip KO ESCs (Figure 2B and E)". Is OCT4 reduced on blastocyst mutants and is OCT4 expression maintained during differentiation in stem cells? In addition, whereas in Figure 2B the authors show downregulation of OCT4 in two separate stem cell clones, the levels of OCT4 protein in one of the clones (H-/- -1) in Figure 3G seem unchanged. How do the authors explain these differences between stem cell experiments? How do the authors interpret these stage-specific (or in vivo versus in vitro) differences in OCT4 regulation?

- It is unclear how do the authors think that HBIXP regulates stem cell renewal and differentiation through activation of mTOR. Are the HBIXP mutant cells dying and/or differentiating into endoderm, either primitive or definitive endoderm? HBIXP immunostainings in Figure 5a seem to indicate that HBIXP is expressed in the visceral endoderm, which derives mostly from the primitive endoderm in the blastocyst. Perhaps the HBIXP mutant embryos lose the visceral endoderm, and it does not allow the embryo to develop further. In stem cells, this effect may not be identifiable, and the authors may need to use extraembryonic ectoderm (XEN) cells.

- As The Ragulator complex is required for the activation of mTORC1 by amino acids, can the authors mimic the phenotype by reducing amino acid levels during pluripotency/differentiation?

Minor concerns:

- The role of HBIXP in trophectoderm (TE) formation at the blastocyst stage is not relevant to understanding the mouse mutant phenotype, as the zygotic mutant dies at post-implantation stages. In addition, the in vitro findings of TE differentiation are likely to be a misinterpretation. In mice, it has been extensively described that embryonic stem cells rarely contribute to trophectoderm-derived lineage (PMID: 33420491). Thus, the interpretation of trophectoderm differentiation using Cdx2 and Lef1 in stem cell differentiation is not accurate. In the context of stem cell differentiation, these two markers are likely markers of primitive streak or gastrulation.

- The authors show that the HBIXP knock-out mice are normal at the blastocyst stage, and they show that this is due to a compensatory effect due to maternal protein expression during pre-implantation. This data is nice but doesn't add anything to the understanding of the mutant phenotype.

- In the text it reads "in undifferentiated ESCs, Hbxip KO suppresses the expression of ectodermal, mesodermal and trophectodermal markers, such as Nestin, Celsr, T, Dlx3, Lef1, and Cdx2, whereas

mesodermal marker Gata6 is activated by Hbxip KO (Figure 2F)." In Figure 2F, Gata 6 is labeled as an endoderm marker. Is Gata 6 an endoderm or a mesoderm marker?

- The authors use pS6K1 as a readout of mTOR activity. mTOR regulates other factors such as ULK1 or 4EBP1. Are these also altered in their stem cell models during pluripotency or differentiation?

- There is a missing figure legend for embryos in "c" in Figure 5.

- There is no validation of loss of protein expression in TSC1, Lamtor 3 and Lamtor 4 mutant stem cells.

- There is no number (n) of embryos used for any experiment. The stem cell experiments (qPCR, western blots) also lack information about the number of biological or experimental repeats.

- The authors could provide the raw western blots as supplemental material to validate the selected results on the figures.

- There is no reference to the EIIa-Cre line they used.

Significance

Understanding the role of genes during development may help us understand fundamental principles about how genes make bodies. In addition, the use of stem cell models allows for the characterization of phenotypes that might be challenging to study in vivo. In this work, the authors show that HBIXP acts through the regulator complex rather than as a transcriptional coactivator. Further, this work suggests that the Raptor complex may be instrumental for gastrulation, and that mTOR signaling may regulate self-renewal and differentiation of epiblast cells. These findings are of potential interest as mTOR mouse mutants do not progress beyond peri-implantation, thus precluding the study of mTOR function during post-implantation development.

I am a developmental biologist with ample expertise in mammalian development and stem cells. I think this work is potentially interesting but major revisions should be made. I would also encourage the authors to carefully revise the labelling of figures and to make sure that the results are accurately interpreted, with no contradictory results in them.

Author response to reviewers' comments

1. General Statements [optional]

We would like to thank all three reviewers for the evaluation of our research and comments to our manuscript. Their comments are highly appreciated and addressed as described below.

2. Description of the planned revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript the authors study the role of Hbxip in early development, by performing a KO of this protein and using ESCs of these embryos, in a mouse model.
They conclude with classical loss of function approaches that Hbxip KO is embryonic lethal, and affects differentiation possibly acting via the mTOR signalling pathway, notably MTORC1. This is interesting work, although several aspects should be considered as there may be simpler explanations to the results than what the authors propose:
1- While the authors show some evidence for limited self renewal in the ESC KO line, cell cycle analyses and apoptosis should also be monitored, as should the stability of the KO line in term of karyotype. This is especially important as Hbxip also seems to interact with microtubules/centrosomes and other biological functions in cells (notably in cancer) that have little to do with differentiation. The authors should control for these aspects, as they may be

skewing the data and actually lead to different interpretations of the data. The fact that the authors perform other loss of function experiments later on (related to MTORC1) function does not resolve this issue. They actually could form the core of a totally different paper not focused on Hbxip at all.

As suggested, we will perform cell cycle, apoptosis and karyotype analyses in the *Hbxip* KO ESC lines.

2- The authors should show EB differentiation and also differentiation markers at the protein level (WB or ICC). as it currently stands this data is not convincing at this level. THe same is true for pulipotency markers as localization (nuclear or not) is equally important, besides levels of expression. Besides the existing WB these ICC images must be shown.

As suggested, WB or ICC will be performed to detect the expression and localization of differentiation and/or pluripotency markers at the protein level, particularly in the embryo.

3-To fully validate the recovery data the WB (3G) should be quantified, and the authors show that differentiation is now normal.

As pointed out by reviewer #3, we will perform more WB experiments to clearly demonstrate the downregulation of Oct4 in *Hbxip*$^{-/-}$ ESCs (Figure 3G). Meanwhile, we will quantify the WB data in Figure 3G.

4- Localization of Hbxip should be shown by ICC in Fig 6.

We will perform immunofluorescence staining to detect the localization of Hbxip in ESCs.

Reviewer #1 (Significance (Required)):

This would be interesting to both people in the pluripotency field and mammalian embryo development, as well as in mTOR signalling and therefore metabolism. This are things I have some expertise in.
Although some aspects need improvement and more experiments this is interesting, if unspectacular, work that will be of interest to a specialized audience.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Qin et al dissect the function of Hbxip in early mouse development and embryonic stem cells. But using a variety of loss-of-function (LOF) models, the authors present an involvement of Hbxip in differentiation and exit from pluripotency in post-implantation embryos. In ES cells, Hbxip KO leads to self-renewal defects and skewed differentiation. The authors show that Hbxip regulates mTORC1 activity in ES cells and argue that this could be the mechanism for embryonic lethality of Hbxip KO embryos. In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function. The results are insightful, however relevant controls and rigorous statistical testing are often missing. Embryo and ES phenotypes contradict each other, and the claim for perturbed differentiation is not sufficiently supported. Overall, the paper could be more streamlined. If the authors focus on key findings and provide definitive supportive evidence, the study would be more informative.

Major comments
o Hbxip is not essential for ES differentiation, since the Hbxip KO ES cells do differentiate. Therefore the title is not correct.

Even though *Hbxip*$^{-/-}$ ESCs are able to differentiate, these ESCs fail to fully activate several differentiation markers, indicating differentiation defects. Thus, Hbxip is required for proper differentiation of ESCs. To be clarified, we have changed the title to “Hbxip (Lamtor5) is essential for embryogenesis and regulates embryonic stem cell differentiation through activation mTORC1”.
o For all KOs, the evidence for the loss of protein is missing. In Fig.1, only genotyping results are shown, but a staining or western blot showing loss of the protein is not presented. In Fig. 2, the shown western blot is not convincing. The authors themselves show higher expression of Hbxip in

these cells (Fig 6A), therefore a better western blot is required. For the KO cell lines presented in Figs. 6 and 7, no evidence of functional KO at the protein level.

Hbxip KO mice leads to embryonic lethality around E7.5. Thus, the evidence for knocking out Hbxip protein requires WB or IHC in early and peri-implantation embryos. Due to limited embryo materials and contamination from maternal tissue, we did not perform WB to demonstrate the knockout of Hbxip protein. Nevertheless, IHC was performed to show the absence of Hbxip protein in Figure 4 of the revised manuscript.

We have provided a better Western blot image in Figure 2B to demonstrate the KO of Hbxip protein.

Many factors, such as the amount of protein loading, the efficiency of antibody binding, and the exposure time, vary among independent Western blot assay. Thus, it is inappropriate to compare the expression level of a given protein between different blots. And our data did not support that Hbxip is expressed higher in the cells shown in Figure 6A than in the cells shown in Figure 2B.

We will perform Western blot to show the knockout of Tsc1, Lamtor3 an Lamtor4 proteins in corresponding KO ESCs.

o Similarly, for the overexpression/rescue shown in Fig. 3G as well as for the siRNA-KD shown in Fig.4 no evidence is provided to show that these methods work. Also in Fig. 4 it is unclear which control is used. In general, the authors need to provide more detailed explanations in the methods section and direct evidence supporting their KO/KD/OE models.

We have provided the WB data to show the overexpression of Hbxip in Figure S2C.

Both reviewer #2 and #3 raised the issue that ESCs do not differentiate into the trophectodermal lineage under our experimental condition, and suggested us to remove Figure 4. As suggested, Figure 4 is removed. Nevertheless, we indeed performed quantitative RT-PCR to demonstrate the knockdown efficiency of Hbxip siRNAs in ESCs (data not shown), before embryo microinjection of these siRNAs.

o qPCRs: Applied statistical tests seem to be T-test, which is not appropriate for the data presented. The authors need to apply two-way ANOVA because of the testing of multiple genes in multiple samples.

As suggested, we have performed stastistical analysis by two-way ANOVA for qPCR data.

o The biggest discrepancy in the paper is the different outcome of Hbxip KO in the embryo and in ES cells. In the embryo, the KO leads to the inability to differentiate and persistence of pluripotency at E8.5. However, the KO ES cells lose self-renewal potential and have to blockage of differentiation. What is the author's take on this? ES data clearly shows that reduced proliferation due to Hbxip LOF is not an obstacle against exit from pluripotency or differentiation. Therefore the authors' argument of a proliferation defect causing persistent pluripotency at E8.5 does not hold. The embryo data points to gastrulation defects. One can envision a scenario where the KO epiblast does not initiate gastrulation and as a result does not exit pluripotency. Staining E7.5 embryos for gastrulation-associated genes would provide a clearer picture.

We agreed that there is some discrepancy in downregulation of Oct4 between *Hbxip*$^{-/-}$ ESCs differentiation and *Hbxip*$^{-/-}$ embryo development. However, *in vitro* differentiation of ESCs cannot capture all the features of *in vivo* embryo development, even though it mimicks *in vivo* development of embryos.

Our point here is that both *Hbxip*$^{-/-}$ ESCs and *Hbxip*$^{-/-}$ epiblast have differentiation defects. *Hbxip*$^{-/-}$ ESCs fail to activate differentiation markers during differentiation, and *Hbxip*$^{-/-}$ epiblast fail to shut down Oct4 at E8.5. Of course, the suggestion from Reviewer #2 is a good one. We will stain E7.5 and E8.5 embryos for gastrulation-associated genes, such as definitive endoderm marker Sox17 and Ectoderm marker Nestin, to demonstrate the differentiation defects of *Hbxip*$^{-/-}$ epiblast.

o Fig. 2/3: Mouse ES cells do not spontaneously differentiate to TE, unless driven by a major TE TF. The shown genes categorized as TE in Fig. 2/3 are not only TE-specific. Therefore the authors should decategorize these genes as TE markers and reinterpret the data as such.

Both reviewer #2 and #3 have the same concern. As suggested, TE markers in Figure 2 and 3 have been removed.

o Fig. 4 is only loosely connected to the rest of the story. The shown si-injected embryos are not blastocysts, it is not indicated which control is used, no evidence of actual KD is presented, no evidence of maternal transcripts in the KO embryos are presented, and as such the whole argument is not convincing. If the authors want to keep this figure, then supporting evidence is necessary. Alternatively, the figure may be removed.

Again, Both reviewer #2 and #3 have the same concern. As suggested, Figure 4 has been deleted.

o Almost all differentiation analysis relies on RNA expression analysis. Supporting evidence at the protein level is necessary to substantiate the claims. The authors can stain for markers of different lineages and quantify.

As suggested, WB or ICC will be performed to detect the expression of differentiation markers at the protein level, particularly in the embryo.

o If mTORC1 activation is mediated by Hbxip function, but there is still mTORC1 activity in Hbxip KO cells, then Hbxip is probably required to promote mTORC1 activity above a certain threshold. mTOR heterozygous KO is not lethal, similar to Hbxip heterozygous KO. So mTOR dosage does not seem to be very critical for embryonic development. Since the authors' argument is that Hbxip functions by regulating TORC1 activity, it would be helpful to see the levels of mTORC1 activity in Hbxip homozygous vs heterozygous KOs vs wt embryos. The authors need to at least discuss how Hbxip/mTOR dosage regulates development in the discussion.

We have added Figure S3 to show the reduced mTORC1 activity in *Hbxip*$^{-/-}$ E8.5 embryos, indicated by IF of p-S6K1. However, we could not distinguish heterozygous KO from WT embryos by IHC staining of Hbxip.

mTOR heterozygous KO might reduce the mTORC1 activity by half, while the effect of *Hbxip* heterozygous KO on the mTORC1 activity is unpredictable. Presumably, *Hbxip* heterozygous KO reduces the level of Hbxip protein by half. However, it is unknown whether the endogenous Hbxip is in excess or in the minimum requirement for the activation of mTORC1. If it is in excess, *Hbxip* heterozygous KO might not affect the mTORC1 activity. If it is at the minimum level, *Hbxip* heterozygous KO might reduce the mTORC1 activity by half. Given that both *mTOR* heterozygous KO and *Hbxip* heterozygous KO mice have no obvious phenotype, we only focus on *Hbxip* homozygous KO effect.

Minor comments
o For westerns please provide size markers and uncropped images (at least as a reviewer figure).

We have marked size markers in Western blots. And uncropped images will be provided if the journal requires so.

o In general, the differences in the expression levels of lineage markers are quite small and hard to read as presented in the figures. The data could be better understood if the authors plot log2FC to show fold change in expression.

We have plotted Log_2FC to show fold change in expression.

o In Fig. 3a, b, it is better to plot expression of each gene relative to its levels in ES cells. This way the reader can see that lineage markers are actually upregulated during differentiation of wt cells, but perturbed in KO cells if that is the case.

We have plotted the expression of each gene relative to its levels in ESCs in Figure 3A and 3B.

o Please show individual data points for qPCR graphs.

It is widely acceptable to show the average and standard deviation for qPCR graphs. We prefer not to add individual data points, because it will make the plots crowded.

o What is the staining in Fig. 1D? No explanation is provided.

Figure 1D just shows dissected E6.5-9.5 embryos without any staining. We have clarified this point in the figure legend.

o Please explain the reason for using EIIa-Cre in the text. This is crucial information to understand the nature of the KO in the embryo.

EIIa-Cre mice were used to generate whole body and germ line knockout of floxed allele. We have described it in the methods now.

o Fig. 2H shows GO terms for downregulated genes. Please also show the same analysis for upregulated genes, as this subset comprises a large portion of the differentially expressed genes.

We have showed GO analysis of upregulated genes in Figure S2A and S2B.

o Fig. 6D is a bit misleading because in 6E and F we see substantial pS6K1 in Hbxip KO, although it may be reduced compared to wt. But definitely not as reduced as in 6A.

To be clarified, we have changed the WB images with similar exposure time. Nevertheless, it is inappropriate to compare the expression level of a given protein between different blots.

Reviewer #2 (Significance (Required)):

In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function.

Role of different aspects of mTOR biology in early development is not well understood. Therefore the advance here could be useful.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:
Qin, Ni, et al. investigate the role of Hepatitis B X-interacting protein (HBXIP, also known as LAMTOR5) during development. To do so, they use a conditional Hbxip mouse model and delete exon 2 of the gene upon recombination with a constitutive Cre. They find that Hbxip mutant embryos are delayed and die ~E7.5. They also make use of Hbxip knock out mouse embryonic stem cells to investigate the potential differentiation defects, and through co-immunoprecipitation coupled to proteomics and epistatic experiments, they conclude that Hbxip plays a role in epiblast differentiation, interacts with ragulator complex proteins, and activates mTORC1 (pS6K1) signalling pathway.
Their findings suggest that Hbixp impacts differentiation at early stages of development and deletion of Hbixp diminishes mTOR signaling activity. I particularly like their proteomic approach to identifying HBXIP interacting partners. This allows them to show clearly and convincingly that HBXIP defects can be recapitulated by mutating other ragulator complex proteins. However, it is not clear what specific effects are primary or secondary to the phenotype, and how does mTOR regulate stem-cell renewal or differentiation.

Major concerns:

- The phenotype of the mouse post-implantation mutants is not clear and the pattern of expression of Hbxip at E7.5 and E8.5 in wild-type embryos is not well described (Figure 5). The authors could

co-stain HBIXP at the onset of the phenotype (E7.5) together with markers of ectoderm and endoderm to demonstrate that HBIXP is expressed in the epiblast and whether there is a downregulation of ectoderm and endoderm markers in the mutant embryos. In addition, it would be good that the authors perform statistics on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios.

As suggested, we will co-stain HBIXP together with markers of three germ layers, such as Nestin, Sox17 and T, in WT and *Hbxip*$^{-/-}$ embryos at E7.5.

We have performed $\chi 2$ test on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios (Figure 1C).

- The role of HBIXP in the regulation of OCT4 seems contradictory in the mouse model in comparison with their claims/findings in mouse stem cells. Mutant embryos seem to arrest/delay their development from ~E7.5 and they observe that embryos at E8.5 retain OCT4 expression (Figure 5). In mouse stem cells the authors state that "The mRNA levels of pluripotency genes, Nanog, Oct4, and Sox2, as well as the protein levels of Nanog and Oct4, are declined in Hbxip KO ESCs (Figure 2B and E)". Is OCT4 reduced on blastocyst mutants and is OCT4 expression maintained during differentiation in stem cells? In addition, whereas in Figure 2B the authors show downregulation of OCT4 in two separate stem cell clones, the levels of OCT4 protein in one of the clones (H-/- -1) in Figure 3G seem unchanged. How do the authors explain these differences between stem cell experiments? How do the authors interpret these stage-specific (or in vivo versus in vitro) differences in OCT4 regulation?

In vitro differentiation of ESCs cannot capture all the features of *in vivo* embryo development, even though it mimicks *in vivo* development of embryos. Thus, the seemly discrepancy of Oct4 regulation in ESC differentiation and embryo development might be explained by the different experimental setting.

The downregulation of Oct4 in *Hbxip*$^{-/-}$-1 ESCs (Figure 3G) was subtle. We will perform more WB experiments to clearly demonstrate the downregulation of Oct4 in *Hbxip*$^{-/-}$ ESCs (Figure 3G).

- It is unclear how do the authors think that HBIXP regulates stem cell renewal and differentiation through activation of mTOR. Are the HBIXP mutant cells dying and/or differentiating into endoderm, either primitive or definitive endoderm? HBIXP immunostainings in Figure 5a seem to indicate that HBIXP is expressed in the visceral endoderm, which derives mostly from the primitive endoderm in the blastocyst. Perhaps the HBIXP mutant embryos lose the visceral endoderm, and it does not allow the embryo to develop further. In stem cells, this effect may not be identifiable, and the authors may need to use extraembryonic ectoderm (XEN) cells.

Our data suggested that knockout of Hbxip reduces mTORC1 activity, consequently leading to slower self-renewal and differentiation defects of ESCs.

It has been reported that inhibition of mTOR leads to embryonic diapause (Nature, 2016, 540: 119–123). Given that knockout of Hbxip reduces mTORC1 activity, we speculated that *Hbxip*$^{-/-}$ epiblast fails to exit from pluripotency and initiate gastrulation, in addition to cell proliferation defect. So far, we only provided the evidence that Oct4 is not down-regulated in E8.5 *Hbxip*$^{-/-}$ embryo. To demonstrate gastrulation defects in *Hbxip*$^{-/-}$ embryos, we will perform IHC or IF for markers of three germ layers, such as Nestin, Sox17 and T, in WT and *Hbxip*$^{-/-}$ embryos at E7.5.

- As The Ragulator complex is required for the activation of mTORC1 by amino acids, can the authors mimic the phenotype by reducing amino acid levels during pluripotency/differentiation?

As suggested, we will try to test whether reduced amino acid levels will yield similar phenotypes of *Hbxip*$^{-/-}$ ESCs.

Minor concerns:

- The role of HBIXP in trophectoderm (TE) formation at the blastocyst stage is not relevant to

understanding the mouse mutant phenotype, as the zygotic mutant dies at post-implantation stages. In addition, the in vitro findings of TE differentiation are likely to be a misinterpretation. In mice, it has been extensively described that embryonic stem cells rarely contribute to trophectoderm-derived lineage (PMID: 33420491). Thus, the interpretation of trophectoderm differentiation using Cdx2 and Lef1 in stem cell differentiation is not accurate. In the context of stem cell differentiation, these two markers are likely markers of primitive streak or gastrulation.

Both reviewer #2 and #3 have the same concern. As suggested, TE markers in Figure 2 and 3 have been removed.

- In the text it reads "in undifferentiated ESCs, Hbxip KO suppresses the expression of ectodermal, mesodermal and trophectodermal markers, such as Nestin, Celsr, T, Dlx3, Lef1, and Cdx2, whereas mesodermal marker Gata6 is activated by Hbxip KO (Figure 2F)." In Figure 2F, Gata 6 is labeled as an endoderm marker. Is Gata 6 an endoderm or a mesoderm marker?

Thanks for pointing out our mistake. Gata6 is an endoderm marker. We have corrected it.

- The authors use pS6K1 as a readout of mTOR activity. mTOR regulates other factors such as ULK1 or 4EBP1. Are these also altered in their stem cell models during pluripotency or differentiation?

We have added the data of 4EBP1 and p-4EBP1 in Figure S2D. It is consistent with the data of p-S6K1.

- There is a missing figure legend for embryos in "c" in Figure 5.

We have revised the figure legend to annotate a, a', b, b', c, and c'.

- There is no validation of loss of protein expression in TSC1, Lamtor 3 and Lamtor 4 mutant stem cells.

We will perform Western blot to show the knockout of Tsc1, Lamtor3 an Lamtor4 proteins in corresponding KO ESCs.

- There is no number (n) of embryos used for any experiment. The stem cell experiments (qPCR, western blots) also lack information about the number of biological or experimental repeats.

We have added the information about the number (n) of embryos and experimental repeats in figure legends.

- The authors could provide the raw western blots as supplemental material to validate the selected results on the figures.

Uncropped raw Western blots will be provided if the journal requires so.

- There is no reference to the EIIa-Cre line they used.

EIIa-Cre mice were used to generate whole body and germ line knockout of floxed allele. We have described it and cited the reference in the methods.

Reviewer #3 (Significance (Required)):

Understanding the role of genes during development may help us understand fundamental principles about how genes make bodies. In addition, the use of stem cell models allows for the characterization of phenotypes that might be challenging to study in vivo. In this work, the authors show that HBIXP acts through the regulator complex rather than as a transcriptional coactivator. Further, this work suggests that the Raptor complex may be instrumental for gastrulation, and that mTOR signaling may regulate self-renewal and differentiation of epiblast cells. These findings are of potential interest as mTOR mouse mutants do not progress beyond peri-implantation, thus

precluding the study of mTOR function during post-implantation development.

I am a developmental biologist with ample expertise in mammalian development and stem cells. I think this work is potentially interesting but major revisions should be made. I would also encourage the authors to carefully revise the labelling of figures and to make sure that the results are accurately interpreted, with no contradictory results in them.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Reviewer #2

Major comments
o Hbxip is not essential for ES differentiation, since the Hbxip KO ES cells do differentiate. Therefore the title is not correct.

Even though *Hbxip*$^{-/-}$ ESCs are able to differentiate, these ESCs fail to fully activate several differentiation markers, indicating differentiation defects. Thus, Hbxip is required for proper differentiation of ESCs. To be clarified, we have changed the title to “Hbxip (Lamtor5) is essential for embryogenesis and regulates embryonic stem cell differentiation through activation mTORC1”.

o For all KOs, the evidence for the loss of protein is missing. In Fig.1, only genotyping results are shown, but a staining or western blot showing loss of the protein is not presented. In Fig. 2, the shown western blot is not convincing. The authors themselves show higher expression of Hbxip in these cells (Fig 6A), therefore a better western blot is required. For the KO cell lines presented in Figs. 6 and 7, no evidence of functional KO at the protein level.

We have provided a better Western blot image in Figure 2B to demonstrate the KO of Hbxip protein.

o Similarly, for the overexpression/rescue shown in Fig. 3G as well as for the siRNA-KD shown in Fig.4 no evidence is provided to show that these methods work. Also in Fig. 4 it is unclear which control is used. In general, the authors need to provide more detailed explanations in the methods section and direct evidence supporting their KO/KD/OE models.

We have provided the WB data to show the overexpression of Hbxip in Figure S2C.

Both reviewer #2 and #3 raised the issue that ESCs do not differentiate into the trophectodermal lineage under our experimental condition, and suggested us to remove Figure 4. As suggested, Figure 4 is removed.

o qPCRs: Applied statistical tests seem to be T-test, which is not appropriate for the data presented. The authors need to apply two-way ANOVA because of the testing of multiple genes in multiple samples.

As suggested, we have performed stastistical analysis by two-way ANOVA for qPCR data.

o Fig. 2/3: Mouse ES cells do not spontaneously differentiate to TE, unless driven by a major TE TF. The shown genes categorized as TE in Fig. 2/3 are not only TE-specific. Therefore the authors should decategorize these genes as TE markers and reinterpret the data as such.

Both reviewer #2 and #3 have the same concern. As suggested, TE markers in Figure 2 and 3 have been removed.

o Fig. 4 is only loosely connected to the rest of the story. The shown si-injected embryos are not blastocysts, it is not indicated which control is used, no evidence of actual KD is presented, no evidence of maternal transcripts in the KO embryos are presented, and as such the whole argument is not convincing. If the authors want to keep this figure, then supporting evidence is necessary. Alternatively, the figure may be removed.

Again, Both reviewer #2 and #3 have the same concern. As suggested, Figure 4 has been deleted.

o If mTORC1 activation is mediated by Hbxip function, but there is still mTORC1 activity in Hbxip KO cells, then Hbxip is probably required to promote mTORC1 activity above a certain threshold. mTOR heterozygous KO is not lethal, similar to Hbxip heterozygous KO. So mTOR dosage does not seem to be very critical for embryonic development. Since the authors' argument is that Hbxip functions by regulating TORC1 activity, it would be helpful to see the levels of mTORC1 activity in Hbxip homozygous vs heterozygous KOs vs wt embryos. The authors need to at least discuss how Hbxip/mTOR dosage regulates development in the discussion.

We have added Figure S3 to show the reduced mTORC1 activity in *Hbxip*$^{-/-}$ E8.5 embryos, indicated by IF of p-S6K1. However, we could not distinguish heterozygous KO from WT embryos by IHC staining of Hbxip.

Minor comments
o For westerns please provide size markers and uncropped images (at least as a reviewer figure).

We have marked size markers in Western blots. And uncropped images will be provided if the journal requires so.

o In general, the differences in the expression levels of lineage markers are quite small and hard to read as presented in the figures. The data could be better understood if the authors plot log2FC to show fold change in expression.

We have plotted Log_2FC to show fold change in expression.

o In Fig. 3a, b, it is better to plot expression of each gene relative to its levels in ES cells. This way the reader can see that lineage markers are actually upregulated during differentiation of wt cells, but perturbed in KO cells if that is the case.

We have plotted the expression of each gene relative to its levels in ESCs in Figure 3A and 3B.

o What is the staining in Fig. 1D? No explanation is provided.

Figure 1D just shows dissected E6.5-9.5 embryos without any staining. We have clarified this point in the figure legend.

o Please explain the reason for using EIIa-Cre in the text. This is crucial information to understand the nature of the KO in the embryo.

EIIa-Cre mice were used to generate whole body and germ line knockout of floxed allele. We have described it in the methods now.

o Fig. 2H shows GO terms for downregulated genes. Please also show the same analysis for upregulated genes, as this subset comprises a large portion of the differentially expressed genes.

We have showed GO analysis of upregulated genes in Figure S2A and S2B.

o Fig. 6D is a bit misleading because in 6E and F we see substantial pS6K1 in Hbxip KO, although it may be reduced compared to wt. But definitely not as reduced as in 6A.

To be clarified, we have changed the WB images with similar exposure time. Nevertheless, it is inappropriate to compare the expression level of a given protein between different blots.

Reviewer #3

Major concerns:

- The phenotype of the mouse post-implantation mutants is not clear and the pattern of expression

of Hbxip at E7.5 and E8.5 in wild-type embryos is not well described (Figure 5). The authors could co-stain HBIXP at the onset of the phenotype (E7.5) together with markers of ectoderm and endoderm to demonstrate that HBIXP is expressed in the epiblast and whether there is a downregulation of ectoderm and endoderm markers in the mutant embryos. In addition, it would be good that the authors perform statistics on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios.

We have performed $\chi 2$ test on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios (Figure 1C).

Minor concerns:

- The role of HBIXP in trophectoderm (TE) formation at the blastocyst stage is not relevant to understanding the mouse mutant phenotype, as the zygotic mutant dies at post-implantation stages. In addition, the in vitro findings of TE differentiation are likely to be a misinterpretation. In mice, it has been extensively described that embryonic stem cells rarely contribute to trophectoderm-derived lineage (PMID: 33420491). Thus, the interpretation of trophectoderm differentiation using Cdx2 and Lef1 in stem cell differentiation is not accurate. In the context of stem cell differentiation, these two markers are likely markers of primitive streak or gastrulation.

Both reviewer #2 and #3 have the same concern. As suggested, TE markers in Figure 2 and 3 have been removed.

- In the text it reads "in undifferentiated ESCs, Hbxip KO suppresses the expression of ectodermal, mesodermal and trophectodermal markers, such as Nestin, Celsr, T, Dlx3, Lef1, and Cdx2, whereas mesodermal marker Gata6 is activated by Hbxip KO (Figure 2F)." In Figure 2F, Gata 6 is labeled as an endoderm marker. Is Gata 6 an endoderm or a mesoderm marker?

Thanks for pointing out our mistake. Gata6 is an endoderm marker. We have corrected it.

- The authors use pS6K1 as a readout of mTOR activity. mTOR regulates other factors such as ULK1 or 4EBP1. Are these also altered in their stem cell models during pluripotency or differentiation?

We have added the data of 4EBP1 and p-4EBP1 in Figure S2D. It is consistent with the data of p-S6K1.

- There is a missing figure legend for embryos in "c" in Figure 5.

We have revised the figure legend to annotate a, a', b, b', c, and c'.

- There is no number (n) of embryos used for any experiment. The stem cell experiments (qPCR, western blots) also lack information about the number of biological or experimental repeats.

We have added the information about the number (n) of embryos and experimental repeats in figure legends.

- There is no reference to the EIIa-Cre line they used.

EIIa-Cre mice were used to generate whole body and germ line knockout of floxed allele. We have described it and cited the reference in the methods.

4. Description of analyses that authors prefer not to carry out

Reviewer #2

o Please show individual data points for qPCR graphs.

It is widely acceptable to show the average and standard deviation for qPCR graphs. We prefer not to add individual data points, because it will make the plots crowded.

Original submission

First decision letter

MS ID#: DEVELOP/2022/200527

MS TITLE: Hbxip (Lamtor5) is essential for embryogenesis and regulates embryonic stem cell differentiation through activating mTORC1

AUTHORS: Yan Qin, Peiling Ni, Qingye Zhang, Xiao Wang, Xiaoling Du, Zixi Yin, Lingling Wang, Lihong Ye, and Lingyi Chen

Thank you for submitting your study to Development via Review Commons. I have read the paper, referees' reports and your revision plan.

I agree that the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines you have outlined in your plan, I will be happy to receive a revised version of the manuscript. I will send your fully revised paper to the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make 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 attend to all of the reviewers' comments and 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. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

First revision

Author response to reviewers' comments

We would like to thank all three reviewers for the evaluation of our research and comments to our manuscript. Their comments are highly appreciated and addressed as described below.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript the authors study the role of Hbxip in early development, by performing a KO of this protein and using ESCs of these embryos, in a mouse model.
They conclude with classical loss of function approaches that Hbxip KO is embryonic lethal, and affects differentiation possibly acting via the mTOR signalling pathway, notably MTORC1. This is

interesting work, although several aspects should be considered as there may be simpler explanations to the results than what the authors propose:

1- While the authors show some evidence for limited self renewal in the ESC KO line, cell cycle analyses and apoptosis should also be monitored, as should the stability of the KO line in term of karyotype. This is especially important as Hbxip also seems to interact with microtubules/centrosomes and other biological functions in cells (notably in cancer) that have little to do with differentiation. The authors should control for these aspects, as they may be skewing the data and actually lead to different interpretations of the data. The fact that the authors perform other loss of function experiments later on (related to MTORC1) function does not resolve this issue. They actually could form the core of a totally different paper not focused on Hbxip at all.

As suggested, we performed cell cycle, apoptosis and karyotype analyses in the *Hbxip* KO ESC lines (Fig. S1D-F), and the data excluded the possibility that the phenotype we observed is due to the instability of *Hbxip* KO ESCs. In addition, the rescue experiments (Fig. 3G, H) further argue against the possibility mentioned above.

2- The authors should show EB differentiation and also differentiation markers at the protein level (WB or ICC). as it currently stands this data is not convincing at this level. THe same is true for pulipotency markers as localization (nuclear or not) is equally important, besides levels of expression. Besides the existing WB, these ICC images must be shown.

As suggested, we performed immunofluorescence (IF) to to detect the expression and localization of germ layer markers, Nestin, T, and Gata4, and/or pluripotency marker Oct4 in E7.5 embryos (Fig. 4). And these data clearly demonstrated the defects in ectodermal and mesodermal differentiation.

ESCs were used as an *in vitro* system for mechanistic investigation. Current quantitative RT- PCR data (Fig. 3) provided preliminary evidence that *Hbxip* KO leads to the differentiation defect in ESCs, implying differentiation defect of *Hbxip*$^{-/-}$ epiblast. Given that we have provided IF data in E7.5 embryos to demonstrate the differentiation defect of *Hbxip*$^{-/-}$ epiblast, it is not necessary to perform IF in EBs.

We indeed tried IF to detect Nanog and Oct4 in WT and *Hbxip*$^{-/-}$ ESCs. However, no difference was observed between WT and *Hbxip*$^{-/-}$ ESCs. It is most likely that IF is a less quantitative assay, and unable to detect slightly reduced expression of Nanog and Oct4. Moreover, non-specific binding of Nanog and Oct4 antibodies might enhance the background signal. This issue is more severe in IF than in WB, because most non-specific signals are separated from specific signals due to the difference in molecular weight.

3- To fully validate the recovery data the WB (3G) should be quantified, and the authors show that differentiation is now normal.

As pointed out by reviewer #3, we provided new WB data to clearly demonstrate the downregulation of Oct4 in *Hbxip*$^{-/-}$ ESCs (Fig. 3G). Meanwhile, we quantified the WB data in Fig. 3G.

4- Localization of Hbxip should be shown by ICC in Fig 6.

We performed IF staining to detect the localization of Hbxip in ESCs. Yet, the data is not convincing, most likely due to non-specific binding of Hbxip antibody. Thus, we preferred not to include IF data. In addition, WB with nuclear and cytoplasmic fractions is sufficient to demonstrate the cytoplasmic localization of Hbxip in ESCs (Fig. 5A).
Reviewer #1 (Significance (Required)):

This would be interesting to both people in the pluripotency field and mammalian embryo development, as well as in mTOR signalling and therefore metabolism. This are things I have some expertise in.
Although some aspects need improvement and more experiments this is interesting, if unspectacular, work that will be of interest to a specialized audience.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Qin et al dissect the function of Hbxip in early mouse development and embryonic stem cells. But using a variety of loss-of-function (LOF) models, the authors present an involvement of Hbxip in differentiation and exit from pluripotency in post-implantation embryos. In ES cells, Hbxip KO leads to self-renewal defects and skewed differentiation. The authors show that Hbxip regulates mTORC1 activity in ES cells and argue that this could be the mechanism for embryonic lethality of Hbxip KO embryos. In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function. The results are insightful, however relevant controls and rigorous statistical testing are often missing. Embryo and ES phenotypes contradict each other, and the claim for perturbed differentiation is not sufficiently supported. Overall, the paper could be more streamlined. If the authors focus on key findings and provide definitive supportive evidence, the study would be more informative.

Major comments
o Hbxip is not essential for ES differentiation, since the Hbxip KO ES cells do differentiate. Therefore the title is not correct.

Even though Hbxip ESCs are able to differentiate, these ESCs fail to fully activate several differentiation markers, indicating differentiation defects. Thus, Hbxip is required for proper differentiation of ESCs. To be clarified, we have changed the title to “Hbxip is essential for embryogenesis and regulates embryonic stem cell differentiation through activation mTORC1”

o For all KOs, the evidence for the loss of protein is missing. In Fig.1, only genotyping results are shown, but a staining or western blot showing loss of the protein is not presented. In Fig. 2, the shown western blot is not convincing. The authors themselves show higher expression of Hbxip in these cells (Fig 6A), therefore a better western blot is required. For the KO cell lines presented in Figs. 6 and 7, no evidence of functional KO at the protein level.

Hbxip KO mice leads to embryonic lethality around E7.5. Thus, the evidence for knocking out Hbxip protein requires WB or IHC in early and peri-implantation embryos. Due to limited embryo materials and contamination from maternal tissue, we did not perform WB to demonstrate the knockout of Hbxip protein. Nevertheless, IHC was performed to show the absence of Hbxip protein in Fig. 4A, B.

We have provided a better Western blot image in Figure 2B to demonstrate the KO of Hbxip protein.

Many factors, such as the amount of protein loading, the efficiency of antibody binding, and the exposure time, vary among independent Western blot assay. Thus, it is inappropriate to compare the expression level of a given protein between different blots. And our data did not support that Hbxip is expressed higher in the cells shown in Figure 6A than in the cells shown in Figure 2B.

We have performed Western blot to show the knockout of Tsc1, Lamtor3 an Lamtor4 proteins in corresponding KO ESCs (Figs S4E, F, S5D, H).

o Similarly, for the overexpression/rescue shown in Fig. 3G as well as for the siRNA-KD shown in Fig.4 no evidence is provided to show that these methods work. Also in Fig. 4 it is unclear which control is used. In general, the authors need to provide more detailed explanations in the methods section and direct evidence supporting their KO/KD/OE models.

We have provided the WB data to show the overexpression of Hbxip in Fig. S2C.

Both reviewer #2 and #3 raised the issue that ESCs do not differentiate into the trophectodermal lineage under our experimental condition, and suggested us to remove Figure 4. As suggested, Figure 4 is removed. Nevertheless, we indeed performed quantitative RT-PCR to demonstrate the knockdown efficiency of Hbxip siRNAs in ESCs (data not shown), before embryo microinjection of these siRNAs.

o qPCRs: Applied statistical tests seem to be T-test, which is not appropriate for the data presented. The authors need to apply two-way ANOVA because of the testing of multiple genes in multiple samples.

As suggested, we have performed stastistical analysis by two-way ANOVA for qPCR data.

o The biggest discrepancy in the paper is the different outcome of Hbxip KO in the embryo and in ES cells. In the embryo, the KO leads to the inability to differentiate and persistence of pluripotency at E8.5. However, the KO ES cells lose self-renewal potential and have to blockage of differentiation. What is the author's take on this? ES data clearly shows that reduced proliferation due to Hbxip LOF is not an obstacle against exit from pluripotency or differentiation. Therefore the authors' argument of a proliferation defect causing persistent pluripotency at E8.5 does not hold. The embryo data points to gastrulation defects. One can envision a scenario where the KO epiblast does not initiate gastrulation and as a result does not exit pluripotency. Staining E7.5 embryos for gastrulation-associated genes would provide a clearer picture.

We agreed that there is some discrepancy in downregulation of Oct4 between *Hbxip*$^{-/-}$ ESCs differentiation and *Hbxip*$^{-/-}$ embryo development. However, *in vitro* differentiation of ESCs cannot recapture all the features of *in vivo* embryo development, even though it mimicks *in vivo* development of embryos.

Our point here is that both *Hbxip*$^{-/-}$ ESCs and *Hbxip*$^{-/-}$ epiblast have differentiation defects. *Hbxip*$^{-/-}$ ESCs fail to activate differentiation markers during differentiation, and *Hbxip*$^{-/-}$ epiblast fail to shut down Oct4 at E8.5. Of course, the suggestion from Reviewer #2 is a good one. We performed IF staining in E7.5 embryos for germ layer markers, such as ectodermal marker Nestin, mesodermal marker T and endodermal marker Gata4. The IF data clearly demonstrated the differentiation defects of *Hbxip*$^{-/-}$ epiblast (Fig. 4C).

o Fig. 2/3: Mouse ES cells do not spontaneously differentiate to TE, unless driven by a major TE TF. The shown genes categorized as TE in Fig. 2/3 are not only TE-specific. Therefore the authors should decategorize these genes as TE markers and reinterpret the data as such.

Both reviewer #2 and #3 have the same concern. As suggested, TE markers in Figs 2, 3 have been removed.

o Fig. 4 is only loosely connected to the rest of the story. The shown si-injected embryos are not blastocysts, it is not indicated which control is used, no evidence of actual KD is presented, no evidence of maternal transcripts in the KO embryos are presented, and as such the whole argument is not convincing. If the authors want to keep this figure, then supporting evidence is necessary. Alternatively, the figure may be removed.

Again, Both reviewer #2 and #3 have the same concern. As suggested, Fig. 4 has been deleted.

o Almost all differentiation analysis relies on RNA expression analysis. Supporting evidence at the protein level is necessary to substantiate the claims. The authors can stain for markers of different lineages and quantify.

As suggested, IF have been performed to detect the expression of germ layer markers in the embryo (Fig. 4C).

o If mTORC1 activation is mediated by Hbxip function, but there is still mTORC1 activity in Hbxip KO cells, then Hbxip is probably required to promote mTORC1 activity above a certain threshold. mTOR heterozygous KO is not lethal, similar to Hbxip heterozygous KO. So mTOR dosage does not seem to be very critical for embryonic development. Since the authors' argument is that Hbxip functions by regulating TORC1 activity, it would be helpful to see the levels of mTORC1 activity in Hbxip homozygous vs heterozygous KOs vs wt embryos. The authors need to at least discuss how Hbxip/mTOR dosage regulates development in the discussion.

We have added Figure S3 to show the reduced mTORC1 activity in *Hbxip*$^{-/-}$ E8.5 embryos, indicated by IF of p-S6K1. However, we could not distinguish heterozygous KO from WT embryos by IHC staining of Hbxip.

mTOR heterozygous KO might reduce the mTORC1 activity by half, while the effect of *Hbxip* heterozygous KO on the mTORC1 activity is unpredictable. Presumably, *Hbxip* heterozygous KO reduces the level of Hbxip protein by half. However, it is unknown whether the endogenous Hbxip is in excess or in the minimum requirement for the activation of mTORC1. If it is in excess, *Hbxip* heterozygous KO might not affect the mTORC1 activity. If it is at the minimum level, *Hbxip* heterozygous KO might reduce the mTORC1 activity by half. Given that both *mTOR* heterozygous KO and *Hbxip* heterozygous KO mice have no obvious phenotype, we only focus on *Hbxip* homozygous KO effect.

Minor comments
o For westerns please provide size markers and uncropped images (at least as a reviewer figure).

We have marked size markers in Western blots. And uncropped images will be provided if the journal requires so.

o In general, the differences in the expression levels of lineage markers are quite small and hard to read as presented in the figures. The data could be better understood if the authors plot log2FC to show fold change in expression.

We have plotted Log2FC to show fold change in expression.

o In Fig. 3a, b, it is better to plot expression of each gene relative to its levels in ES cells. This way the reader can see that lineage markers are actually upregulated during differentiation of wt cells, but perturbed in KO cells if that is the case.

We have plotted the expression of each gene relative to its levels in ESCs in Fig. 3a, b.

o Please show individual data points for qPCR graphs.

Individual data points have been plotted for qPCR graphs.

What is the staining in Fig. 1D? No explanation is provided.
0
Fig. 1D just shows dissected E6.5-9.5 embryos without any staining.

o Please explain the reason for using EIIa-Cre in the text. This is crucial information to understand the nature of the KO in the embryo.

EIIa-Cre mice were used to generate whole body and germ line knockout of floxed allele. We have described it in the methods now (line 230-232).

o Fig. 2H shows GO terms for downregulated genes. Please also show the same analysis for upregulated genes, as this subset comprises a large portion of the differentially expressed genes.

GO analysis of upregulated genes have been added in Fig. S2A, B.

o Fig. 6D is a bit misleading because in 6E and F we see substantial pS6K1 in Hbxip KO, although it may be reduced compared to wt. But definitely not as reduced as in 6A.

To be clarified, we have changed the WB images with similar exposure time. Nevertheless, it is inappropriate to compare the expression level of a given protein between different blots.

Reviewer #2 (Significance (Required)):

In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function.

Role of different aspects of mTOR biology in early development is not well understood. Therefore the advance here could be useful.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:
Qin, Ni, et al. investigate the role of Hepatitis B X-interacting protein (HBXIP, also known as LAMTOR5) during development. To do so, they use a conditional Hbxip mouse model and delete exon 2 of the gene upon recombination with a constitutive Cre. They find that Hbxip mutant embryos are delayed and die ~E7.5. They also make use of Hbxip knock out mouse embryonic stem cells to investigate the potential differentiation defects, and through co- immunoprecipitation coupled to proteomics and epistatic experiments, they conclude that Hbxip plays a role in epiblast differentiation, interacts with ragulator complex proteins, and activates mTORC1 (pS6K1) signalling pathway.
Their findings suggest that Hbixp impacts differentiation at early stages of development and deletion of Hbixp diminishes mTOR signaling activity. I particularly like their proteomic approach to identifying HBXIP interacting partners. This allows them to show clearly and convincingly that HBXIP defects can be recapitulated by mutating other ragulator complex proteins. However, it is not clear what specific effects are primary or secondary to the phenotype, and how does mTOR regulate stem-cell renewal or differentiation.

Major concerns:

- The phenotype of the mouse post-implantation mutants is not clear and the pattern of expression of Hbxip at E7.5 and E8.5 in wild-type embryos is not well described (Figure 5). The authors could co-stain HBIXP at the onset of the phenotype (E7.5) together with markers of ectoderm and endoderm to demonstrate that HBIXP is expressed in the epiblast and whether there is a downregulation of ectoderm and endoderm markers in the mutant embryos. In addition, it would be good that the authors perform statistics on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios.

As suggested, IF was performed to detect germ layer markers, Nestin, T and Gata4, in WT and *Hbxip*$^{-/-}$ E7.5 embryos (Fig. 4C).

We have performed $\chi 2$ test on the number of mouse mutants obtained per stage in comparison to the expected Mendelian ratios (Fig. 1C).

- The role of HBIXP in the regulation of OCT4 seems contradictory in the mouse model in comparison with their claims/findings in mouse stem cells. Mutant embryos seem to arrest/delay their development from ~E7.5 and they observe that embryos at E8.5 retain OCT4 expression (Figure 5). In mouse stem cells the authors state that "The mRNA levels of pluripotency genes, Nanog, Oct4, and Sox2, as well as the protein levels of Nanog and Oct4, are declined in Hbxip KO ESCs (Figure 2B and E)". Is OCT4 reduced on blastocyst mutants and is OCT4 expression maintained during differentiation in stem cells? In addition, whereas in Figure 2B the authors show downregulation of OCT4 in two separate stem cell clones, the levels of OCT4 protein in one of the clones (H-/- -1) in Figure 3G seem unchanged. How do the authors explain these differences between stem cell experiments? How do the authors interpret these stage-specific (or in vivo versus in vitro) differences in OCT4 regulation?

In vitro differentiation of ESCs cannot recapture all the features of *in vivo* embryo development, even though it mimicks *in vivo* development of embryos. Thus, the seemly discrepancy of Oct4 regulation in ESC differentiation and embryo development might be explained by the different experimental setting.

The downregulation of Oct4 in *Hbxip*$^{-/-}$-1 ESCs (Fig. 3G) was subtle. New WB data has been provided

to clearly demonstrate the downregulation of Oct4 in *Hbxip*$^{-/-}$ ESCs (Fig. 3G).

- It is unclear how do the authors think that HBIXP regulates stem cell renewal and differentiation through activation of mTOR. Are the HBIXP mutant cells dying and/or differentiating into endoderm, either primitive or definitive endoderm? HBIXP immunostainings in Figure 5a seem to indicate that HBIXP is expressed in the visceral endoderm, which derives mostly from the primitive endoderm in the blastocyst. Perhaps the HBIXP mutant embryos lose the visceral endoderm, and it does not allow the embryo to develop further. In stem cells, this effect may not be identifiable, and the authors may need to use extraembryonic ectoderm (XEN) cells.

Our data suggested that knockout of Hbxip reduces mTORC1 activity, consequently leading to slower self-renewal and differentiation defects of ESCs.

It has been reported that inhibition of mTOR leads to embryonic diapause (Nature, 2016, 540: 119–123). Given that knockout of Hbxip reduces mTORC1 activity, we speculated that *Hbxip*$^{-/-}$ epiblast fails to exit from pluripotency and initiate gastrulation, in addition to cell proliferation defect. In the revised manuscript, we provided not only the evidence that Oct4 is not down- regulated in E8.5 *Hbxip*$^{-/-}$ embryo (Fig. 4B), but also the evidence that ectodermal marker Nestin and mesodermal marker T are not properly expressed in E7.5 *Hbxip*$^{-/-}$ embryo (Fig. 4C), clearly demonstrating the defect in epiblast differentiation.

Of course, the suggestion to look into visceral endoderm defect using XEN cells is a good one. We will further test this possibility in the future.

- As The Ragulator complex is required for the activation of mTORC1 by amino acids, can the authors mimic the phenotype by reducing amino acid levels during pluripotency/differentiation?

Amino acid starvation can only be applied to cultured cells for a short time, such as 24 hours. In our experimental setting, ESC differentiation needs 4 days. Thus, it is difficult to detect how amino acid starvation affects ESC differentiation.

Moreover, it has been well elucidated that the Ragulator complex is required for the activation of mTORC1 by amino acids (Sancak et al., 2010, Bar-Peled et al., 2012). Our focus is how Hbxip regulates embryogenesis and the differentiation of ESCs, rather than the upstream regulator of Hbxip. Thus, we preferred not to address this issue.

Minor concerns:

- The role of HBIXP in trophectoderm (TE) formation at the blastocyst stage is not relevant to understanding the mouse mutant phenotype, as the zygotic mutant dies at post-implantation stages. In addition, the in vitro findings of TE differentiation are likely to be a misinterpretation. In mice, it has been extensively described that embryonic stem cells rarely contribute to trophectoderm-derived lineage (PMID: 33420491). Thus, the interpretation of trophectoderm differentiation using Cdx2 and Lef1 in stem cell differentiation is not accurate. In the context of stem cell differentiation, these two markers are likely markers of primitive streak or gastrulation.

Both reviewer #2 and #3 have the same concern. As suggested, TE markers in Figs 2, 3 have been removed.

- In the text it reads "in undifferentiated ESCs, Hbxip KO suppresses the expression of ectodermal, mesodermal and trophectodermal markers, such as Nestin, Celsr, T, Dlx3, Lef1, and Cdx2, whereas mesodermal marker Gata6 is activated by Hbxip KO (Figure 2F)." In Figure 2F, Gata 6 is labeled as an endoderm marker. Is Gata 6 an endoderm or a mesoderm marker?
Thanks for pointing out our mistake. Gata6 is an endoderm marker. We have corrected it.

- The authors use pS6K1 as a readout of mTOR activity. mTOR regulates other factors such as ULK1 or 4EBP1. Are these also altered in their stem cell models during pluripotency or differentiation?

We have added the data of 4EBP1 and p-4EBP1 in Fig. S3A. It is consistent with the data of p- S6K1.

- There is a missing figure legend for embryos in "c" in Figure 5.

We have revised the figure legend to annote a, a', b, b', c, and c' (line 576-577).

- There is no validation of loss of protein expression in TSC1, Lamtor 3 and Lamtor 4 mutant stem cells.

We have performed Western blot to show the knockout of Tsc1, Lamtor3 an Lamtor4 proteins in corresponding KO ESCs (Figs S4E, F, S5D, H).

- There is no number (n) of embryos used for any experiment. The stem cell experiments (qPCR, western blots) also lack information about the number of biological or experimental repeats.

We have added the information about the number (n) of embryos and experimental repeats in figure legends.

- The authors could provide the raw western blots as supplemental material to validate the selected results on the figures.

Uncropped raw Western blots will be provided if the journal requires so.

- There is no reference to the EIIa-Cre line they used.

EIIa-Cre mice were used to generate whole body and germ line knockout of floxed allele. We have described it and cited the reference in the methods (line 230-232).

Reviewer #3 (Significance (Required)):

Understanding the role of genes during development may help us understand fundamental principles about how genes make bodies. In addition, the use of stem cell models allows for the characterization of phenotypes that might be challenging to study in vivo. In this work, the authors show that HBIXP acts through the regulator complex rather than as a transcriptional coactivator. Further, this work suggests that the Raptor complex may be instrumental for gastrulation, and that mTOR signaling may regulate self-renewal and differentiation of epiblast cells. These findings are of potential interest as mTOR mouse mutants do not progress beyond peri-implantation, thus precluding the study of mTOR function during post-implantation development.

I am a developmental biologist with ample expertise in mammalian development and stem cells. I think this work is potentially interesting but major revisions should be made. I would also encourage the authors to carefully revise the labelling of figures and to make sure that the results are accurately interpreted, with no contradictory results in them.

Second decision letter

MS ID#: DEVELOP/2022/200527

MS TITLE: Hbxip is essential for embryogenesis and regulates embryonic stem cell differentiation through activating mTORC1

AUTHORS: Yan Qin, peiling ni, Qingye Zhang, Xiao Wang, Xiaoling Du, Zixi Yin, Lingling Wang, Lihong Ye, and Lingyi Chen

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

I would like to thank you for engaging with our Ethics Team to clarify the issues and correct the figures.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Both Reviewer 2 and 3 highlight the remaining discrepancy between the in vivo and in vitro findings. The in vivo results point to a delayed differentiation and the in vitro differentiation data suggests differentiation defects. However, only one time point for the in vitro analysis is provided and the question remains whether additional time points would reveal a delay. More discussion on this point and how these two phenotypes can be reconciled would strengthen the study.Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The authors clearly show the importance of Hbxip for embryogenesis and embryonic stem cell1 differentiation, and have strong evidence that it acts via mTORC1.

Comments for the author

The authors have performed extensive revisions to the Manuscript, and have included novel relevant data that address my main queries in relation to the previous version.
I have no further comments.

Reviewer 2

Advance summary and potential significance to field

Qin, Ni, et al. investigate the role of Hepatitis B X-interacting protein
(HBXIP, also known as LAMTOR5) during development in a mouse knock-out model as well as mouse embryonic stem cells. They conclude that Hbxip plays a role in epiblast differentiation, interacts with regulator complex proteins and activates of mTORC1 signalling pathway.
Understanding the role of genes during development may help us understand fundamental principles about how genes make bodies. In addition, the use of stem cell models allows for the characterization of phenotypes that might be challenging to study in vivo. In this work, the authors show that HBIXP acts through the regulator complex rather than as a transcriptional coactivator. Further, this work suggests that the Raptor complex may be instrumental for gastrulation, and that mTOR signaling may regulate self-renewal and differentiation of epiblast cells. These findings are of potential interest as mTOR mouse mutants do not progress beyond peri-implantation, thus precluding the study of mTOR function during post-implantation development.

Comments for the author

The authors have now addressed most of the concerns raised in the previous version of the manuscript. The controls to demonstrate the knockout of specific proteins are solid, the interpretations of the results are clear, and a better characterization of the mouse mutant has been performed. However, there is still a discrepancy between the in vivo expression of OCT4 and their in vitro findings that the authors have failed to discuss or resolve in the manuscript. The in vivo results point to a delayed differentiation defect and the in vitro differentiation data suggests

differentiation defects. As the in vitro differentiation analysis was performed on day 4, I wonder if the authors could include in the discussion how these two phenotypes match together and what would they expect to see if they measured various (earlier) timepoints of differentiation.

Minor comments:
- Fig S1D lacks scale bars
- Fig4 stainings could include blown-up images of the epiblast and annotations
- The previous qPCR data of Cdx2 and Lef genes could be included as primitive streak markers
- EIIa-Cre recombination states "EIIa-cre mice (Lakso et al., 1996), which express Cre in early mouse embryos and is useful for whole-body and germ line deletion of floxed allele". The authors could specify in which cell types (zygote, epiblast) does it recombine and when (pre-implantaion, post-implantation).

Reviewer 3

Advance summary and potential significance to field

The authors address the role of Hbxip in ESC maintenance and differentiation while also characterizing the phenotype of Hbxip KO embryos. The authors show that Hbxip regulates mTORC1 activity in ES cells and argue that this could be the mechanism for embryonic lethality of Hbxip KO embryos. In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function.

Comments for the author

The main discrepancy of the paper remains, namely the different outcomes of Hbxip KO in the embryo and in ES cells. The embryo data show delayed or blocked exit from pluripotency, while the ES data show that the pluripotency markers are readily downregulated. Due to the downregulation of pluripotency markers in ES cells, one would expect upregulation of a variety of differentiation markers. In contrast the authors observe downregulation of lineage markers except for only Gata6. It is not clear from the RNAseq analysis shown in Figure 1 which other genes are upregulated. The main GO terms appear to be associated with metabolic processes. Since there isn't a strong pro-differentiation phenotype in ES cells associated with reduced pluripotent markers, it is unclear what the role of Hbxip is and why it does not match the in vivo phenotype. The authors argue that in vivo and in vitro conditions are not the same, which is true, however, the question remains as to what Hbxip's role is. As such, I am convinced that Hbxip is required for embryonic development but it is not clear to me why it is required.
Further comments:
- Fig 2B, the authors added a new blot for HBXIP however the loading control is still the old one. Since the new HBXIP and the old TUBULIN cannot have been run on the same blot, a new loading control is needed for the HBXIP blot. The same is true for Figure 5E.
- Fig 3G, WB is overexposed. Both OCT4 and NANOG plots look different than before with drastic downregulation of OCT4 this time around and both proteins showing double bands now and not before. The loading control in the previous version was better, so why change this blot now?

Second revision

Author response to reviewers' comments

We would like to thank all three reviewers again for the comments to our revised manuscript. Their comments are highly appreciated and addressed as described below.

Reviewer 1 Advance summary and potential significance to field
The authors clearly show the importance of Hbxip for embryogenesis and embryonic stem cell differentiation, and have strong evidence that it acts via mTORC1.

Reviewer 1 Comments for the author
The authors have performed extensive revisions to the Manuscript, and have included novel relevant data that address my main queries in relation to the previous version.
I have no further comments.

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Qin, Ni, et al. investigate the role of Hepatitis B X-interacting protein (HBXIP, also known as LAMTOR5) during development in a mouse knock-out model as well as mouse embryonic stem cells. They conclude that Hbxip plays a role in epiblast differentiation, interacts with regulator complex proteins and activates of mTORC1 signalling pathway.
Understanding the role of genes during development may help us understand fundamental principles about how genes make bodies. In addition, the use of stem cell models allows for the characterization of phenotypes that might be challenging to study in vivo. In this work, the authors show that HBIXP acts through the regulator complex rather than as a transcriptional coactivator. Further, this work suggests that the Raptor complex may be instrumental for gastrulation, and that mTOR signaling may regulate self-renewal and differentiation of epiblast cells. These findings are of potential interest as mTOR mouse mutants do not progress beyond peri-implantation, thus precluding the study of mTOR function during post-implantation development.

Reviewer 2 Comments for the author
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We have added a paragraph in the discussion section (line 220-233) to discuss to the discrepancy between the in vivo and in vitro expression of Oct4.

As for the measurement of earlier timepoints, we indeed measured the expression of differentiation markers at day 2 of ESC differentiation. Most differentiation genes, except for T, are not activated significantly at day 2, implying that day 4 is a early timepoint of in vitro differentiation, mimicking the formation of three germ layers.

Minor comments:
- Fig S1D lacks scale bars

We have added scale bar in Fig. S1D.

- Fig4 stainings could include blown-up images of the epiblast and annotations

As suggested, annotations of epiblast, ectoderm, mesoderm, and endoderm, have been added in Fig. 4. However, due to the space limit, we did not add blown-up images in Fig. 4.

- The previous qPCR data of Cdx2 and Lef genes could be included as primitive streak markers

We are not confident that Cdx2 and Lef are markers for primitive streak. And our point here is that both Hbxip-/- ESCs and Hbxip-/- epiblast have differentiation defects in the formation of three germ layers, especially ectoderm and mesoderm. Thus, we prefer not to include the qPCR data of Cdx2 and Lef genes.

- EIIa-Cre recombination states "EIIa-cre mice (Lakso et al., 1996), which express Cre in early mouse embryos and is useful for whole-body and germ line deletion of floxed allele". The authors could specify in which cell types (zygote, epiblast) does it recombine and when (pre-implantaion, post-implantation).

In the cited reference (Lakso et al., 1996), the EIIa-Cre transgene was expected to express Cre at a very early stage of preimplantation embryogenesis, most likely at the one-cell stage. While 50% of EIIa-Cre mice excised the floxed NEO gene in all cells, the remaining 50% of EIIa-Cre mice showed some mosaicism, excising the floxed NEO gene in a portion of cells and retaining the floxed NEO gene in the rest cells. This data indicates that Cre mediated recombination may occur after the zygote stage.

Thus, we revised this sentence to “EIIa-cre mice (Lakso et al., 1996), which express Cre at a very early stage of preimplantation embryogenesis, most likely at the zygote stage, and are useful for whole-body and germ line deletion of floxed allele”.

Reviewer 3 Advance summary and potential significance to field
The authors address the role of Hbxip in ESC maintenance and differentiation, while also characterizing the phenotype of Hbxip KO embryos. The authors show that Hbxip regulates mTORC1 activity in ES cells and argue that this could be the mechanism for embryonic lethality of Hbxip KO embryos. In general, the authors study an interesting and relevant question of Hbxip/mTOR-based regulation of embryonic development and stem cell function.

Reviewer 3 Comments for the author
The main discrepancy of the paper remains, namely the different outcomes of Hbxip KO in the embryo and in ES cells. The embryo data show delayed or blocked exit from pluripotency, while the ES data show that the pluripotency markers are readily downregulated. Due to the downregulation of pluripotency markers in ES cells, one would expect upregulation of a variety of differentiation markers. In contrast the authors observe downregulation of lineage markers except for only Gata6. It is not clear from the RNAseq analysis shown in Figure 1 which other genes are upregulated. The main GO terms appear to be associated with metabolic processes. Since there isn’t a strong pro-differentiation phenotype in ES cells associated with reduced pluripotent markers, it is unclear what the role of Hbxip is and why it does not match the in vivo phenotype. The authors argue that in vivo and in vitro conditions are not the same, which is true, however, the question remains as to what Hbxip’s role is. As such, I am convinced that Hbxip is required for embryonic development but it is not clear to me why it is required.

We have added a paragraph in the discussion section (line 220-233) to discuss to the discrepancy between the in vivo and in vitro expression of Oct4. In addition, we also provided explanation why pluripotency markers and differentiation markers are both downregulated in Hbxip-/- ESCs. It is consistent with the role of Hbxip in activating mTORC1. Our main conclusion is that Hbxip is required for the activation of mTORC1, which is essential for the activation of lineage markers during embryonic development and ESC differentiation.

Further comments:
- Fig 2B, the authors added a new blot for HBXIP however the loading control is still the old one. Since the new HBXIP and the old TUBULIN cannot have been run on the same blot, a new loading control is needed for the HBXIP blot. The same is true for Figure 5E.

Fig. 2B, to make the figure layout consistent with previous version, we omitted the exact matched Tubulin blot for the new Hbxip blot. In the revised Fig 2, we added the Tubulin blot matching with the the Hbxip blot.
In response to the last comment from Reviewer #2 (Fig. 6D is a bit misleading because in 6E and F we see substantial pS6K1 in Hbxip KO, although it may be reduced compared to wt. But definitely not as reduced as in 6A), we tried to select blots showing weaker p-S6K1 in Hbxip KO and stronger p-S6K1 in WT in Fig. 5E, even though we have explained that it is inappropriate to compare the expression level of a given protein between different blots. During the revision, images were not matched correctly. We have revised Fig. 5E to show matched blots. In addition, a WT control was added in the right panel to show the reduced level of p-S6K1 in Hbxip-/- ESCs.

- Fig 3G, WB is overexposed. Both OCT4 and NANOG plots look different than before, with drastic downregulation of OCT4 this time around and both proteins showing double bands now and not before. The loading control in the previous version was better, so why change this blot now?

In response to the 3rd comment from Reviewer #1 (To fully validate the recovery data the WB (3G) should be quantified, and the authors show that differentiation is now normal) and the 2nd comment from Reviewer #3 (whereas in Figure 2B the authors show downregulation of OCT4 in two separate stem cell clones, the levels of OCT4 protein in one of the clones (H-/--1) in Figure 3G seem unchanged), we provided new WB data to clearly demonstrate the downregulation of Oct4 in Hbxip-/- ESCs (Fig. 3G). The Oct4 and Nanog bands seems different from previous blots. The reason is that both Oct4 and Nanog are phosphorylated proteins. Due to experimental variations, such as gel separation and exposure time, multiple bands are detected for Oct4 and Nanog occationally (Fig. 2B, Mech Dev, 2005, 122: 67-79, DOI: 10.1016/j.mod.2004.08.008; Fig. 2A, PNAS, 2020, 117:2519-2525, DOI: 10.1073/pnas.1915079117; Fig. 1C and D, Oncology Reports, 2015, 33:1621–1629, DOI: 10.3892/or.2015.3752; Fig. 1D, Cell Research, 2009, 19: 1052-1061, DOI: 10.1038/cr.2009.79). Indeed, in Fig. 2B, multiple bands of Oct4 and Nanog were also detected, even though not as obvious as those in Fig. 3G. Therefore, the Oct4 and Nanog blots in the current Fig. 3G reflect the expression levels of Oct4 and Nanog, and thus are acceptable.

Third decision letter

MS ID#: DEVELOP/2022/200527

MS TITLE: Hbxip is essential for embryogenesis and regulates embryonic stem cell differentiation through activating mTORC1

AUTHORS: Yan Qin, peiling ni, Qingye Zhang, Xiao Wang, Xiaoling Du, Zixi Yin, Lingling Wang, Lihong Ye, and Lingyi Chen
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

The authors show the tole of Hbxip in development and stem cell differentiation through the activation of mTORC1.

The authors have included an additional paragraph in the discussion about the discrepancy in their in vivo versus in vitro data.

Comments for the author

I would recommend the authors remove or explain further the sentence "It is most likely due to that in vitro differentiation of ESCs cannot recapture all the features of in vivo embryo development, even though it mimics in vivo development of embryos.", as I am not sure what it means.
On the statement: "whereas the repression of Oct4 could be triggered by LIF withdrawal during ESC differentiation, regardless whether differentiation genes are activated or not.", it would be great if they could support their statements with references that justify this interpretation.

Reviewer 3

Advance summary and potential significance to field

Role of Hbxip is ESC biology and mouse development is shown.

Comments for the author

The authors have addressed the concerns sufficiently.