nature portfolio

Peer Review File

Cell-context response to germ layer differentiation signals is predetermined by the epigenome in regionalized epiblast populations.

Corresponding Author: Dr Elisabetta Ferretti

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study by Menezes et al. investigated the primed nature of epiblast cells. They evaluated their self-renewal properties, assessed their epigenetic code, and challenged their context-specific predisposition to WNT and BMP signaling cues using in vitro and in vivo experimental models. The authors used single-cell RNAseq (scRNAseq), spatial transcriptomics, chromatin accessibility, and DNA methylation analyses to identify molecular mechanisms influencing epiblast signaling response. The study provides valuable insights into the mechanisms underlying stem cell plasticity and lineage fate acquisition, crucial to mammalian tissue diversity. However, there are some concerns listed below.

The authors observed that CLDN6Low EpiSCs retained low CLDN6 levels over time and express mesenchymal genes, whereas CLDN6High EpiSCs reverted to their initial unsorted state and exhibited higher plasticity and potency by having elevated levels of epithelial genes (Figure 1D). However, these observations may not be entirely accurate, as cells with high plasticity (CLDN6High) generally express higher levels of mesenchymal genes (in contrast to the authors' claim) to attain motile or migratory characteristics. It would be beneficial to know the authors' thoughts on this.
Additionally, instead of GO terms for all DEGs (both up and down genes), it would be interesting to see GO terms for upregulated and downregulated genes separately to infer activated and suppressed functional pathways between these cell types, CLDN6Low and CLDN6High.

The in vitro differentiation profiling of EpiSCs towards APSD using single-cell RNA-seq doesn't seem to be clear, as the subpopulations of DE, NMPs, and cardiac mesoderm cell types don't appear to be transcriptionally distinct on the UMAP plot (Supplementary Fig. 2a). Moreover, cell type-specific markers are not specific to each cluster (Supplementary Fig. 2b), which does not add much to the story.

The chromatin accessibility (ATAC-Seq) profile aimed to investigate if different chromatin landscapes influence CLDN6Low and CLDN6High EpiSCs lineage biases. However, the investigation doesn't show strong changes in the open chromatin landscape between these cell types. For example, very few differentially accessible peaks (n=110 DAPs) were found, and none of them are linked with specific germ-layer lineages (Figure 3a), contrary to a previous report (Metzis, V. et al., 2018). Although this observation might be due to the culturing of EpiSCs under WNT-inhibited conditions, upon WNT stimulation, the gain in open chromatin in CLDN6Low APSD cells is minimal (Figure 3b). Furthermore, it was not clear which functional pathways these 110 DAPs were involved in.

The results in figure 3d show that the addition of WNT leads to epigenetic remodeling and differentiation. It would be helpful if the authors could provide previous reports that observed similar findings in the EpiSCs or similar lines.

Similarly, it would be beneficial if the authors could provide references to findings showing that higher gene body methylation leads to restricted potency in EpiSCs or similar lines.

Reviewer #2

(Remarks to the Author)

The manuscript by Menezes and colleagues describe different subpopulation of the post-implantation epiblast (and subpopulations of EpiSCs) displaying differential responses to signalling molecules.
The authors used CLDN6 expression levels to sort EpiSCs, finding that CLDN6 High cells display also increased pERK levels and a biased towards DE and anterior neuroectoderm.
Conversely, CLDN6 low cells are less plastic and can form mainly NMPs.
ATACseq did not reveal differences that could explain the alternative cell fate acquisition, while WGBS was more informative, potentially providing a molecular explanation.
The authors then used Pbx1 KO models to study the role of pERK, finding differences in accessibility and methylome connected to BMP responsiveness e extra embryonic mesoderm differentiation.

The manuscript presents a large number of datasets and is quite "dense". Several concepts are repeated multiple times, making the reading rather complex.
This reviewer suggests to streamline the text of manuscript, in particular the Results section.

One point should be clarified, concerning the role of FGF/pERK.
The authors put forward the idea that levels of pERK control the responsiveness to differentiation cues. To test their model they then used Pbx1-KO EpiSCs, in which they observed a partial reduction in pERK. Still, the effects observed in Pbx1-KO could be due to Pbx1 itself, rather than FGF/pERK.
However, one aspect is rather confusing, the presence of FGF ligands both on EpiSC expansion medium and in their differentiation protocols (e.g. APSD).
If the levels of pERK are really crucial it should be sufficient to lower the concentration of FGF in the medium, measure pERK, and observe a more "posterior/NMP biased" phenotype.
Can the authors change the responsiveness to differentiation cues by directly modulating FGF signal intensity?

Minor point:
line 126-128 ", the ability of CLDN6Low EpiSCs to self-renew and retain its initial stem-state can be attributed to the expression of stemness genes"
This statement is not clear. CLDN6 Low and High EpiSCs express comparable levels of pluripotency factors Oct4 and Nanog, so there is not reason why they should not self-renew and maintain pluripotency.
Why should the expression of Sox9 and Snail2, which are expressed in mammary stem cells, support pluripotency? Please delete / clarify.

Reviewer #3

(Remarks to the Author)
This paper by Menezes and colleagues perform various genome-scale analyses on Claudin-high and Claudin-low EpiSCs. They use this data to claim that Erk signaling alters various aspects of chromatin state prior to germ layer acquisition. While the datasets are of potential value to the community, I find that the interpretations in this paper often go far beyond what the data shows.

Major concerns:

My main concerns with this paper stem from loosely correlated observations that are used to make exceptionally strong causal statements. I point out some examples below, but there are few - if any - causal statements about connections between FGF/Erk, BMP or Wnt signaling and changes to methylation that can be justified based on the data shown.

(1) The authors begin by staining for Claudin-high and Claudin-low subpopulations in the E7.5 embryo, and report an A-P bias in this signal. What makes Claudin a good marker for anterior vs posterior cells compared to the numerous other factors that show a clear (and in some cases, even clearer) A-P bias? The authors should justify why a new A-P marker is necessary or an improvement over well characterized existing ones. Why not sort for the many existing makers with known A-P bias, or directly sort for Erk-high and Erk-low cells using a ppErk antibody if Erk is what one is interested in?

There is also something logically inconsistent in the early analysis of Fig 1 relative to the common understanding of the mouse embryo. FGF/Erk signaling is typically thought to be associated with the posterior domain of the embryo during gastrulation, yet the authors report that Claudin is associated with both anterior positions and high Erk phosphorylation. Is there any literature to support an anterior-to-posterior gradient of FGF/Erk activity in the mouse epiblast? I would at the minimum require co-staining of pErk and Claudin in the embryo to show a reasonable spatial overlap between them, and also some explanation of why the authors obtain results that are contrary to the expected FGF/Erk activity pattern. Also, the correlation between Claudin expression and pErk is quite mild in Figure 1F. The two different pErk peaks having highly overlapping Claudin distributions. From this data it is not obvious that sorting Claudin levels is a good proxy for separating pErk-low and pErk-high cells at all. Fig 1F is also misleading because it shows the results of gating ERK-high and ERK-low

cells to obtain different Claudin levels, which is quite different than gating Claudin-high and Claudin-low cells to obtain different ERK levels (the premise of this part of the paper). Finally, why does the range of Claudin expression look so much more compressed and on a different scale in Figure 1F than it does in Figure 1C and Figure S1D?

(2) Pbx2 knockout might have extremely broad effects, of which a change in ERK signaling is at best one among many. Once again, this loose correlation is interpreted causally throughout the paper.

Line 304: “FGF/ERK is known to regulate site-specific DNA methylation in different contexts, but whether it has a similar role in post-implantation epiblast stages remains uncertain. A significant complication is the absence of a suitable model to challenge FGF/ERK function without inducing spontaneous differentiation. The three-amino acid loop extension (TALE) homeodomain proteins (PBX, MEIS, and PREP) are alternative candidates as they regulate FGF/ERK signalling in diverse developmental contexts and cancers.”

Line 361: “Thus, our data suggest a systematic network where PBX1 regulation of Spry2 controls differential ERK/ETS activity, which shapes the epigenetic landscape across lineage-specific genes in primed epiblast cells before their transcriptional induction (Fig. 4i). Altogether, we established an ERK-deficient model to study the link between FGF/ERK, DNA methylation and lineage preprogramming.”

This paper repeatedly presents exceptionally convoluted ways to study Erk signaling, using loosely correlated markers (Claudin) and perturbations (Pbx2). Why not just simply treat EpiSCs with a MEK inhibitor and look for a direct role of ERK signaling, or collect cells from embryos using validated Erk-associated markers? And if doing so gives completely different results, then isn’t that evidence that the Claudin and Pbx2 results have nothing to do with FGF/Erk signaling at all?

(3) The authors frequently ascribe effects to just one factor in an experiment when many factors are varied. For example, the Wnt agonist CHIR is one of many signaling-related components of the APS media (along with activin and bFGF). Yet the authors repeatedly use APS media experiments to claim that Wnt stimulation is *the* determinant of differentiation to different fates. For example:

Line 278: “However, WNT stimulation induces both NMPs and DE progenitors, which arise after epiblast cells ingress through the PS. Upon exposure to WNT agonists, CLDN6Low EpiSCs differentiate towards NMPs while CLDN6High EpiSCs form DE (Fig. 2), supporting the existence of different epigenetically primed epiblast populations generating the mesoderm and endoderm cells.”

Line 291: “Altogether, DNA methylation patterns precedes WNT-driven chromatin accessibility changes influencing innate priming and lineage acquisition, by safeguarding distal-posterior epiblast cells (CLDN6Low EpiSCs) from DE and anterior neural differentiation”

The authors should treat with CHIR on its own if they wish to make claims about Wnt induced differentiation or compare media with and without CHIR to assess its effect. Similar claims are made about BMP (Line 377, Line 398), again in a cocktail with many other signaling agonists. This mirrors my similar concerns about ascribing effects in Claudin-high and Claudin-low cells to the ERK pathway.

Minor comments:

Figure 4C: From this data it is unclear whether Erk pathway activity is maintained but only total Erk1/2 protein is decreased, or if Erk protein levels are constant but phosphorylation is reduced. The authors should blot for both phospho and total Erk.

Line 182: “Higher TBX6 and Msgn1 (Fig. 2a,b,c) indicated that CLDN6Low EpiSCs differentiated more efficiently towards NMPs.” TBX6 is a marker of mesoderm differentiation away from NMPs, not a marker of the NMP state.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have appropriately addressed most of my concerns. The only thing I would suggest if they could include a more inclusive model to connect all key findings of this study.

Reviewer #2

(Remarks to the Author)
The authors did not address in a satisfactory way the concerns raised by this reviewer, and by reviewer 3, about the causal role of FGF in the mechanism described.

There are many correlative pieces of evidence pointing at FGF signalling being regulated by Pbx1, potentially via Spry2. The authors claim that somehow it is not possible to test this link directly, by inhibiting FGF, or, more specifically, by reducing pERK levels, because EpiSCs might die or differentiate when FGF/pERK are reduced.

There are multiple ways to address this point, some already suggested by reviewer 3:

1. Titrate the inhibitor PD03, in order to reduce only partially pERK, at the levels shown in Figure 4c. Such concentration of PD03 should phenocopy Pbx1 KO
2. Use heterozygous mutants or just knockdown components of FGF pathway, to reduce pERK levels, without ablating them.
3. If Spry2 is the key regulator, modulate the levels of Spry2 to reduce pERK and see if the phenotype is the same of Pbx1 KO
4. Use validated Erk-associated markers, rather than Claudin, to isolate cells with different levels of pERK.

I will quote reviewer 3:
'Why not just simply treat EpiSCs with a MEK inhibitor and look for a direct role of ERK signaling … ? And if doing so gives completely different results, then isn't that evidence that the Claudin and Pbx2 results have nothing to do with FGF/Erk signaling at all? '

Although the language is quite direct, I totally agree with the reasoning behind it.

The authors claim that in Pbx1 KO there are reduced levels of FGF signaling, which, in turn affect epigenetics and differentiation potential. But they now show that blocking FGF actually causes cell death and differentiation, which is very different from the behavior of Pbx1 KO cells.
So, the only fair conclusion is that differences in FGF signalling do NOT explain the phenotype of Pbx1 KO cells.

Reviewer #3

(Remarks to the Author)
This response to our prior reviews offered many explanations but few changes based on my and other reviewers' concerns about cell signaling connections. I still do not agree that the manuscript demonstrates an effect through ERK signaling. There are correlative data (signatures associated with ETS factors; correlations between dpERK and claudin), but no causal results that establish changes driven by ERK. Additionally, the authors' responses to my previously raised concerns argue that the Wnt and BMP signaling dependencies are that these connections should simply be known/assumed from literature, and are not directly demonstrated in this study. I am still not comfortable with the authors' conclusive statements in the title, abstract, and summaries of experiments that make claims about these discoveries (e.g. "Erk-driven lineage specification"; "chromatin accessibility and methylome states prime BMP-dependent fate decisions", "DNA methylation… safeguards WNT response"). As stated by me and another reviewer, Pbx perturbations don't just affect Erk but affect many other processes. Also: PI3K/Akt is not part of the MAPK/Erk cascade, as stated in the response to Reviewer 2, but is exactly one of those parallel signaling pathways that could lead to confounding results.

In contrast, I am very convinced by the authors' perturbations to Pbx proteins, sorting of Claudin proteins, and assessment of chromatin state and accessibility. I would be very happy to see that data published without further revisions, but am disappointed that the manuscript can't rest on those demonstrated results but instead has to expand claims to shakier ground.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I read the "point-by-point response to Reviewers" and unfortunately my concerns about the functional role of FGF have not been addressed.
As shown in figure 1A of the response, in Pbx1-KO cells over 1018 genes (596+422) are differentially expressed. Only 15 of them are associated with FGF signalling, corresponding to ~1.5% of differentially expressed genes.
So, very likely Pbx1 regulates additional processes, beyond FGF.
It is very unlikely that the remaining 1003 genes (~98.5%) are completely irrelevant to the observed phenotype.

This reviewer is not questioning that in Pbx1 KO cells FGF signalling (pERK1/2) is affected, but many other processes and signalling pathways might be affected as well.
So, alternative experiments, in which FGF signalling is directly inhibited, are needed to show a direct causal relationship between FGF and the observed phenotype.

There is a wide range of techniques (DOX-inducible shRNA, CRISPRi, conditional KOs, chemical inhibitors) available nowadays, with whom Spry1 or FGF5 or Erk1/2 could be inhibited during differentiation, aiming for a partial inhibition like the one observed by the authors.

Without any additional experimental evidence it would not be fair to conclude that FGF signalling is the cause of the observed phenotype.

Version 4:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors addressed the doubts about the functional involvement of ERK in the mechanism proposed.
They also edited extensively the manuscript, improving the clarity of their message.
For these reasons I support publication of the revised manuscript.

Point-by-point response to the Reviewers

We express our sincere appreciation to the referees for their favorable evaluation of our paper, as well as for their valuable comments and suggestions that have significantly enhanced the quality of our manuscript. In response to their feedback, we have incorporated substantial changes, including the addition of new experiments for your perusal (included in the letter to reviewers) and a thorough revision of the manuscript. These revisions, coupled with our original findings, collectively contribute novel insights into the epigenetic machinery regulating lineage priming in stem cells. We have undertaken a comprehensive editing of the manuscript to offer a more lucid and coherent explanation of our data and the novel insights it presents. In the manuscript, all the changes introduced in the text have been highlighted in red.

We have streamlined the results section, added further details and clarifications in the M&M, and modified the panels in the Figures as follows:
In Supplementary Fig. 2b, NMPs have been updated with NMPs/PSM.
Fig. 2 has been similarly modified from NMPs to NMPs/PSM.
Supplementary Fig. 5h. The Western blot of total-ERK, which was already included, has now been labeled as "t-ERK" to enhance clarity.
Supplementary Fig. 5j. We have corrected the abbreviation from "EpiSCS" to "EpiSCs" for accuracy and consistency.

A point-by-point response to the reviewers' comments is provided below. Our comments are highlighted in blue.

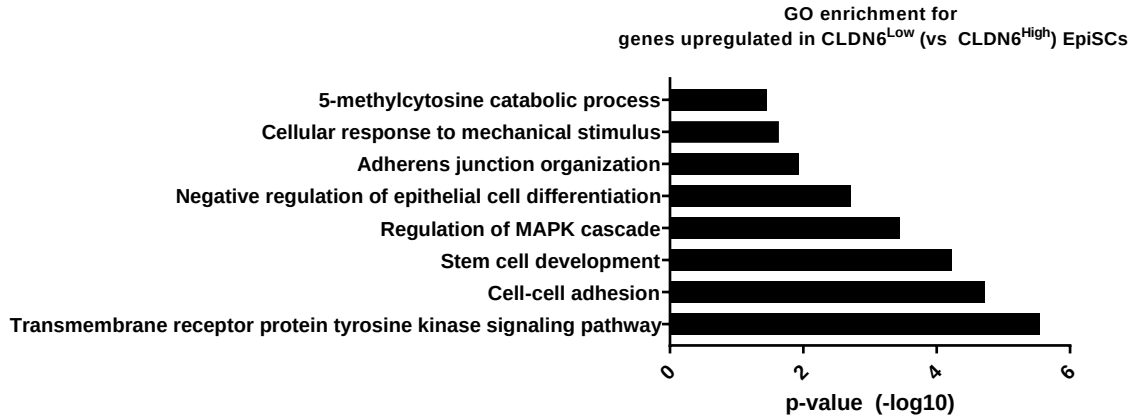
Reviewer #1 (Remarks to the Author):
The study by Menezes et al. investigated the primed nature of epiblast cells. They evaluated their self-renewal properties, assessed their epigenetic code, and challenged their context-specific predisposition to WNT and BMP signaling cues using in vitro and in vivo experimental models. The authors used single-cell RNAseq (scRNAseq), spatial transcriptomics, chromatin accessibility, and DNA methylation analyses to identify molecular mechanisms influencing epiblast signaling response. The study provides valuable insights into the mechanisms underlying stem cell plasticity and lineage fate acquisition, crucial to mammalian tissue diversity. However, there are some concerns listed below.
We express our gratitude to the reviewer for their positive remarks about our work and their insightful feedback. Their constructive input has significantly improved the clarity of our results and allowed us to emphasize the key findings in our study.

The authors observed that CLDN6Low EpiSCs retained low CLDN6 levels over time and express mesenchymal genes, whereas CLDN6High EpiSCs reverted to their initial unsorted state and exhibited higher plasticity and potency by having elevated levels of epithelial genes (Figure 1D). However, these observations may not be entirely accurate, as cells with high plasticity (CLDN6High) generally express higher levels of mesenchymal genes (in contrast to the authors' claim) to attain motile or migratory characteristics. It would be beneficial to know the authors' thoughts on this.

We thank the reviewer for highlighting this point. When we said that CLDN6^{High} EpiSCs have higher plasticity, we meant that they revert back to becoming a mix of CLDN6^{High} EpiSCs and CLDN6^{Low} EpiSCs after one passage: a salt-and-pepper state. CLDN6^{High} EpiSCs possess the potency to form all the lineages, while CLDN6^{Low} EpiSCs have reduced potency as they do not differentiate towards neuroectoderm or definitive endoderm. We apologize for the misunderstanding, as we were referring to the differentiation potential of stem cells, rather than their migratory characteristics. In the revized manuscript, we have edited the results indicating plasticity or potency where appropriate.

Additionally, instead of GO terms for all DEGs (both up and down genes), it would be interesting to see GO terms for upregulated and downregulated genes separately to infer activated and suppressed functional pathways between these cell types, CLDN6Low and CLDN6High.
We thank the reviewer for recommending this specific type of analysis. Indeed, the Gene Ontology (GO) terms associated with upregulated and downregulated genes yield highly intriguing findings, as illustrated in the two figures provided below (Reviewer Fig. 1-2).

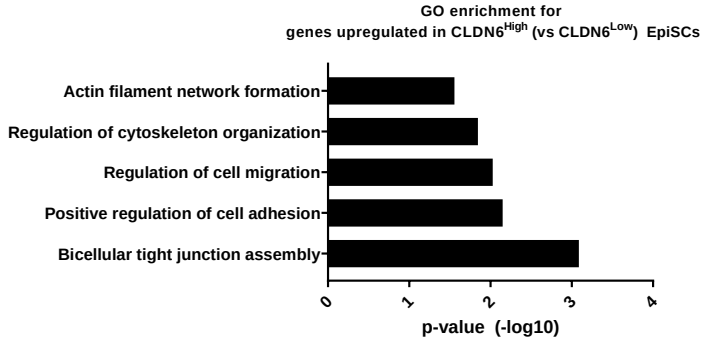
The GO terms for upregulates genes in CLDN6^{Low} EpiSCs (232 genes), illustrated in Rev. Fig. 1 below, show association with MAPK/tyrosine kinase signalling cascades, cell adhesion and more interestingly to "stem cell development" and DNA methylation, which strengthen our results and interpretation of our work.



Rev. Fig. 1 GO-term enrichment analysis of genes upregulated in CLDN6^{Low} EpiSCs displays significant overreprentation of terms associated with MAPK/tyrosine kinase signalling cascades, cell adhesion, stem cell development, and DNA methylation.

In the manuscript (Fig. 1), we showed that genes associated with stemness such as *Sox9* and *Snai2* are upregulated in CLDN6^{Low} EpiSCs, and these genes have been associated with maintaining stem cell identity (Guo *et al.,* Cell 2012). DNA methylation machinery in the GO enrichment analysis validates our findings on DNA methylation playing a crucial role in restricting the fate of CLDN6^{Low} EpiSCs, which we showed to specifically differentiate towards neuromesoderm/presomitic mesoderm (NMPs/PSM) but not towards definitive endoderm or neuroectoderm.

However, the GO analysis for upregulated genes in CLDN6^{High} EpiSCs (194 genes) did not yield statistically significant term enrichment, illustrated in Rev. Fig. 2, even when assessed using the Binomial test with Bonferroni correction for multiple testing or Fisher's test with calculated False Discovery Rates (FDRs) ($P \leq 0.05$)). This outcome can be attributed to the limited number of genes displaying differential expression, which consequently results in an insufficient quantity of input genes for a statistically significant interpretation.



Rev. Fig. 2 GO-term enrichment analysis of genes upregulated in CLDN6^{High} EpiSCs does not show statistically significant term enrichment.

In the manuscript, we included the GO terms associated with differentially expressed genes without separating them. We acknowledge the reviewer's viewpoint that the separated data is more intriguing, particularly given the CLDN6^{Low} EpiSC enrichment results, suggesting an effect on DNA methylation, which is lost in the combined analysis. However, we believe that this limitation is inherent in the GO analysis methodology.

The in vitro differentiation profiling of EpiSCs towards APSD using single-cell RNA-seq doesn't seem to be clear, as the subpopulations of DE, NMPs, and cardiac mesoderm cell types don't appear to be transcriptionally distinct on the UMAP plot (Supplementary Fig. 2a). Moreover, cell type-specific markers are not specific to each cluster (Supplementary Fig. 2b), which does not add much to the story.

We appreciate the reviewer's comment. It may appear that the clustering in Supplementary Figure 2a lacks the robustness typically seen when profiling different cell types using scRNA-Seq. However, it's important to clarify that the primary objective of our experiment is to induce *in vitro* differentiation of EpiSCs into mesoderm and endoderm progenitors. These cellular states (progenitors) possess a high degree of plasticity and do not represent fully committed cell lineages.

Given the asynchronous division of the seeded cells and the short duration of the differentiation protocol (only 24 hours of incubation with differentiation medium), it is reasonable to assume that the profiled cells represent transitory states towards germ layer differentiation, rather than being definitively committed. This is evident from the presence of cells at different levels of differentiation, ranging from less differentiated APS cells to nascent mesoderm, and progenitors exhibiting biases toward different cell types.

Although the UMAP plot does not completely segregate these subpopulations, an unbiased analysis still identifies them as distinct clusters. The UMAP plot thus clearly reflects the plasticity of the progenitors generated *in vitro*, which are on the path to acquiring a specific cell fate, as suggested by the expression of tissue-specific markers in Supplementary Figure 2b, e.g.: *Foxa2* and *Sox17* in definitive endoderm progenitors, *Msgn1* and *Tbx6* in NMPs/PSM, *Mesp1* in cardiac mesoderm progenitors. Importantly, these markers exhibit specificity to their respective progenitor classes: *Foxa2* and *Sox17* are expressed in definitive endoderm but not in NMPs or cardiac mesoderm progenitors; *Msgn1* and *Tbx6* are enriched in NMPs/PSM but not in definitive endoderm or cardiac mesoderm progenitors; and *Mesp1* is detected in cardiac mesoderm but not in definitive endoderm or NMPs.
It's worth noting that the continuous nature of the clusters on the UMAP plot and the trends in marker expression are consistent with findings from other studies conducted on pluripotent stem cells and embryo-derived progenitors, as reported in references Edri *et al.,* Development 2019, and Pijuan-Sala *et al.*, Nature 2019.

The chromatin accessibility (ATAC-Seq) profile aimed to investigate if different chromatin landscapes influence CLDN6Low and CLDN6High EpiSCs lineage biases. However, the investigation doesn't show strong changes in the open chromatin landscape between these cell types. For example, very few differentially accessible peaks (n=110 DAPs) were found, and none of them are linked with specific germ-layer lineages (Figure 3a), contrary to a previous report (Metzis et al., 2018). Although this observation might be due to the culturing of EpiSCs under WNT-inhibited conditions, upon WNT stimulation, the gain in open chromatin in CLDN6Low APSD cells is minimal (Figure 3b). Furthermore, it was not clear which functional pathways these 110 DAPs were involved in.

Regarding the functional pathways associated to the 110 DAPs, there were too few genes to do any statistically significant GO analyses, and a targeted approach did not reveal any lineage genes.

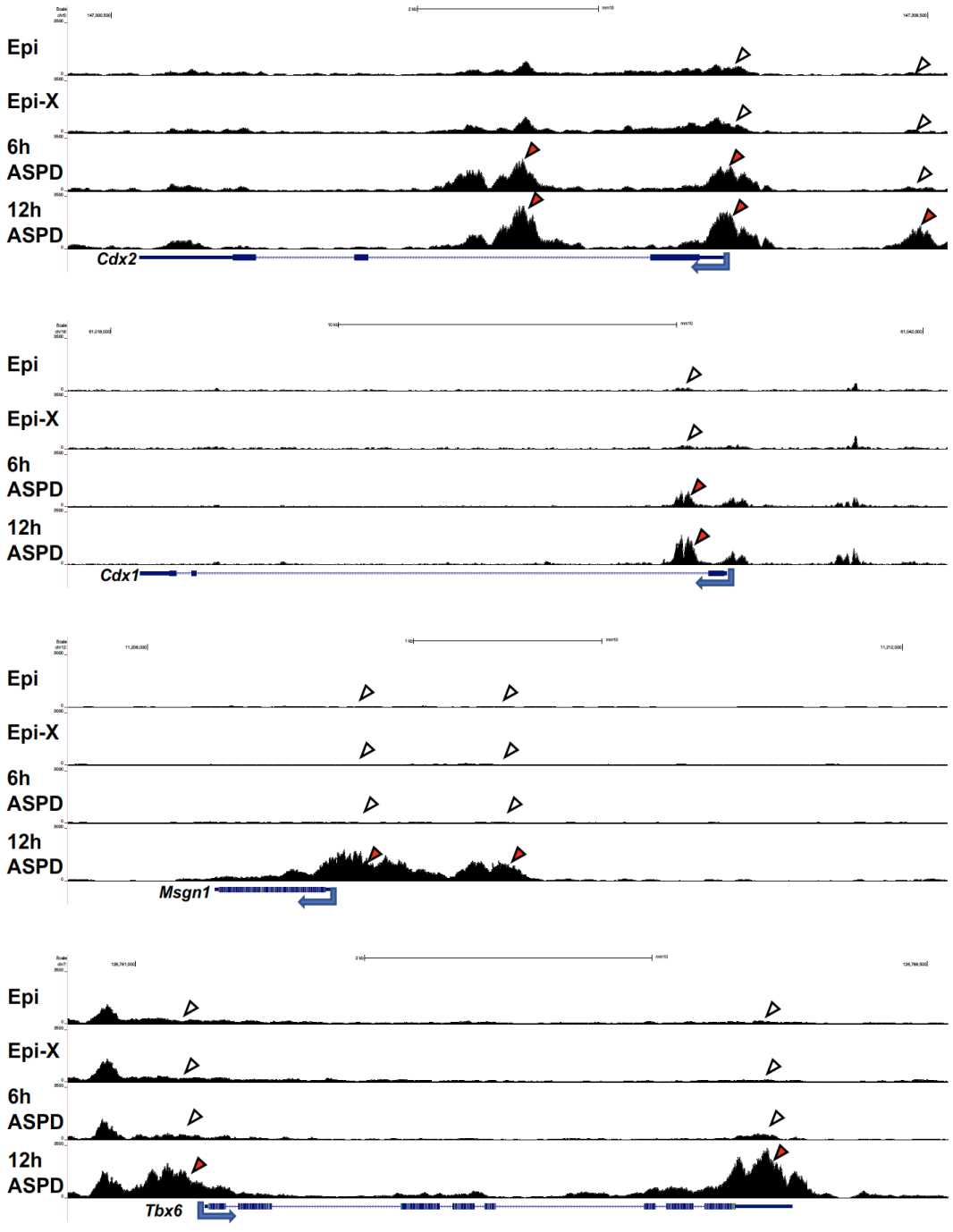
Furthermore, the reviewer's broader point on the chromatin landscape is indeed significant, and we appreciate them raising it. We have undertaken an investigation into how the intrinsic primed state of $CLDN6^{Low}$ and $CLDN6^{High}$ epiblast cells influences their response to WNT signaling. We addressed this point by culturing these cells under conditions that inhibit WNT signaling and then assessing their self-renewal capabilities and their potential to differentiate when exposed to WNT stimulation.

As accurately pointed out by the reviewer, our work challenges results from Metzis *et al.,* Cell 2018, who proposed that regional neural identity is set by chromatin accessibility in the epiblast. However, Metzis *et al.*, (2018) used "epiblast cells" treated with WNT agonist, which induces WNT signalling in these cells. It is well-established in the field, supported by numerous seminal studies (Kurek *et al.,* Stem Cell Reports 2015; Arnold and Robertson, Nat Rev Mol Cell Biol. 2009; Morgani *et al.,* BMC Developmental Biology 2017), that epiblast cells exposed to WNT signaling differentiate towards the primitive streak. Furthermore, it's important to highlight that Metzis *et al.* (2018) did not offer substantial evidence demonstrating that their

"epiblast cells" exposed to WNT agonists retained their epiblast characteristics. In fact, they did not provide convincing proof regarding the self-renewal and expansion capabilities of their "regionalized epiblast populations." This aspect raises doubts about whether the "posterior epiblast cells" treated with WNT in their study genuinely maintained an epiblast identity or had instead undergone differentiation towards the primitive streak.
Significantly, in our work, we expand CLDN6^{Low} EpiSCs over multiple passages, and demonstrated that they retain a CLDN6^{Low} state with restricted potency towards NMP fate. Notably, our work represents the first instance of *in vitro* culture of a self-renewing musculoskeletal progenitor population (NMPs). While these findings are reflected in the discussion, we deliberately refrained from engaging in a debate with the Metzis *et al.* (2018) study for obvious reasons.
Another aspect in the Metzis *et al.* (2018) study that causes confusion is their choice of starting cellular model. In their research, they used mouse embryonic stem cells (ESCs) that represent the initial naive phase of pluripotency, while making assertions regarding post-implantation epiblast regionalization and primed pluripotency. In contrast, our study focuses on post-implantation epiblast cells (EpiSCs), which are primed and represent the developmental stage that precedes germ-layer specification.

To further clarify that the variation in chromatin accessibility between our experimental conditions and those in the study by Metzis *et al.* is not a result of the WNT-inhibited culture conditions, we conducted ATAC-seq analysis on EpiSCs cultured both with and without WNT inhibition. These EpiSC lines were derived separately and never exposed to WNT agonists or antagonists.
In Rev. Fig. 3 below, Epi-X refers to EpiSCs cultured with WNT inhibitor (XAV939) which are used in this study, while Epi refers to EpiSCs not exposed to WNT agonists or antagonists. As can be observed, there are no discernible differences in chromatin accessibility across NMP genes (like *Cdx2, Cdx1*) or NMP-to-PSM genes (*Msgn1, Tbx6*). Notably, we can track the dynamic chromatin changes upon WNT stimulation during APSD differentiation. Chromatin accessibility of regulator regions associated with NMP-to-PSM genes increases upon WNT stimulation at 6 hours and 12 hours respectively.
Furthermore, Metzis *et al.*, 2018 do not provide an explanation or elucidate the biological basis for how regional identity is assigned to the WNT+ and WNT- epiblast populations, aside from citing evidence from late chick embryos (although the Metzis *et al.* paper is focused on mouse).

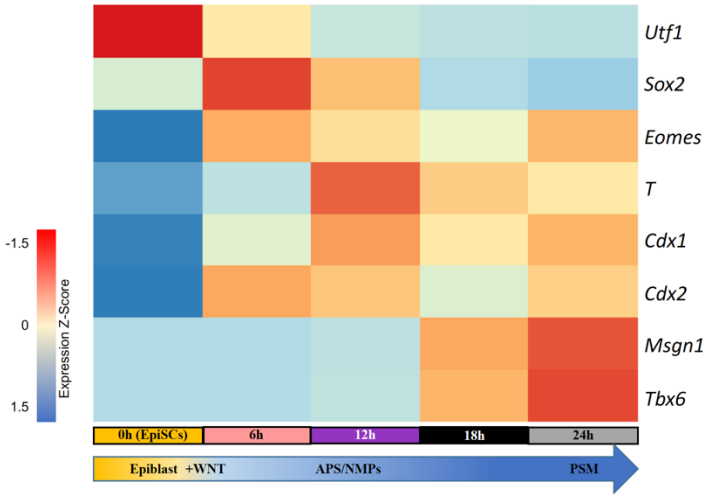
Significantly, in our work, we expand CLDN6^{Low} EpiSCs over multiple passages, and Metzis *et al.* mentioned that CDX2 present in the “WNT+ posterior epiblast” is responsible for posterior neural priming, citing two papers (van der Akker *et al.,* Development 2002; Mallo *et al.*, Dev. Biol. 2010). However, it's important to note that neither of these two references mentions the expression of *Cdx2* in the epiblast. Instead, both sources clearly state that *Cdx2* is expressed in the primitive streak and nascent paraxial mesoderm, as initially reported by Meyer and Kruss, Development 1993 and Beck *et al.,* Dev. Dyn. 1995. Indeed, we directly cite here from Van der Akker et al., 2002: “*Cdx1 starts to be expressed at the late primitive streak stage (day 7.2) in ectodermal and mesodermal cells of the primitive streak* (Meyer and Gruss, 1993).



Rev. Fig. 3 ATAC-seq of *Cdx2*, *Cdx1*, *Msgn1* and *Tbx6* loci in EpiSCs not exposed to WNT agonists or antagonists (Epi) and EpiSCs cultured with WNT inhibitor XAV939 (Epi-X). The ATAC-seq data reveals similar accessibility patterns over the NMP and PSM (Presomitic Mesoderm) genes in Epi and Epi-X conditions. Chromatin accessibility increased at 6 hours post-WNT induction for the NMP genes *Cdx1* and *Cdx2*, and at 12 hours for the early PSM genes *Msgn1* and *Tbx6*. Accessible and open regions are indicated by the red arrows, while closed regions are with white arrows.

Expression of Cdx2 begins at day 3.5 in the trophectoderm of the blastocyst and later continues in tissues derived from the extra-embryonic ectoderm. Cdx2 transcripts are detected in the embryo proper slightly later than Cdx1 (Beck et al., 1995) (E. Tolner, B. Roelen and J. D., unpublished)."

We conducted RNA sequencing (RNAseq) to examine the differentiation of EpiSCs towards NMPs and presomitic mesoderm (PSM) at various time points, with samples collected every 6 hours (Rev. Fig. 4). Our analysis revealed a notable pattern: the absence of *Cdx2* expression in EpiSCs, followed by an increased expression of NMP genes *(Sox2*, *Cdx1*, and *Cdx2*), and NMP-to-PSM (*Msgn1* and *Tbx6*) genes upon WNT stimulation during the differentiation process.



Rev. Fig. 4 Heatmap of RNAseq analysis, displaying relative expression of selected differentially expressed genes across NMP towards PSM differentiation. *Utf1* is a marker of undifferentiated state, marking EpiSCs. Temporal analysis showed no expression of *Cdx2* in EpiSCs and an increased expression of APS/NMP (*Sox2, Cdx1*, and *Cdx2*) and PSM (*Msgn1* and *Tbx6*) genes upon WNT stimulation.

The Metzis 2018 Cell paper has influenced the field, but the premise and evidence are not properly demonstrated. The data presented in our manuscript challenge the biological basis of epiblast stem cell nature and the overall findings published by Metzis *et al*. 2018. We briefly reflected on this aspect in the discussion but did not divulge it at a deeper level. We believe that our results are impactful to the field, and force a rethink of how epiblast regionalization impacts lineage priming.

"Although this observation might be due to the culturing of EpiSCs under WNT-inhibited conditions, upon WNT stimulation, the gain in open chromatin in CLDN6Low APSD cells is minimal (Figure 3b)."

As the reviewer pointed out, the gain in open chromatin in CLDN6^{Low} APSD cells is minimal when looking at PSM genes. This is due to the fact that many of these PSM lineage genes open up later, as described in Mariani *et al.,* Nature Communications 2021. Looking specifically at the WNT-response genes bound by the WNT-effector LEF1 in NMPs (Supplementary Figure 4a), we can see a significant rewiring of the chromatin landscape upon WNT-induced differentiation towards presomitic mesoderm.

The results in figure 3d show that the addition of WNT leads to epigenetic remodeling and differentiation. It would be helpful if the authors could provide previous reports that observed similar findings in the EpiSCs or similar lines.

The effect of WNT induction over epigenetic remodeling is a noteworthy observation that aligns with prior research findings. We appreciate the reviewer for highlighting this point. Notably, the seminal study conducted by Smith *et al.,* Nature 2017, also reported comparable observations in inner cell mass (ICM) outgrowths subjected to various combinations of WNT and FGF agonists and antagonists.

As indicated by Smith *et al.,* Nature 2017 - *"In combination, PD and CHIR comprise the "2i" condition, an FGF-impeded, WNT-activated state that maintains pre-implantation-like global hypomethylation. Alternatively, exogenous FGF is sufficient to drive genome and CGI methylation to nonphysiologically high levels (Fig. 2d). Surprisingly, when coupled with FGF, WNT agonism effectively blocks genome remethylation but redirects CGI-level methylation to a broader subset of extraembryonic targets (Fig. 2e, f)."*

We observed a similarly fascinating epigenetic rewiring, as the fate-restricted CLDN6^{Low} EpiSCs exhibited higher levels of intragenic methylation, whereas the CLDN6^{Low} APSD (NMPs) displayed the lowest intragenic methylation among all the samples analyzed. This finding suggests that CLDN6^{Low} EpiSCs respond to WNT-induced differentiation drastically differently from CLDN6^{High} EpiSCs. Furthermore, we also observed a similar chromatin remodeling upon WNT stimulation in the later stages of paraxial mesoderm differentiation, as described in Mariani *et al.,* Nature Communications 2021.

Similarly, it would be beneficial if the authors could provide references to findings showing that higher gene body methylation leads to restricted potency in EpiSCs or similar lines.
While, to the best of our knowledge, there are no studies directly addressing this aspect in EpiSCs, we have taken the reviewer's feedback into consideration.
As a response to their concern, we have incorporated references from analogous studies conducted in different systems into our current manuscript.
Below, there is an example of intragenic methylation implicated in hematopoietic differentiation and lineage potency. This is a paragraph taken from Deaton *et al.*, Genome Research 2011 from the lab of Adrian Bird, one of the pioneers in the field of DNA methylation, highlighting the association between intragenic methylation and cell fate acquisition, where they also assessed *in vivo* functionality.
"Cell type–specific methylation preferentially occurs at intragenic CGIs and this negatively correlates with gene expression.
A key immune cell regulator showing a negative correlation between intragenic CGI methylation and expression is the Gata3 gene, which is expressed specifically in Th2-cells (Fig. 3E) and determines Th2 cell identity (Nawijn et al. 2001). The only methylation difference between Th1 and Th2-cells occurred at an intragenic CGI in the Gata3 gene (Fig. 3F). However, IL4-producing Th2 cells isolated from a Th2-inducing infection with Schistosoma mansoni showed complete

demethylation of this CGI after an 8-wk infection (8% methylation) (Fig. 3G). This confirmed that DNA methylation changes identified at intragenic CGIs using in vitro T-cell differentiation are relevant in vivo."
As the reviewer suggested, we have added this and similar citations in our manuscript, Lorincz *et al.,* Nat Struct Mol Biol 2004 and Rönnerblad *et al.,* Blood 2014.

Altogether we thank Reviewer #1 for the feedback and helping us increase the clarity of the manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript by Menezes and colleagues describe different subpopulation of the post-implantation epiblast (and subpopulations of EpiSCs) displaying differential responses to signalling molecules.
The authors used CLDN6 expression levels to sort EpiSCs, finding that CLDN6 High cells display also increased pERK levels and a biased towards DE and anterior neuroectoderm.
Conversely, CLDN6 low cells are less plastic and can form mainly NMPs.
ATACseq did not reveal differences that could explain the alternative cell fate acquisition, while WGBS was more informative, potentially providing a molecular explanation.
The authors then used Pbx1 KO models to study the role of pERK, finding differences in accessibility and methylome connected to BMP responsiveness e extra embryonic mesoderm differentiation.
The manuscript presents a large number of datasets and is quite "dense". Several concepts are repeated multiple times, making the reading rather complex. This reviewer suggests to streamline the text of manuscript, in particular the Results section.

We appreciate the reviewer for bringing up this matter. In response, we have streamlined the presentation of our results by eliminating redundancy, such as repetitive phrases or sentences conveying the same information, and breaking down lengthy and complex sentences into shorter, more easily understood ones in the revised version of the manuscript.

One point should be clarified, concerning the role of FGF/pERK.
The authors put forward the idea that levels of pERK control the responsiveness to differentiation cues. To test their model they then used Pbx1-KO EpiSCs, in which they observed a partial reduction in pERK. Still, the effects observed in Pbx1-KO could be due to Pbx1 itself, rather than FGF/pERK.

We thank the reviewer for raising this point. Here, we will outline our rationale towards why the observed effect in *Pbx1-KO* is due to pERK modulation, rather than a direct role of PBX1 on ExM lineage genes.

RNAseq analysis of WT vs *Pbx1-KO* EpiSCs revealed a strong effect on FGF/ERK signalling pathway (Figure 4b), but there were no transcriptional effects on lineage genes at this stage,

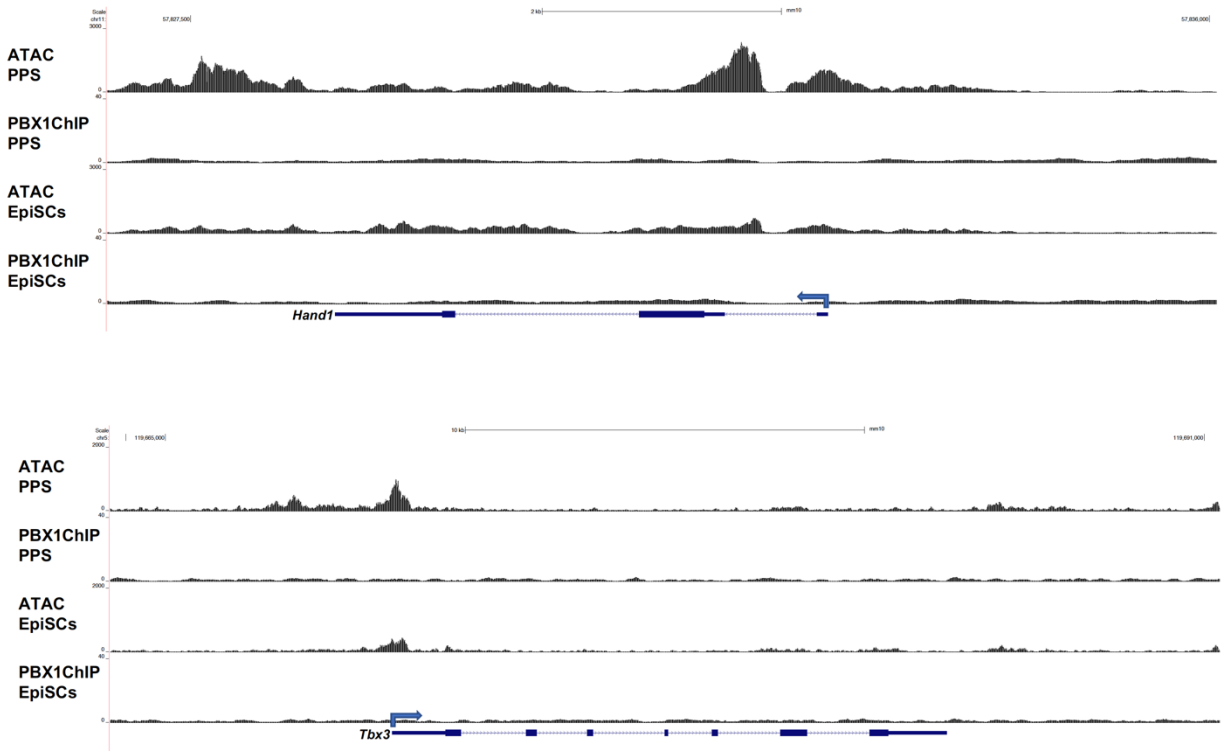
including *Hand1* and *Tbx3* which are known early extraembryonic mesoderm (ExM) markers, on which we focus in our manuscript.
GO of differentially expressed genes, WT vs *Pbx1-KO* EpiSCs, revealed terms associated to MAP Kinase and ERK signalling (Supplementary Fig. 5g).
RNAseq and Western blot analyses narrowed down our focus to pERK modulation, suggesting an effect of PBX1 on ERK feedback regulation.
We did ChIPseq for PBX1 in the EpiSCs. GO analysis of the PBX1-bound sites in EpiSCs revealed terms associated to regulation of MAPK/ERK cascade (Supplementary Fig. 5j). Western blot analysis with phospho-ERK (Figure 4c), total-ERK (Supplementary Figure 5h), phospho-MEK (Supplementary Fig. 5i), phospho-AKT (Reviewer Fig. 13) antibodies, narrowed down the effect to phospho-ERK and its downstream effectors. Focusing further on how PBX1 could be regulating the FGF/ERK signalling cascade, we observed an EpiSC-specific binding of PBX1 on *Spry2* putative enhancer region (Supplementary Fig. 5k). This PBX1-bound site was only accessible in EpiSCs. We performed EMSA that confirmed *in vitro* assembly of PBX1-PREP multimeric complex on *Spry2* epiblast-specific PBX1-bound region (Supplementary Fig. 5l). These data point towards PBX1 regulation of the ERK feedback loop via *Spry2* modulation.

In this study we focused on how the FGF-driven epiblast methylome regulates lineage priming, using *Pbx1-KO* as a model to study ExM differentiation as *Pbx* mutant embryos have severe ExM defects (Fig. 5b, Supplementary Fig. 5d). Furthermore, epiblast-specific deletion of *Pbx* (via $Sox2^{Cre}$) versus primitive streak deletion (T^{Cre}) demonstrated the epiblast-specific role of *Pbx* in regulating ExM differentiation *in vivo* (Supplementary Fig. 5c,d,e).

We observed an intriguing effect on DNA methylation of ExM genes in the EpiSC stage (Fig. 5g, i), where these lineage genes were not transcriptionally active. When we compared the DNA methylation patterns of these ExM lineage genes in WT and *Pbx1-KO* EpiSCs, we observed a distinct correlation with their transcriptional activity during differentiation (as shown in Figure 5, panels d, e, and f). These findings suggest that the regulation of ExM genes is mediated through DNA methylation established at EpiSC stage.

We further focused on the regulation of two key ExM genes: *Tbx3* and *Hand1*.

To probe whether PBX1 was directly regulating transcription of *Tbx3* and *Hand1*, we did ATACseq and ChIPseq against PBX1. We found no direct binding of PBX1 to *Hand1* or *Tbx3* in either EpiSCs or PPS/ExM cells (Rev. Fig. 5). It's worth noting that ATACseq data indicated accessible regulatory regions in PPS, but PBX1 did not exhibit any binding activity in these regions. Thus, PBX1 does not directly influences the transcription of PPS/ExM genes.

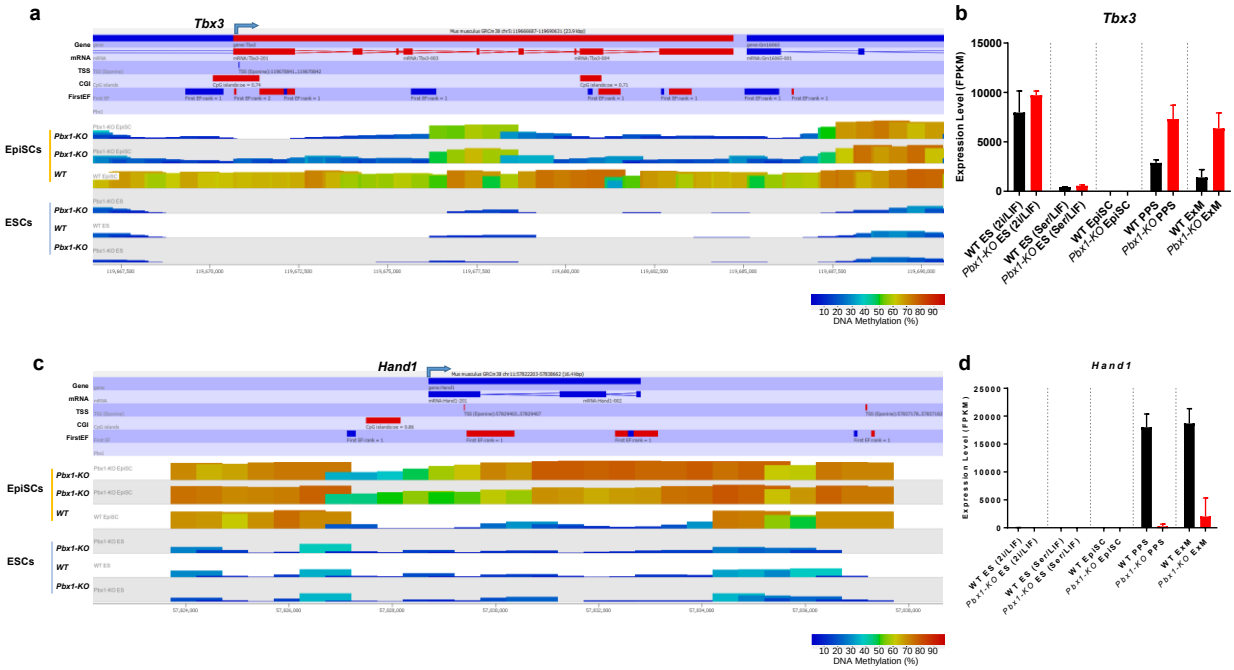


Rev. Fig. 5 ChIPseq and ATACseq coverage tracks showing absence of PBX1 recruitment over *Hand1* and *Tbx3* genes, even when the regulatory regions open upon differentiation towards PPS.

Tbx3 is expressed in mouse ES cells and is a key part of the pluripotency machinery (Ivanova *et al.,* Nature 2006; Russell *et al.,* Stem Cell Reports 2015). However, *Tbx3* was not differentially expressed in WT vs *Pbx1-KO* ES (ser/LIF or 2i/LIF) (Supplementary Fig. 5f, Reviewer Fig. 6), despite both *Tbx3* and *Pbx1* being expressed in ES cells.

Probing the DNA methylation status of *Tbx3* locus, we observed consistent hypomethylation in both WT and *Pbx1-KO* ES cells (Rev. Fig. 6), validating observations regarding the hypomethylated nature of mouse ES cells which is dependent on inhibition of FGF signalling (Ficz *et al.,* Cell Stem Cell 2013; Habibi *et al.,* Cell Stem Cell 2013). In contrast, the *Tbx3* locus stays hypomethylated in the *Pbx1-KO* EpiSCs but gets hypermethylated in WT EpiSCs (Rev. Fig. 6). Upon latter transcriptional activation of *Tbx3* during ExM differentiation, we observed a significant upregulation in *Pbx1-KO* PPS and ExM cells.

Conversely, *Hand1*, which is also not expressed in the EpiSC stage, gets specifically hypermethylated in the *Pbx1-KO* EpiSCs, and significantly downregulated in *Pbx1-KO* PPS and ExM cells upon differentiation towards ExM fate (Rev. Fig. 6).



Rev. Fig. 6 a) WGBS (SeqMonk) visualization indicating differentially methylated regions (DMRs) across the *Tbx3* locus in WT and *Pbx1-KO* ESCs and EpiSCs, showing hypermethylation in WT EpiSCs but not in ESCs and *Pbx1-KO* EpiSCs. The bar shows CpG methylation levels (%). **b**) Gene expression level (normalized FPKM data from RNAseq) of *Tbx3* across ESCs (2i/LIF and serum/LIF), EpiSCs, PPS and ExM in WT and *Pbx1-KO*, shows no difference between WT and *Pbx1-KO* ESCs and no expression, but *Tbx3* gets upregulated in *Pbx1-KO* PPS and ExM. **c**) WGBS (SeqMonk) visualization showing DMRs across *Hand1* locus in WT and *Pbx1-KO* ESCs and EpiSCs, reveals hypermethylation *in Pbx1-KO* EpiSCs and hypomethylation in WT EpiSCs and across the ESCs. Bar shows CpG methylation levels (%). **d**) Analysis of normalized FPKM data from RNAseq reveals distinct expression patterns for Hand1 in different cell types and genotypes. Hand1 is not expressed in ESCs and EpiSCs, but gets specifically expressed in WT but not *Pbx1-KO* upon BMP-mediated differentiation towards PPS and ExM.

These observations are particularly intriguing because they underscore the differences in signalling requirements between these two distinct cell states. EpiSCs require FGF stimulation, while ESCs rely on FGF inhibition. The methylome of mouse ESCs resembles pre-implantation blastocyst cells, which are dependent on FGF inhibition (Ficz *et al.,* Cell Stem Cell 2013; Habibi *et al.,* Cell Stem Cell 2013). The role of PBX1 functionally originates in the post-implantation epiblast embryonic developmental stage (as shown in Fig. 4b), coinciding with FGF/ERK signalling requirement and a transition towards a global hypermethylated state (Borgel *et al.,* Nature Genetics 2010; Auclair *et al.,* Genome Biology 2014; Smith *et al.,* Nature 2017).

Altogether these multiple evidences point towards an epiblast-originating role of PBX1 regulating pERK modulation, influencing site-specific DNA methylation in EpiSCs that primes cell signalling response to differentiation cues.

However, one aspect is rather confusing, the presence of FGF ligands both on EpiSC expansion medium and in their differentiation protocols (e.g. APSD).
If the levels of pERK are really crucial it should be sufficient to lower the concentration of FGF in the medium, measure pERK, and observe a more "posterior/NMP biased" phenotype.
Can the authors change the responsiveness to differentiation cues by directly modulating FGF signal intensity?
We thank the reviewer for highlighting this aspect. The presence of FGF ligands both in the EpiSC expansion medium and differentiation protocols does raise an intriguing question about how FGF signal intensity might influence the cellular response to differentiation signals. However, it's crucial to recognize that the relationship between FGF signalling and differentiation cues can be quite complex, going beyond simply adjusting FGF concentration in the medium. Therefore, altering FGF levels may not have a straightforward impact on ERK activity. Indeed, we found that in EpiSCs, not adding FGF in the EpiSC medium led to an increased expression of *Fgf5* ligand, suggesting an immediate compensatory response (Rev. Fig. 9). However, upon multiple passages, EpiSCs cultured without FGF (EpiSCs-bFGF) became heterogeneous with a mix of epiblast cells and differentiated cells. This approach is not ideal because we cannot maintain the undifferentiated epiblast state, as we did with the CLDN6-sorted EpiSCs and the *Pbx1-KO* EpiSCs.

Furthermore, attempts to modulate FGF/ERK activity in primed pluripotent stem cells (mouse and human) via RNAi or selective cell-permeable chemical inhibitors have been challenging as they do not maintain their primed state (Goke *et al.,* Molecular Cell 2013; Sterneckert *et al.,* Stem Cells 2010).

Altogether our data points towards modulation of the ERK feedback loop rather than changes in FGF levels that play a direct role. For instance, we observed a significant increase in FGF ligands in *Pbx1-KO* EpiSCs (Fig. 4b), yet paradoxically, the levels of phosphorylated ERK (pERK) were reduced (Fig. 4c).

Minor point:
line 126-128 ", the ability of CLDN6Low EpiSCs to self-renew and retain its initial stem-state can be attributed to the expression of stemness genes"
This statement is not clear. CLDN6 Low and High EpiSCs express comparable levels of pluripotency factors Oct4 and Nanog, so there is no reason why they should not self-renew and maintain pluripotency. Why should the expression of Sox9 and Snail2, which are expressed in mammary stem cells, support pluripotency? Please delete/clarify.

We thank the reviewer for raising this point. It's essential to clarify the distinction between stemness genes, embryonic state, and pluripotency markers in the context of the ability of $CLDN6^{Low}$ EpiSCs to self-renew and maintain their stem-like state.

Stemness genes are a group of genes that are associated with maintaining the self-renewal capacity of stem cells, thus playing a crucial role in preserving their stem cell state and suppressing differentiation regardless of stage. *Sox9* and *Snai2* have been shown to together

cooperatively regulate stemness properties across different stem cell types, like skin epithelial cells and carcinomas (Badarinath *et al.*, Cell Reports 2022); Drosophila intestinal stem cells (Korzelius *et al.*, EMBO Journal 2014); colorectal cancer cells (Avendaño-Felix *et al.,* Journal of Gastrointestinal Oncology 2023); not just in mammary stem cells (Guo *et al.*, Cell 2012). Interestingly, these cell states where the stemness properties of *Sox9* and *Snai2* have been studied have many similarities with EpiSCs and share common epithelial traits. The aforementioned publications provide direct functional proof of the connection between the function of stemness genes (*Sox9* and *Snai2*) and subsequent passage through an epithelial-to-mesenchymal transition (EMT). In our study, EpiSCs undergo EMT which is crucial to form NMPs, but not definitive endoderm (arising through a mechanistically different partial-EMT process (Scheibner *et al.,* Nature Cell Biology 2021) or neuroectoderm (that retain their epithelial characteristics). Indeed, as we demonstrated, unlike CLDN6^{High} EpiSCs, CLDN6^{Low} EpiSCs possess the ability to self-renew and maintain a stem-like state (marked by *Sox9* and *Snail2* expression), with restricted potency towards NMPs. CLDN6^{Low} EpiSCs retain their CLDN6^{Low} nature and NMP-restricted potency upon multiple passages, while CLDN6^{High} EpiSCs revert back to a salt-and-pepper state of CLDN6 expression, resembling untreated EpiSCs, and regain potency to form NMPs upon passaging. Indeed, it would be interesting to study the mechanism by which *Sox9* and *Snai2* modulate the transition from fate-restricted self-renewing CLDN6^{Low} EpiSCs towards NMPs upon WNT activation, but that is beyond the scope of this manuscript.

Thus, unlike CLDN6^{Low} EpiSCs, CLDN6^{High} EpiSCs are truly “pluripotent”. “Pluripotent” stem cell markers are expressed in both CLDN6^{High} and CLDN6^{Low} EpiSCs, but stemness genes are specifically expressed in CLDN6^{Low} EpiSCs, which we show maintain their CLDN6^{Low} state upon multiple passages, and have restricted differentiation potential. Embryonic state “pluripotency” markers, like *Oct4, Sox2*, and *Nanog* identify pre-implantation embryonic stem cells like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) as well as post-implantation EpiSCs. These markers are indicative of a broader differentiation potential compared to stemness genes. However, these genes also have pleiotropic functions during differentiation. For example, *Sox2* is also a marker of NMP state (Gouti *et al.,* Dev. Cell 2014; Koch *et al.,* Dev. Cell 2017).

Thus, our work demonstrates a need to rethink how we define pluripotency markers, and the term pluripotency in general. As we show, CLDN6^{Low} EpiSCs are not pluripotent, but in bulk, EpiSCs are thought to be pluripotent. Studying the potency of individual cells via single-cell NGS or FACS-sorting provides a deeper insight into the true potency of cells compared to bulk analysis.

We extend our gratitude to Reviewer #2 for their valuable feedback, which has contributed to enhancing the clarity of our manuscript.

Reviewer #3 (Remarks to the Author):

This paper by Menezes and colleagues perform various genome-scale analyses on Claudin-high and Claudin-low EpiSCs. They use this data to claim that Erk signaling alters various aspects of chromatin state prior to germ layer acquisition. While the datasets are of potential value to the community, I find that the interpretations in this paper often go far beyond what the data shows.

Major concerns:
My main concerns with this paper stem from loosely correlated observations that are used to make exceptionally strong causal statements. I point out some examples below, but there are few - if any - causal statements about connections between FGF/Erk, BMP or Wnt signaling and changes to methylation that can be justified based on the data shown.

(1) The authors begin by staining for Claudin-high and Claudin-low subpopulations in the E7.5 embryo, and report an A-P bias in this signal. What makes Claudin a good marker for anterior vs posterior sompared to the numerous other factors that show a clear (and in some cases, even clearer) A-P bias? The authors should justify why a new A-P marker is necessary or an improvement over well characterized existing ones. Why not sort for the many existing makers with known A-P bias, or directly sort for Erk-high and Erk-low cells using a ppErk antibody if Erk is what one is interested in?

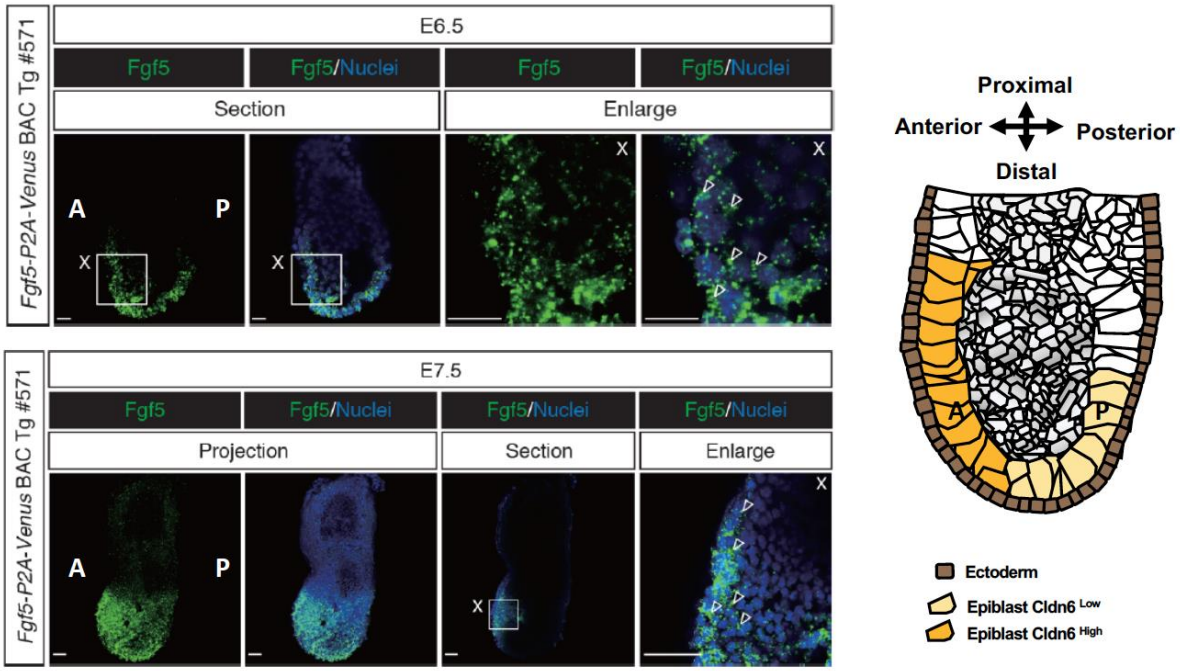
We thank the reviewer for pointing this aspect out, and we will gladly reiterate the logic behind using CLDN6 as the basis for our study. Our study aims to identify factors regulating the primed nature of epiblast cells, influencing how they transition towards specific germ-layer lineages. Using a system that enables sorting and re-seeding of EpiSCs enables us to use *in vitro* differentiation systems as a proof-of-concept. There are two ways to do this, via a reporter system or using a surface marker. A reporter system while elegant in its own right, raises the question of clonality and furthermore has disadvantages especially when the focus of the study is on dosage and temporal transitions (rather than on/off gene expression). Thus, a surface marker-based FACS sorting system (like CLDN6) offers us the flexibility to quickly sort based on protein expression, and re-seed back the cells into culture. Using an intracellular pERK antibody would not allow us to re-seed the cells upon sorting.

We have identified CLDN6 as the most suitable surface marker for this purpose based on our observations and prior research that suggests anterior-posterior (A-P) expression of CLDN6 (Scheibner *et al.,* Nature Cell Biology 2021; Anderson *et al.,* Dev. Dyn. 2008). We coupled our CLDN6-based sorting system with the MACS-Tyto sorter that allows quick, efficient and contamination-free sorting directly into the differentiation medium when required.
In summary, the purpose of our study was not to identify novel A-P markers, as there are well-established markers available, as mentioned by the reviewer. Instead, our study aimed to explore the factors that connect epiblast regionalization, bias in germ-layer lineage priming, and subsequent differentiation.

In addition to showing CLDN6 A-P expression *in vivo*, we also looked to see how its expression compares to other existing markers using single-cell spatial transcriptomics and compared to previously published bulk spatial GEOseq data (Peng *et al.,* Dev. Cell 2016).

There is also something logically inconsistent in the early analysis of Fig 1 relative to the common understanding of the mouse embryo. FGF/Erk signaling is typically thought to be associated with the posterior domain of the embryo during gastrulation, yet the authors report that Claudin is associated with both anterior positions and high Erk phosphorylation. Is there any literature to support an anterior-to-posterior gradient of FGF/Erk activity in the mouse epiblast? I would at the minimum require co-staining of pErk and Claudin in the embryo to show a reasonable spatial overlap between them, and also some explanation of why the authors obtain results that are contrary to the expected FGF/Erk activity pattern.

What the reviewer points out is true with regards to the expression of FGF4 and FGF8, which at the onset of gastrulation, are highly expressed in the posterior primitive streak (PS) relative to the anterior end and are required for migration away from the PS (Sun *et al.,* Genes and Development 1999). However, FGF5, an epiblast marker (Greber *et al.,* Cell Stem Cell 2010; Kojima *et al.,* Cell Stem Cell 2014), is expressed in the post-implantation pre-gastrulation epiblast and retains an A-P expression pattern at E6.5 and E7.5 in the epiblast region (Khoa *et al.,* PLoS One 2016). Indeed, FGF8 is not expressed in EpiSCs, and gets expressed in the primitive streak. Thus, our observations are not contrary to the expected FGF/ERK activity pattern in the epiblast. Please find in the Rev. Fig. 7 below the data from Khoa *et al.,* PLoS One 2016, showing the anterior-posterior gradient of FGF5.



Rev. Fig. 7 FGF5 localization in the anterior epiblast as shown by the *Fgf5* reporter generated by Khoa *et al*., PLoS One 2016. Panels are extracted from Khoa *et al*., 2016.

Also, the correlation between Claudin expression and pErk is quite mild in Figure 1F. The two different pErk peaks having highly overlapping Claudin distributions. From this data it is not obvious that sorting Claudin levels is a good proxy for separating pErk-low and pErk-high cells at all. Fig 1F is also misleading because it shows the results of gating ERK-high and ERK-low cells to obtain different Claudin levels, which is quite different than gating Claudin-high and Claudin-low cells to obtain different ERK levels (the premise of this part of the paper). Finally, why does the range of Claudin expression look so much more compressed and on a different scale in Figure 1F than it does in Figure 1C and Figure S1D?

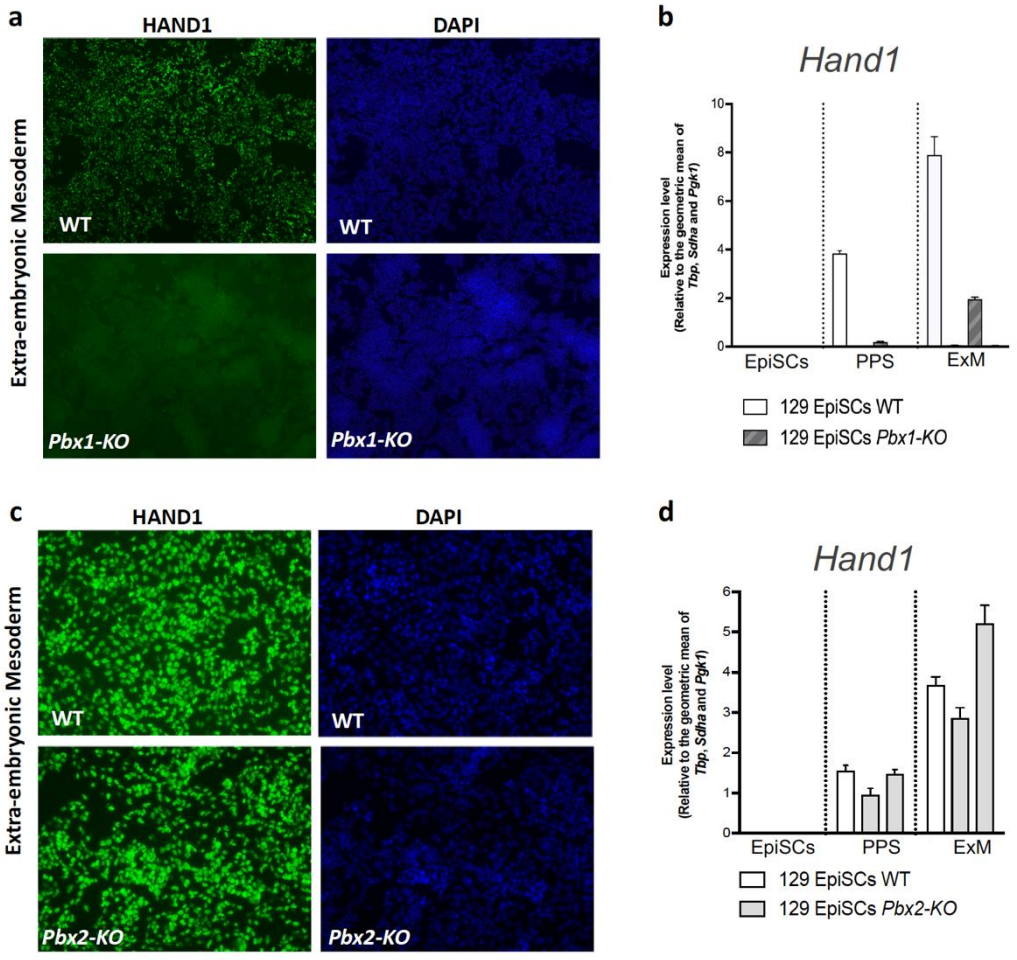
We acknowledge the observation of the reviewer. Fig. 1C and Fig. S1D are based on FACS analyses done using a MACS-Tyto sorter. The MACS-Tyto sorter uses a patented microchip-based technology which allowed us to take advantage of high-speed flow sorting in the safety of a fully enclosed cartridge. This technology allowed us to seamlessly sort and reseed millions of EpiSCs in sterile conditions. Fig. 1F was performed on a BD LSRFortessa™ Cell Analyzer which was more suitable for analyses, and required limited cell numbers. Additionally, the cells were treated differently for sorting on the BD Fortessa, as pERK is an intracellular staining that required cell permeabilization. Since we were analyzing for differences in pERK levels, we used that as the starting gating point. Ultimately, while the ranges and scales might differ due to technical approaches, the interpretation remains the same.

(2) Pbx2 knockout might have extremely broad effects, of which a change in ERK signaling is at best one among many. Once again, this loose correlation is interpreted causally throughout the paper.
Line 304: “FGF/ERK is known to regulate site-specific DNA methylation in different contexts, but whether it has a similar role in post-implantation epiblast stages remains uncertain. A significant complication is the absence of a suitable model to challenge FGF/ERK function without inducing spontaneous differentiation. The three-amino acid loop extension (TALE) homeodomain proteins (PBX, MEIS, and PREP) are alternative candidates as they regulate FGF/ERK signalling in diverse developmental contexts and cancers.”

Line 361: “Thus, our data suggest a systematic network where PBX1 regulation of Spry2 controls differential ERK/ETS activity, which shapes the epigenetic landscape across lineage-specific genes in primed epiblast cells before their transcriptional induction (Fig. 4i). Altogether, we established an ERK-deficient model to study the link between FGF/ERK, DNA methylation and lineage preprogramming.”

In our manuscript we showed collective evidence indicating that PBX1 plays a crucial role in the epiblast, modulating pERK activity, thereby influencing site-specific DNA methylation in EpiSCs and priming the cellular signaling response to differentiation cues. The PBX1/ERK relationship was raised also by reviewer 2. Please see our answer addressing the link between Pbx1 and ERK signalling in the above question by Reviewer 2 (Rev. Fig. 5 and 6) in detail, and further focused on pERK below (Rev. Fig. 11).
Furthermore, we thank the reviewer for suggesting a possible compensatory effect of PBX2. In the TALE family, there are indeed proteins with a similar pattern of expression and DNA binding

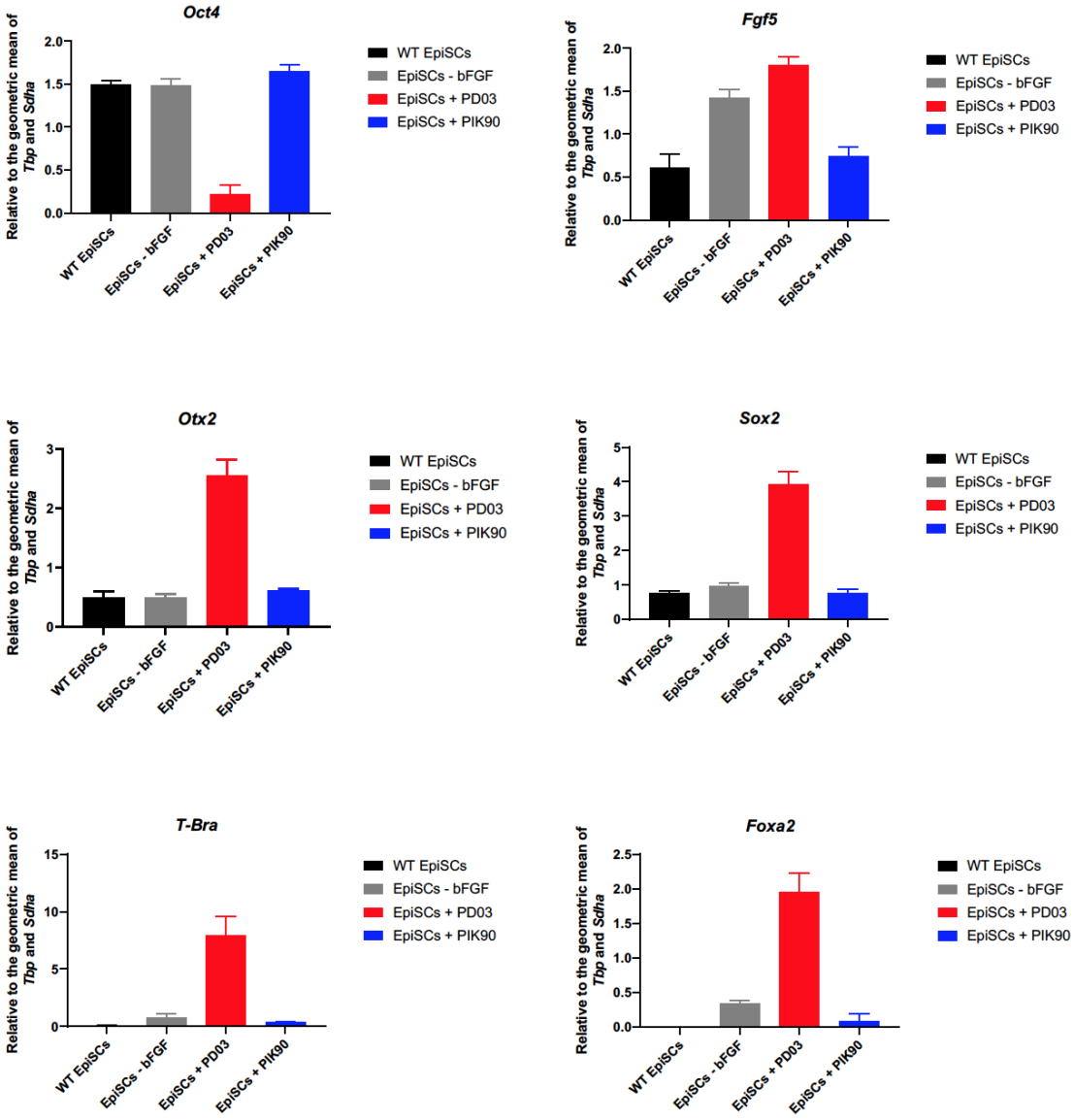
capacity as PBX1, indicating a potential compensatory effect. Consequently, we have investigated the role of *Pbx2* in ExM differentiation. *Pbx2-KO* EpiSCs differentiated towards ExM show no effect on *Hand1* expression, as shown in the Rev. Fig. 8 below, by IF and qPCR. Thus, PBX2 is not responsible for modulating ExM specification.



Rev. Fig. 8 a) IF of HAND1 (green) and DAPI (blue) showing loss of HAND1 expression in *Pbx1-KO* ExM cells. **b**) RT-qPCR mRNA analysis showing loss of *Hand1* expression in *Pbx1-KO* EpiSCs upon PPS and ExM differentiation. **c**) IF and qPCR **d**) analysis of HAND1 showing no difference in protein and mRNA levels in WT and *Pbx2-KO* across ExM differentiation.

This paper repeatedly presents exceptionally convoluted ways to study Erk signaling, using loosely correlated markers (Claudin) and perturbations (Pbx2). Why not just simply treat EpiSCs with a MEK inhibitor and look for a direct role of ERK signaling, or collect cells from embryos using validated Erk-associated markers? And if doing so gives completely different results, then isn't that evidence that the Claudin and Pbx2 results have nothing to do with FGF/Erk signaling at all?

We agree with the reviewer that PBX2 has nothing to do with FGF/ERK signalling. Our paper is focused on using CLDN6 as a methodology for sorting EpiSC populations, and using the genetic perturbation of *Pbx1* as a tool to study ERK feedback loop.
As the reviewer suggested, we did treat the EpiSCs with a MEK inhibitor (also known as PD03) and the results were already in the manuscript (Supplementary Fig. 1g, h). As can be observed by us (Supplementary Fig. 1g, 1h) and others (Greber *et al.,* Cell Stem Cell 2010; Goke *et al.,* Molecular Cell 2013; Sterneckert *et al.,* Stem Cells 2010) who have attempted to study the role of ERK signalling in this manner, primed pluripotent stem cells differentiate upon MEK inhibition.

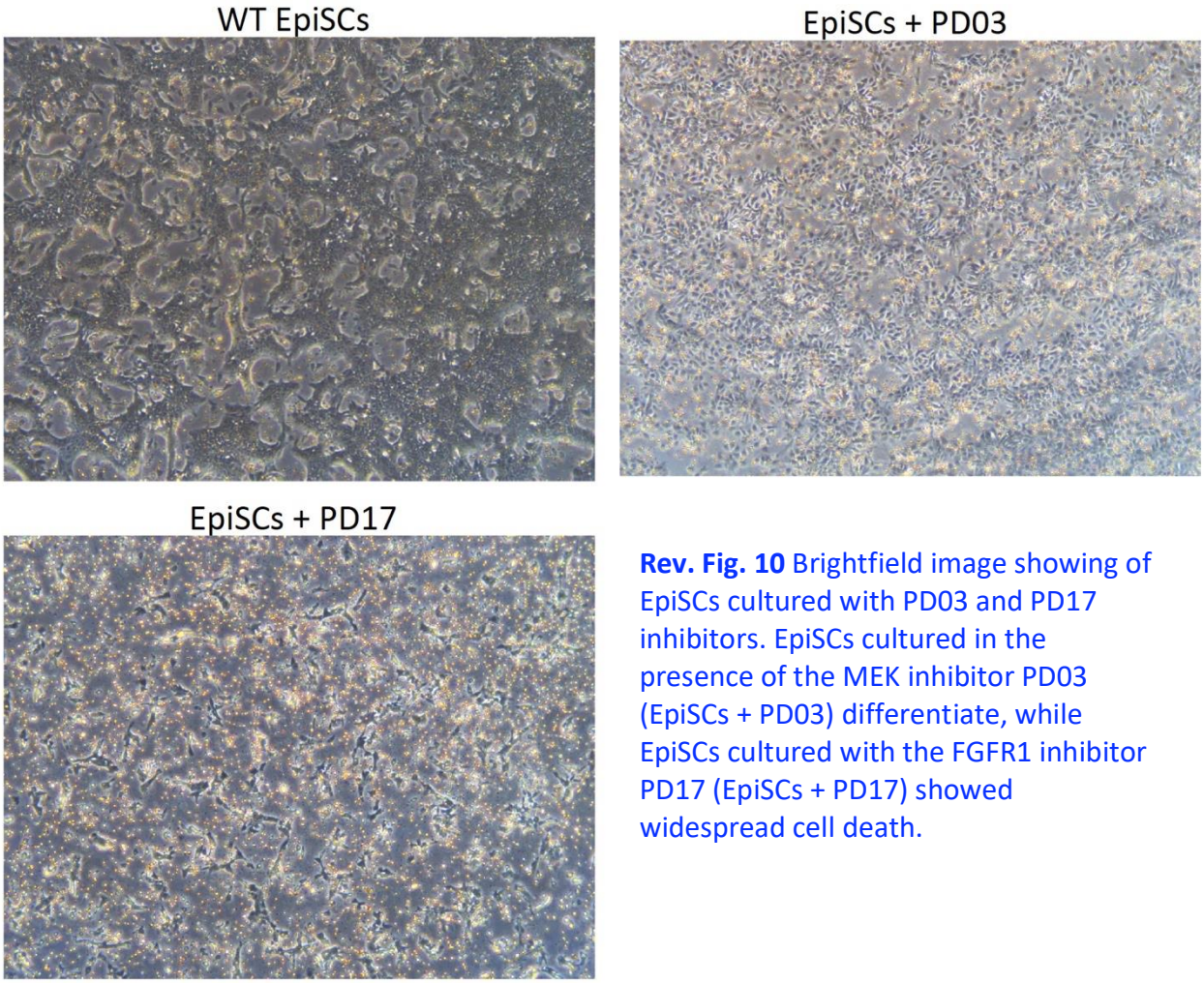


Rev. Fig. 9 Relative expression of *Oct4* (pluripotency marker), *Fgf5*, *Otx2* and *Sox2* (neural markers), *T-Bra*, and *Foxa2* (PS markers), revealing EpiSCs differentiation upon PD03 treatment.

We also attempted to culture the EpiSCs without FGF (EpiSCs – bFGF), with the MEK inhibitor PD03 (EpiSCs + PD03) and using the PI3K inhibitor PIK90 (EpiSCs + PIK90). As can be seen in the Rev. Fig. 9, the addition of the PI3K inhibitor PIK90 does not have any effect in the EpiSC stage. Adding PD03 leads to the differentiation of EpiSCs suggested by upregulation of *T-Bra* and *Foxa2* (mesendoderm), as well as increased levels of *Sox2* and *Otx2* (neural). Interestingly, when bFGF is not added to the EpiSC medium, the FGF ligand FGF5 gets upregulated, possibly as a compensatory mechanism. We observed a slight degree of spontaneous differentiation in EpiSCs when they were cultured without FGF. This tendency became more pronounced with successive passages. Thus, modulating bFGF led to heterogeneity (mix of undifferentiated and differentiated cells) rather than differential undifferentiated primed state. The CLDN6-sorted and *Pbx1-KO* EpiSCs offer a model where pERK and the ERK feedback loop (and downstream effectors) are innately different, within two differently primed undifferentiated states.

We also conducted a comparison between EpiSCs cultured with the MEK inhibitor PD03 (referred to as EpiSCs + PD03) and EpiSCs cultured with the FGFR1 inhibitor PD17 (referred to as EpiSCs + PD17). Notably, when PD17 was added to the EpiSC culture, we observed massive cell death, as illustrated below in the brightfield image in Rev. Fig. 10. Due to the widespread cell death, quality RNA could not be retrieved for the EpiSCs + PD17 cells. This outcome aligns with previous findings, particularly those reported by Ciruna and Rossant, Dev. Cell 2001, which suggested that FGFR1 plays a critical role in maintaining E-Cadherin expression and epiblast state, and this function appears to be MEK/ERK-independent.
On the other hand, EpiSCs cultured with PD03 exhibited spontaneous differentiation and could not be passaged, which is consistent with observations made by Greber *et al.,* Cell Stem Cell 2010; Goke *et al.,* Molecular Cell 2013; Sterneckert *et al.,* Stem Cells 2010 and others. The data showing differentiation of EpiSCs + PD03 is already in the manuscript (Supplementary Fig. 1f). Also, IF demonstrating downregulation of CLDN6 upon PD03 addition (MEK/ERK inhibition) is shown in Supplementary Fig. 1e), validating the link between CLDN6 and pERK.

Thus, inhibiting MEK or perturbation of ERK by RNAi or any other selective cell-permeable chemical inhibitors is not a suitable model to study the role of ERK signalling because, based on our experiments and work in this area, it is difficult to modulate ERK dosage while maintaining an undifferentiated state. The role of PBX1 in modulating the ERK feedback loop resulting in ERK-dependent differently primed epiblast populations offers a better alternative where we can directly see differences in pERK levels without the side effect of differentiation. Similarly, CLDN6 sorting is a suitable strategy to study how differently primed epiblast populations with innate FGF/ERK signalling differences respond to differentiation cues giving rise to different germ layers. Moreover, in both the *Pbx1-KO* and CLDN6-sorting model, we could assess the effect of lineage priming starting with EpiSCs that could self-renew and maintain their epiblast state.



Rev. Fig. 10 Brightfield image showing of EpiSCs cultured with PD03 and PD17 inhibitors. EpiSCs cultured in the presence of the MEK inhibitor PD03 (EpiSCs + PD03) differentiate, while EpiSCs cultured with the FGFR1 inhibitor PD17 (EpiSCs + PD17) showed widespread cell death.

(3) The authors frequently ascribe effects to just one factor in an experiment when many factors are varied. For example, the Wnt agonist CHIR is one of many signaling-related components of the APS media (along with activin and bFGF). Yet the authors repeatedly use APS media experiments to claim that Wnt stimulation is *the* determinant of differentiation to different fates. For example:

Line 278: “However, WNT stimulation induces both NMPs and DE progenitors, which arise after epiblast cells ingress through the PS. Upon exposure to WNT agonists, CLDN6Low EpiSCs differentiate towards NMPs while CLDN6High EpiSCs form DE (Fig. 2), supporting the existence of different epigenetically primed epiblast populations generating the mesoderm and endoderm cells.”

Line 291: “Altogether, DNA methylation patterns precedes WNT-driven chromatin accessibility changes influencing innate priming and lineage acquisition, by safeguarding distal-posterior epiblast cells (CLDN6Low EpiSCs) from DE and anterior neural differentiation”

The authors should treat with CHIR on its own if they wish to make claims about Wnt induced differentiation or compare media with and without CHIR to assess its effect.

We agree with the reviewer that our differentiation protocols require addition of cocktails of cytokines and inhibitors to efficiently induce differentiation towards specific germ layer lineages. These specific differentiation mediums were designed to induce robust and efficient differentiation towards PPS or APS derivatives, so that we could study posterior primitive streak or anterior primitive streak specific lineages.

The EpiSC medium contains FGF and ACTIVIN, alongside a WNT inhibitor (XAV939) to maintain the EpiSC state. To induce differentiation, we go from a medium containing a WNT inhibitor, to adding a WNT agonist (CHIR). The APS medium is a cocktail containing FGF, ACTIVIN and CHIR.

Seminal studies have shown that WNT and ACTIVIN/NODAL agonism are crucial for anterior primitive streak (APS) differentiation (Gadue *et al.,* PNAS 2006; Liu *et al.,* Nature Genetics 1999; Brennan *et al.,* Nature 2001).
Further studies have shown that it is the addition of WNT agonists that shifts the role of ACTIVIN/NODAL from regulating self-renewal to promoting differentiation (Funa *et al.,* Cell Stem Cell 2015; Yoney *et al.,* eLife 2018; Yoney *et al.,* Development 2022).

However, the signalling hierarchy inducing gastrulation is well established and evolutionarily conserved in mice *in vivo* and even hESCs. Here is an excerpt from Martyn *et al.,* Nature 2018.
"*During mouse gastrulation BMP4 signalling activates the WNT pathway which in turn activates the ACTIVIN/NODAL pathway (BMP4→WNT→ACTIVIN/NODAL, Fig. 1A), both at the transcriptional and signalling level (Ben-Haim et al., Dev. Cell 2006). Since it has been shown in mouse and other vertebrates that these three pathways are the most critical for organizer formation (Crease et al., PNAS 1998; Gritsman et al., Development 2000), we first asked if this hierarchy was conserved in human gastruloids. Using RNA-Seq, we found that out of all the 19 WNT ligands present in the human genome, WNT3 is the only one that is significantly and immediately induced upon BMP4 presentation (Fig. 1B). qPCR analysis shows that activation of WNT signalling directly induces NODAL expression (Fig. 1C). Further qPCR analysis showed that NODAL induction was reduced when the NODAL inhibitor SB was present, and taken together with the observation that ACTIVIN induces NODAL expression, suggests the presence of a NODAL feedback loop, as also noted in the mouse (Brennan et al., Nature 2001). Additionally, no direct BMP4 induction by either WNT or NODAL signalling was observed (Extended Data Fig. 1B). Thus the transcriptional hierarchy of BMP→WNT→NODAL is evolutionarily conserved in hESCs.*"

Given that WNT is the major driver for induction of both APS derivatives (neuromesoderm and definitive endoderm) *in vitro* (Gadue *et al.,* PNAS 2006; Tsakiridis *et al.,* Development 2014) in the absence of BMP signalling, we refer to APS differentiation as WNT-driven.

Furthermore, we previously demonstrated that signalling response during NMP differentiation is dictated by transcription factors unlocking the WNT-responsive elements (dictated by LEF1) rather than by WNT ligand dosage, questioning the significance of titrating morphogen concentrations (Mariani *et al.,* Nature Communications 2021).

Similar claims are made about BMP (Line 377, Line 398), again in a cocktail with many other signaling agonists.
BMP is crucial for the specification of the posterior primitive streak derivatives that give rise to the extraembryonic mesoderm (ExM) (Arnold and Robertson, Nat Rev Mol Cell Biol. 2009). Please also refer above to the excerpt from Martyn *et al.,* Nature 2018, showing BMP as the main driver of PS signalling hierarchy. WNT is required for primitive streak maintenance, and BMP for posteriorization (posterior spatial identity).

In the context of the *Pbx1* mutant, we emphasize the significance of BMP because it exerts a widespread effect on well-established BMP-responsive genes, such as *Hand1* (Zheng *et al.,* Int J Mol Sci. 2021; Morikawa and Cserjesi, Development 2004; Barnes *et al.,* Dev. Dyn. 2010) and *Tbx3* (Yang *et al.,* Development 2006; Davenport *et al.,* Development 2003) already in the epiblast stages at the chromatin and DNA methylation level. Thus, we refer to the effect on ExM genes as a BMP-response. Indeed, one of the most impactful review papers in gastrulation and developmental biology by Arnold and Robertson, Nat Rev Mol Cell Biol. 2009, refer to "*The earliest, most posterior mesoderm subpopulations, which are patterned in response to BMP4 signalling from the ExE, give rise to the extraembryonic tissues, including the mesodermal layer of the chorion and the visceral yolk sac mesoderm and blood islands.*" More recently, single-cell analysis validated the important role of BMP signalling in ExM specification (Scialdone *et al.,* Nature 2016) - "*Tbx3 and Bmp4 are expressed in the more posterior portion of cluster 4 and in the embryo are expressed in the more posterior region of the primitive streak around the amnion and into the extra-embryonic mesoderm*."

Additionally, we can detail further evidences from our work that point towards the role of FGF/ERK regulation in EpiSCs influencing BMP responsive genes.
1. *Tbx3* expression is observed in mESCs cultured with a WNT agonist (CHIR) and a MEK/ERK antagonist (PD03). However, there is no difference in *Tbx3* expression between wild-type (WT) and *Pbx1-KO* ESCs. In contrast, Hand1 is not expressed in ESCs. It's worth noting that both *Hand*1 and *Tbx3* loci exhibit a state of hypomethylation in ESCs, as depicted in Revised Figure 6. *Tbx3* is a crucial component of the transcriptional circuity in maintenance of ESCs (Niwa *et al.,* Nature 2009; Russell *et al.,* Stem Cell Reports 2015). BMP signalling also plays a crucial role in ESCs (Ying *et al.,* Nature 2003; Li *et al.,* Cell Stem Cell 2012), and BMP pathway components are expressed in the 16-cell stage in ICM (Graham *et al.,* Nature Communications 2014). Interestingly, although ESCs resemble the preimplantation epiblast progenitors, a requirement for Wnt/β-catenin signalling requirement has not been reported at this stage (Muñoz-Descalzo, Hadjantonakis and Arias, Semin Cell Dev Biol. 2015).

2. Neither *Tbx3* nor *Hand1* are expressed in EpiSCs cultured in medium containing FGF and a WNT antagonist (XAV939). Here, *Tbx3* locus gets hypomethylated in *Pbx1-KO* EpiSCs, and *Hand1* locus gets hypermethylated in ERK-deficient *Pbx1-KO* EpiSCs [Rev. Fig. 6].

3. Upon PPS differentiation (PPS medium containing high BMP doses in addition to FGF, WNT, ACTIVIN), *Tbx3* gets upregulated in *Pbx1-KO* PPS cells, while *Hand1* gets downregulated in *Pbx1-KO* PPS cells. This transcriptional activation and repression correlate to the respective methylation status of *Tbx3* (hypomethylated in *Pbx1-KO* EpiSCs) and *Hand1* (hypermethylated in *Pbx1-KO* EpiSCs) in EpiSCs [Fig. 5f, Rev. Fig. 6] .

4. Upon ExM differentiation (ExM medium containing high BMP doses, alongside ACTIVIN and WNT inhibition), the same gene expression observed in PPS conditions persist. Thus emphasizing that it is BMP and not WNT or ACTIVIN driving the expression of *Tbx3* and *Hand1*. [Fig. 5f, Rev. Fig. 6].

Ultimately, the focus of our manuscript is on how the epiblast methylome predetermines specific germ layer differentiation. Using two systems (CLDN6 sorted cells and *Pbx1-KO*), we show that differential ERK activity corresponds to different methylation statuses; and that these separate populations when exposed to the same signalling cues (same cocktail of cytokines and inhibitors) give rise to divergent lineages. Thus, these epiblast populations are differently primed to respond to these differentiation cocktails.

With regards to both WNT and BMP response, we have cited the Martyn *et al.,* Nature 2018 paper in the manuscript. We thank the reviewer for raising this discussion which brought to our attention a need for this reference.

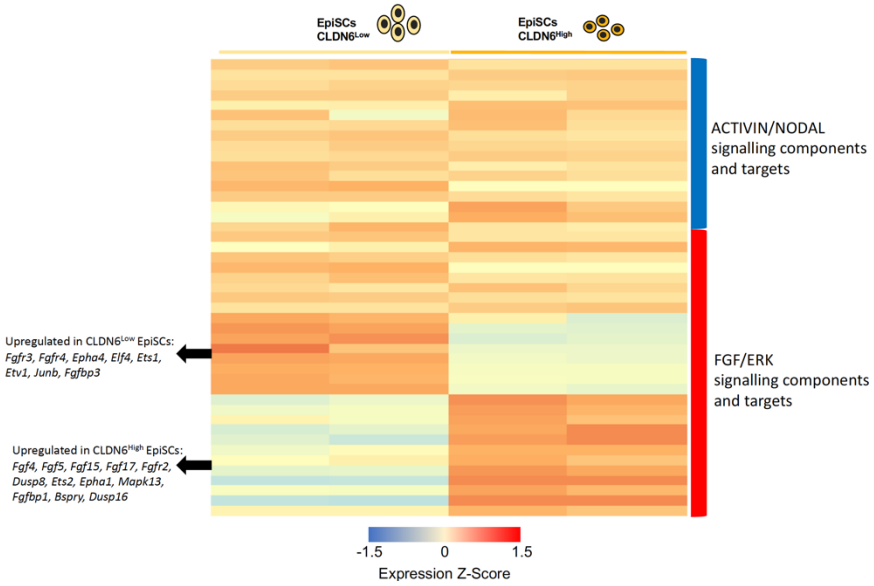
This mirrors my similar concerns about ascribing effects in Claudin-high and Claudin-low cells to the ERK pathway.

We focus on the ERK pathway based on the following logic.

1. EpiSCs require FGF and ACTIVIN signalling to maintain their primed pluripotent state (Brons *et al.,* Nature 2007; Tesar *et al.,* Nature 2007; Greber *et al.,* Cell Stem Cell 2010). EpiSCs are maintained under WNT-inhibited conditions, and WNT-responsive genes are not expressed in EpiSCs.
2. GO analysis of the differentially expressed genes in $CLDN6^{High}$ vs $CLDN6^{Low}$ EpiSCs showed significant enrichment over terms associated with FGF/ERK signalling (Fig. 1e and above response to reviewer #1). Notably, there were no hits associated with ACTIVIN/NODAL signaling.
3. While ACTIVIN signalling maintains pluripotency by controlling *Nanog* expression in EpiSCs and hESCs (Vallier *et al.,* Development 2009; Xu *et al.,* Cell Stem Cell 2008), there was no difference in *Nanog* expression in $CLDN6^{High}$ vs $CLDN6^{Low}$ EpiSCs (Supp. Fig 1c).

To provide further clarity regarding our focus on ERK signalling, in the Reviewer Figure 11 and 12 below, made for the reviewer's perusal, we analyzed FGF/ERK and ACTIVIN/NODAL signalling components in $CLDN6^{High}$ vs $CLDN6^{Low}$ EpiSCs. As can be clearly observed in Rev. Fig. 11, there is

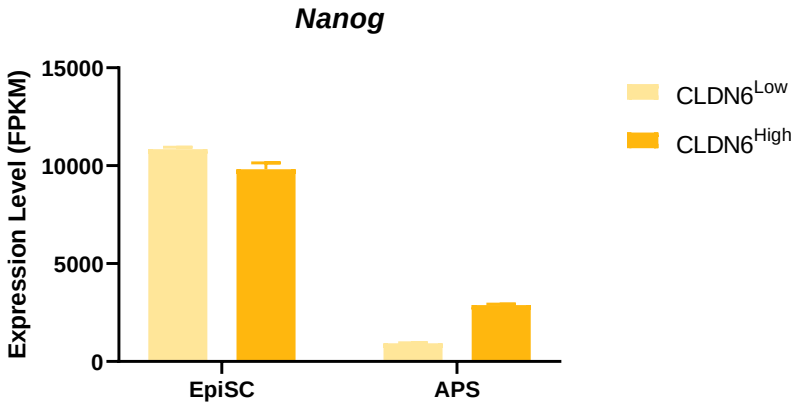
a distinct enrichment of components related to the FGF/ERK signaling pathway in the two CLDN6-sorted EpiSC populations. However, there are no discernible differences in terms of ACTIVIN/NODAL signaling.



Rev. Fig. 11 Heatmap of RNAseq analysis, displaying relative expression of ACTIVIN/NODAL and FGF/ERK signalling components and target genes in CLDN6^{High} and CLDN6^{Low} EpiSCs. While CLDN6-sorted EpiSC populations exhibit distinct enrichment patterns in the FGF/ERK signaling pathway, there is no significant disparity in the ACTIVIN/NODAL signaling pathway between these populations.

To further clarify the roles of ERK in EpiSCs and WNT in APSD differentiation, we specifically examined the regulation of *Nanog*, a gene responsive to ACTIVIN/NODAL signaling, as documented in studies by Vallier *et al.,* Development 2009; Xu *et al.,* Cell Stem Cell 2008. *Nanog* is not differentially expressed in CLDN6^{Low} EpiSCs vs CLDN6^{High} EpiSCs, and gets upregulated in CLDN6^{High} APS compared to CLDN6^{Low} APS. (Rev. Fig. 12). These observations demonstrate that FGF/ERK signalling is the main difference between CLDN6^{Low} vs CLDN6^{High} EpiSCs. In contrast, WNT-stimulated differentiation towards APS alters ACTIVIN/NODAL response from pluripotency towards differentiation (Funa *et al.,* Cell Stem Cell 2015; Yoney *et al.,* eLife 2018; Yoney *et al.,* Development 2022).

Altogether, the goal of this paper was to investigate factors modulating the primed nature of epiblast stem cells, influencing lineage priming. Multiple studies have shown that directly modulating FGF/ERK activity via genetic perturbations or agonist/antagonist additions leads to spontaneous differentiation (Greber *et al.,* Cell Stem Cell 2010; Goke *et al.,* Molecular Cell 2013; Sterneckert *et al.,* Stem Cells 2010). Here, we demonstrate two systems with innate FGF/ERK signalling differences in differently primed epiblast populations. Moreover, our paper also highlights the impact of employing the right system/model to study embryonic development. Unfortunately to date, most of the epigenetic studies attempting to study lineage priming have focused on embryonic stem cells (ESCs) rather than epiblast stem cells (EpiSCs) *in vitro*.

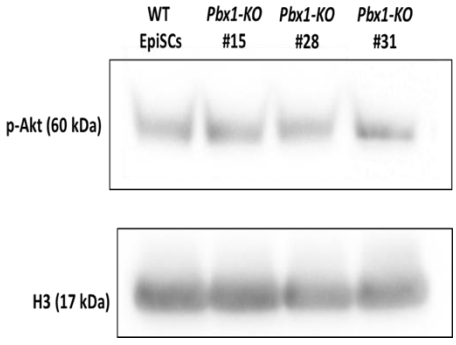


Rev. Fig. 12 Relative expression of *Nanog* revealing no difference in CLDN6^{Low} and CLDN6^{High} EpiSCs. Upon WNT-induced APSD differentiation *Nanog* is upregulated in CLDN6^{High} APS.

Minor comments:

Figure 4C: From this data it is unclear whether Erk pathway activity is maintained but only total Erk1/2 protein is decreased, or if Erk protein levels are constant but phosphorylation is reduced. The authors should blot for both phospho and total Erk.

The authors would like to note that the Immunoblots for both phospho- and total-ERK were in the initial submission. The phospho-ERK (pERK) Western blot can be found in Fig. 4c. The total-ERK blot in Supplementary Fig. 5h, alongside a blot for pMEK (Supplementary fig. 5i). To avoid misunderstanding, in Supplementary Fig. 5h, we indicated total-ERK as tERK instead of ERK.
To narrow down a specific effect on pERK and not on PIK/AKT pathway which is also downstream of FGF signaling, we performed Western blot with pAKT antibody, which is showed for the perusal of the reviewer in Rev. Fig. 13, showing no level variation in *Pbx1-KO* EpiSCs.



Rev. Fig. 13 Western Blot analysis showing comparable levels of pAKT in control EpiSCs and in the three *Pbx1-KO* clones. Histone H3 was used as loading control.

Line 182:
“Higher TBX6 and Msgn1 (Fig. 2a,b,c) indicated that CLDN6Low EpiSCs differentiated more efficiently towards NMPs.” TBX6 is a marker of mesoderm differentiation away from NMPs, not a marker of the NMP state.

We agree with the reviewer that TBX6 is indeed an early marker of presomitic (paraxial) mesoderm identity away from NMPs rather than a marker of NMP state itself. In Figure 2, we used TBX6 to differentiate the lineages: Presomitic mesoderm versus definitive endoderm. Since the early presomitic mesoderm markers (*Msgn1, Tbx6*) identify cells transitioning from NMPs towards PSM (Mariani *et al.,* Nature Communications 2021), we indicated it as NMPs. We edited the manuscript to accurately mention NMPs/PSM where appropriate.

We thank Reviewer #3 for their constructive feedback, which has contributed to enhancing the clarity of our manuscript.

A point-by-point response to the Reviewers

We express our appreciation to the referees for their valuable comments and suggestions.

To provide a complete picture, we want to share our current working situation with the reviewers. The research center where we worked for the past 9 years was unexpectedly closed, leading to the unfortunate shutdown of our laboratory at the end of 2022. This unforeseen circumstance has left us in a challenging situation, as we now find ourselves without the essential resources and financial support required to continue our research. Consequently, our ability to conduct experiments and contribute to the revision of this manuscript is limited. Despite the setbacks, we remain committed to answering the raised critiques and fulfilling the standards set by Nature Communications.

Based on the valuable feedback of Reviewer 2 and 3, we have changed the title and manuscript of the paper to more accurately represent our findings, toning down the claim about the causal role of FGF.
New Title: Cell-context response to germ layer differentiation signals is predetermined by the epigenome in regionalized epiblast populations.
We hope you find the revised manuscript comprehensive and compelling.

A point-by-point response to the reviewers' comments is provided below. Our comments are highlighted in blue.

Reviewer #1 (Remarks to the Author):
The authors have appropriately addressed most of my concerns. The only thing I would suggest if they could include a more inclusive model to connect all key findings of this study.
We extend our appreciation to the reviewer for their favorable comments regarding our work and their perceptive feedback. Their constructive input has greatly enhanced the clarity of our results and empowered us to highlight the key findings of our study. As suggested, we have modified our previous model making it more inclusive and connecting it to our key findings (revised, Fig. 6), and added a new Supplementary Fig. 5 to the manuscript.

Reviewer #2 (Remarks to the Author):
The authors did not address in a satisfactory way the concerns raised by this reviewer, and by reviewer 3, about the causal role of FGF in the mechanism described.
There are many correlative pieces of evidence pointing at FGF signalling being regulated by Pbx1, potentially via Spry2.
We apologize if the content was not as clear as intended, and we are fully prepared to rephrase and tone down our claims regarding the association between PBX and FGF. While acknowledging the significance of FGF signaling in cellular processes, it's important to note that our paper does not primarily focus on FGF activity itself or PBX. We introduced the *Pbx1* mutant as an optimal model to study lineage priming in the epiblast state in the context of reduced ERK activity. The main focus of the paper is not how PBX1 regulates *Spry2*. Our paper delves into the identification of distinct primed cell populations of post-implantation pluripotent cells (EpiSCs). Our findings revealed that these EpiSC populations are differently primed and have differential expression of ERK components, with distinct fate biases upon differentiation. Each population boasts a unique DNA methylation signature dictating their responses to signaling cues (like WNT and BMP), determining their cellular fates. Our research does not primarily focus on the intricacies of FGF-ERK signaling itself or their links to DNA methylation in the epiblast stage, which is already established[1-3] (Smith *et al.,* 2017; Ficz *et al*., Cell Stem Cell 2013; Bar *et al.,* Nature Communications

2021, Habibi E. *et al.*, Cell Stem Cell 2013). Instead, we emphasize the context-dependent nature of the DNA methylation signatures, particularly their correlation with ERK activity and signaling effectors, and their profound impact on cellular responses to subsequent differentiation signals. Our work sheds light on the interplay between epigenetics and signaling pathways in determining cell fate.
Modulating ERK activity in the contest of primed pluripotency is extremely complex because it results in cellular heterogeneity and differentiation[4,5] (Greber *et al.*, Cell Stem Cell 2010: Goke *et al.*, Molecular Cell 2013). To address these challenges, we introduced the *Pbx1-KO* model. This model exhibits reduced ERK levels and differential DNA methylation while retaining the undifferentiated epiblast state.
Given that we used *Pbx1-KO* as a model for studying ERK activity, we explored the underlying molecular mechanisms by approaching key questions from various angles.

Below, we present a detailed outline of the evidence supporting PBX1's regulation of ERK. Additionally, we have analyzed influential papers in the field, examining their approach to elucidating the causal relationship between transcription factors (TFs) and signaling pathway outcomes (see answer to reviewer 3).

Provided below is a comprehensive overview of the evidence substantiating PBX1's regulation of ERK signaling, including experimental findings, molecular mechanisms, and any relevant supporting data.
1) Pbx1 begins to have a role in EpiSCs (where ERK signalling is crucial), but is dispensable in ESCs (where ERK is inhibited).
2) PBX1 regulates ERK activity by modulating the ERK feedback loop via *Spry2* regulation.
3) Observation of FGF ligand upregulation in *Pbx1-KO* EpiSCs, corroborating effects seen with PD03 treatment or absence of bFGF in the medium.
4) Pivotal role of PBX1 recruitment to *Spry2,* specifically in EpiSCs, verified through ChIP, ATAC and EMSA.

1) We demonstrated that PBX1 has a crucial role starting in EpiSCs, which depend on ERK signalling. RNA-seq (Figure 1A) and GO analyses (Figure 1B) in *Pbx1-KO* EpiSCs, showed that *Pbx1* regulates ERK pathway components in EpiSCs. On the contrary, in ESCs (dependent on ERK inhibition), despite being expressed, *Pbx1* is dispensable with no impact on any transcripts, including ERK-FGF components (Figure 1).
FGF/ERK is important for maintaining the EpiSC state, preventing reversion backward or differentiation forwards[4] (Greber *et al.,* Cell Stem Cell 2010). ERK signalling has been hypothesized to be the main driver of lineage priming within EpiSCs. However, no study has effectively elucidated how ERK activity regulates the primed nature of EpiSCs, since genetic modifications such as knockout of ERK proteins and downstream effectors, including PD03 treatment, lead to spontaneous differentiation[4,5] (Goke *et al.* Molecular Cell 2013, Greber *et al.*, Cell Stem Cell 2010), causing the cells to exit a primed state.
We demonstrated that PBX1 impacts specifically ERK activity in the EpiSCs (primed epiblast state) (Figure 1). *Pbx1-KO* EpiSCs maintain their undifferentiated epiblast state, implying that PBX1 influences cell fate acquisition (via priming rather than directly inducing spontaneous differentiation).
Thus, with *Pbx* mutant, we can examine the role of FGF/ERK specifically in influencing cell fate acquisition, as the cells retain their undifferentiated epiblast state despite alterations in the ERK pathway.
This aspect makes *Pbx1-KO* EpiSCs a unique model, unlike tampering with ERK inhibitors (PD03) or direct FGF pathway components, which promote spontaneous differentiation.

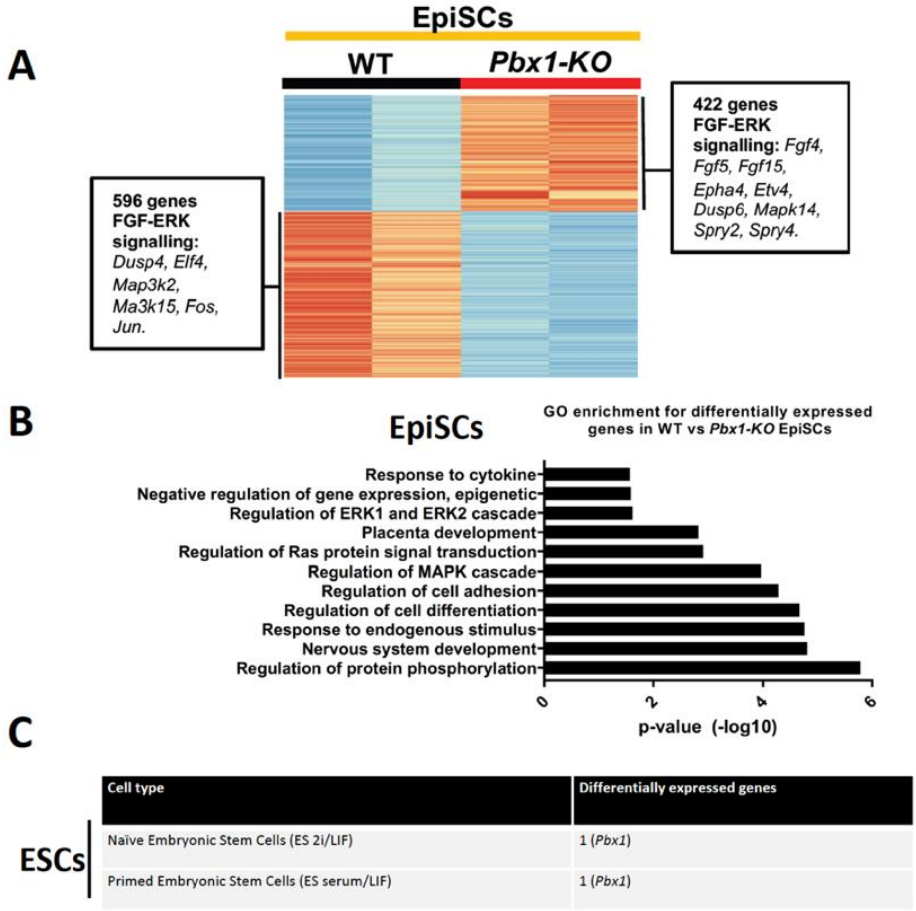


Figure 1. PBX1 is dispensable in ESCs, which rely on ERK inhibition. However, in EpiSCs where ERK is required, PBX1 plays a crucial role (modified from Supp Fig. 5f-g and Fig4b in the manuscript). **A**) Heatmap showing higher expression of FGF/ERK signalling components like *Fgf4* and *Fgf5 and Fgf15*, ERK feedback inhibitors such as *Spry2, Spry4* and *Dusp6*, and the ERK downstream TFs, *Etv4* in *Pbx1-KO* EpiSCs, with downregulation of *Jun* and *Elf4*. **B**) In WT versus *Pbx1-KO* EpiSCs, significant overrepresentation of terms associated with the MAPK/ERK cascade was observed in the GO term enrichment analysis. **C**) RNAseq analysis comparing WT and *Pbx1-KO* ES cells cultured in 2i/LIF or serum/LIF conditions did not detect any differentially expressed genes.

2) In *Pbx1-KO* EpiSCs, we saw specific upregulation of *Spry2* transcript, a negative regulator of ERK signalling (Figure 2), implicated in ERK feedback regulation[6] (Mason *et al.,* Trends in Cell Biol. 2006). Furthermore, in *Pbx1-KO* EpiSCs, we observed a downregulation of pERK protein (Figure 2A), while total ERK (tERK) and pAKT were unaffected (Figure 2B-C). We also provided evidence that pMEK is downregulated (Figure 2D), indicating a specific impact on ERK activity, mirroring SPRY2 functional role in the ERK feedback loop[6-8] (Mason *et al.,* Trends in Cell Biol. 2006; Shukla *et al.,* Blood 2016; Hanafusa *et al*., Nature Cell Biology 2002).

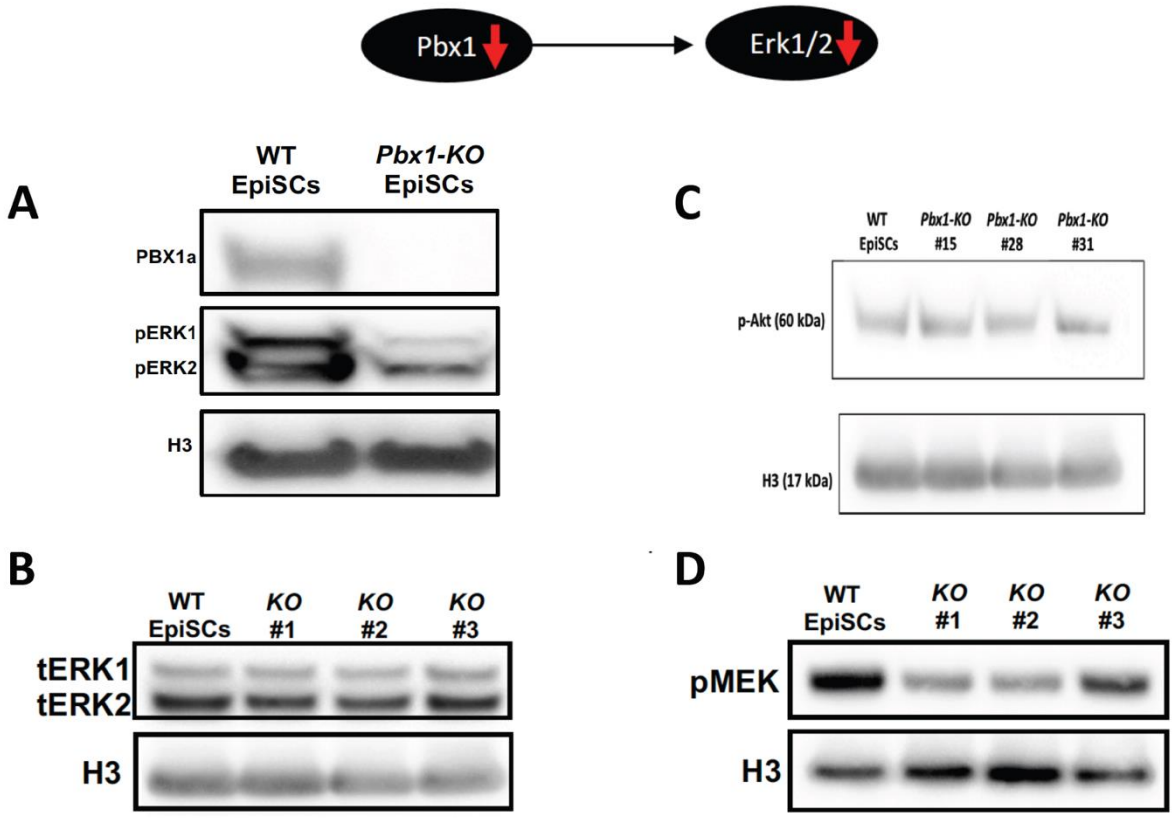


Figure 2. Loss of *Pbx1* results in reduced pERK activity without altering tERK and pAKT levels. (Modified from Fig 4c, and Supp Fig 5h-I in the manuscript). **A**) Western blot analysis confirms the absence of PBX1 and reduction of pERK1/2 in *Pbx1-KO* EpiSCs. Western blot analyses showing. **B**) unchanged levels of total ERK1/2 proteins and pAKT **C**), and reduced levels of pMEK **D**) in three *Pbx1-KO* EpiSC cell lines compared with WT.

3) We observed an upregulation of *Fgf* ligands in *Pbx1-KO* EpiSCs (Figure 1A), suggesting compensation for FGF signaling due to the absence of ERK (Figure 3). This phenomenon was also observed when PD03 was added or when bFGF was not included in the EpiSC medium (Figure 3). Furthermore, these data corroborate the findings that treatment with PD03 in EpiSCs result in differentiation Figure 3).

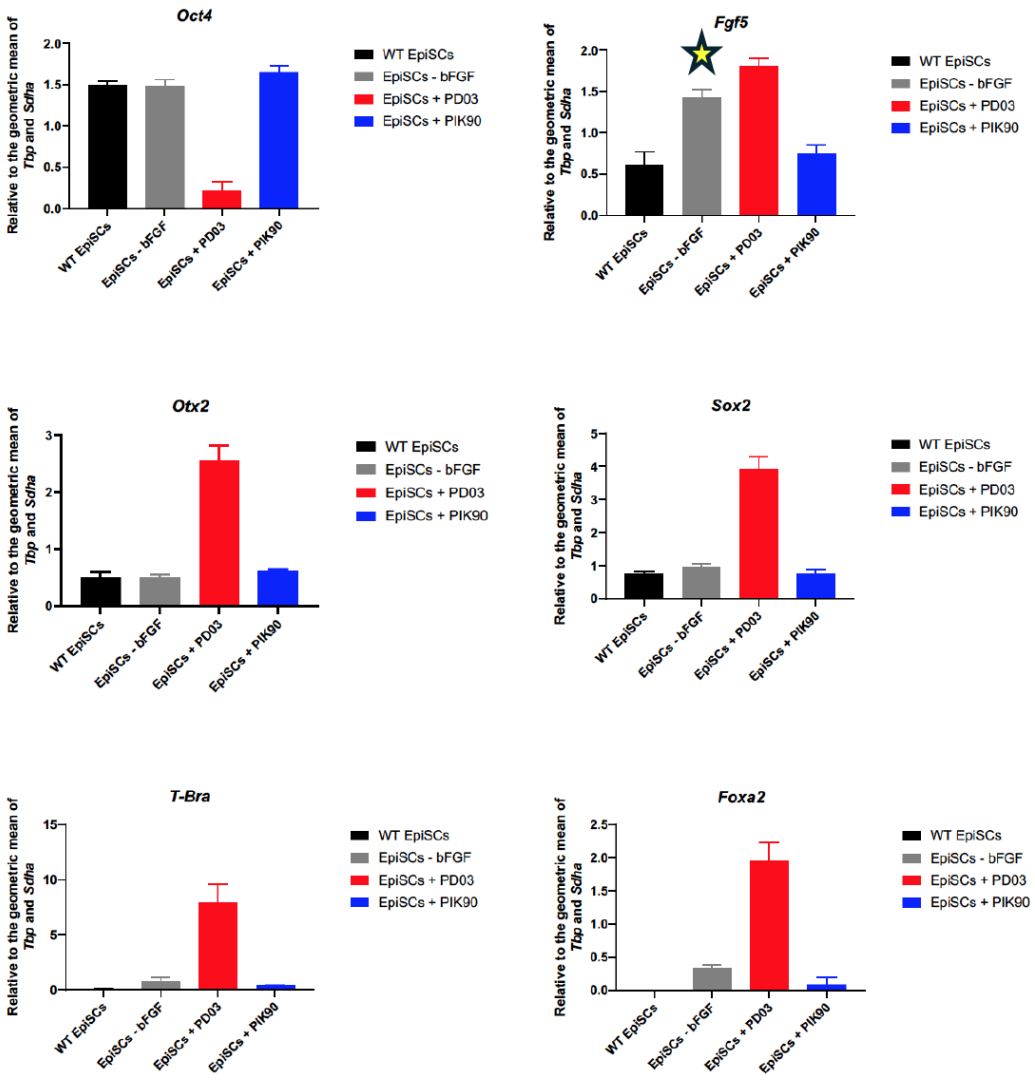


Figure 3. Treatment of PD03 in EpiSCs leads to differentiation and loss of epiblast state. Not adding bFGF (see *, as initially suggested by Reviewers 2 and 3, leads to upregulation of *Fgf5* ligands with some cells spontaneously differentiation (increasing level of *T-Bra* and *Foxa2*).

4) Through ChIP and ATAC-seq, we demonstrated that PBX1 is recruited to *Spry2* specifically in EpiSCs, not in ESCs (Figure 4A). EMSAs confirmed the PBX complex's affinity and composition for the identified binding site (Figure 4B). Similar approaches to prove a causative effect of TFs over signalling regulators have been performed in seminal work investigating gene regulatory networks[9-11] (Scheibner *et al.,* Nature Cell Biology 2021; Metzis *et al.,* Cell 2017; Harland *et al.,* Nature Cell Biology 2022), with a few exceptions (Mariani *et al.,* Nature Communications 2021)[12].

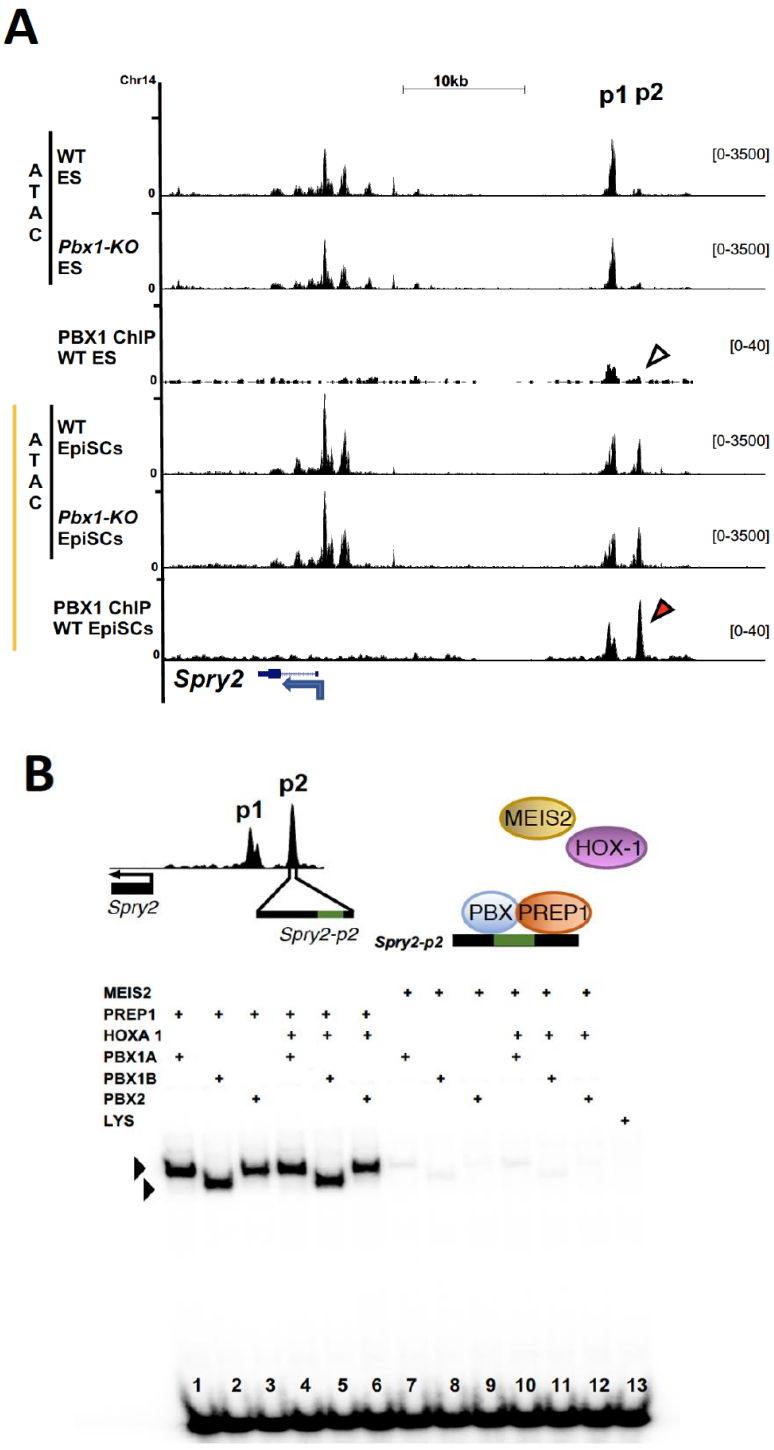


Figure 4. PBX1 is recruited to *Spry2* specifically in EpiSCs, and not in ESCs (Modified from Suppl Fig.5 k-l in the manuscript). **A**) ATACseq and ChIPseq data demonstrating specific recruitment of PBX1 on *Spry2* enhancer site that gets specifically accessible in EpiSCs, but is closed in ES cells (p2). **B**) EMSA showing affinity of PBX-PREP1 complex over *Spry2* enhancer site (p2) that is specifically accessible in EpiSCs.

Notably, ERK feedback regulators have been shown to modulate DNA methylation machinery in pluripotent stem cells[1,13] (Ficz *et al.*, Cell Stem Cell 2013; Smith *et al.*, Nature 2017), further corroborating the altered DNA methylation signatures in both our cellular models. We observed site-specific DNA methylation corresponding to EpiSC populations (CLDN6^{Low} vs CLDN6^{High} and WT vs *Pbx1-KO*) with ERK differential expression and the associated ETS family members. We expanded on the significance of these findings based on feedback from Reviewer 1 in the latest version of the manuscript.
Altogether, based on this substantial body of data, we have robust indications supporting that PBX regulates ERK levels in EpiSCs.

The authors claim that somehow it is not possible to test this link directly, by inhibiting FGF, or more specifically, by reducing pERK levels, because EpiSCs might die or differentiate when FGF/pERK are reduced. There are multiple ways to address this point, some already suggested by reviewer 3:
1. Titrate the inhibitor PD03, to reduce only partially pERK, at the levels shown in Figure 4c. Such concentration of PD03 should phenocopy Pbx1 KO
The challenges of using PD03 with EpiSCs are threefold. The primary reasons why treating EpiSCs with PD03 is impracticable are as follows:

1. PD03 administration induces differentiation, and no study, to the best of our knowledge, shows that modulating PD03 levels will maintain an undifferentiated state or result in different cellular/epigenetic states (with differing lineage outcomes).
2. MEK inhibition via PD03 treatment for prolonged time results in chromosomal aberrations.
3. MEK inhibition via PD03 treatment results in the reactivation of ERK activity.

1) Modulating ERK by adding the inhibitor PD03 in post-implantation epiblast cells is impractical due to EpiSCs differentiation (Figure 3, and 5). Accordingly, when we treated EpiSCs with PD03, we observed differentiation towards neuroectoderm and ExM lineages (Figure 5). Thus, under PD03 treatment, EpiSCs differentiate, making it challenging to address priming or cell fate bias since the cells are already

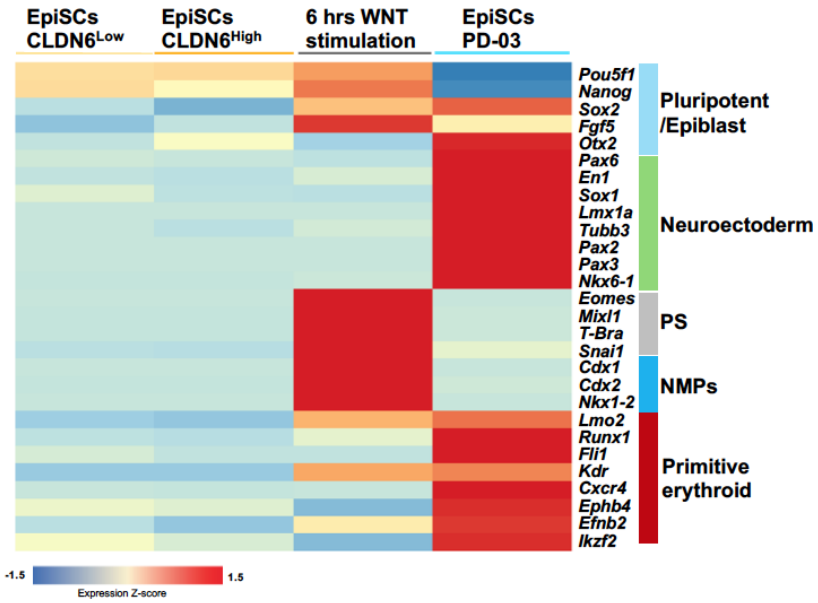


Figure 5. Treating EpiSCs with PD03 prompts differentiation towards neuroectoderm and ExM lineages (Modified from Supp Fig 1h in the manuscript).

differentiated. Similar observations have also been published by others[4] (Greber *et al.,* Cell Stem Cell 2010).

2) The suppression of the ERK pathway using PD03 has been demonstrated to erode genomic imprints, leading to chromosomal abnormalities and compromising the developmental potential of mouse ESCs. Two seminal reports[14,15] (Choi J *et al.,* Nature 2017, and Yagi *et al.,* Nature 2017) provide substantial evidence in this regard. Similar consequences were investigated by titrating PD03 in hESCs, which exhibit transcriptional and signaling responses closely resembling those of EpiSCs. As highlighted by [16]Di Stefano *et al.*, Nature Methods 2018, this examination revealed an unexpected sensitivity of pluripotent stem cells to ERK signal inhibition. Consequently, culturing primed stem cells such as hESCs and EpiSCs with PD03 substantially impacts chromosomal stability. These data prove detrimental to maintaining these cells in an undifferentiated primed state and hinder their successful differentiation. As a result, it is widely avoided to culture these cells with PD03 due to these adverse consequences.

3) The impact of PD03 treatment on "non-embryonic stem cells", such as cancer cells, is complex and context-dependent, leading to modulation of ERK activation or inhibition. Multiple studies have demonstrated that inhibition of MEK via PD03 leads to ERK activity reactivation, depending on the cellular context[2,3] (Lake *et al*. Cell Mol Life Sci 2016; Samatar and Poulikakos, Nat Rev Drug Discov 2014). These findings suggest that utilizing PD03 to study the priming function of ERK activity may not be the most optimal approach.

To overcome all these limitations, we introduced the *Pbx1-KO* model, characterized by reduced ERK levels and the ability to maintain an undifferentiated epiblast state, all while avoiding chromosomal aberrations or reactivation of ERK. *Pbx1-KO* EpiSCs can be expanded and differentiated upon adding a differentiation-inducing medium. Furthermore, PBX1 regulation of pERK is specific to EpiSCs, particularly influencing how primed pluripotent cells react to differentiation signals. Thus, PBX1 stands out as a more robust and dependable system than manipulating PD03 concentration.

The impact of PD03 on gene regulation and differentiation resembles the effect observed with *Pbx1* deletion.

While treatment of EpiSCs with PD03 fails to preserve their state, we noticed that *Pbx1-KO* cells and PD-treated cells exhibit comparable characteristics:

A) Influence on imprinted genes
B) Analogous cell fate bias during differentiation

A) Multiple studies dissected the role of PD03 regulating FGF/ERK-mediated DNA methylation in primed pluripotent cells[1,17-19] (Ficz *et al*., Cell Stem Cell 2013; Bar *et al.,* Nature Communications 2021, Habibi E. *et al*., Cell Stem Cell 2013, Li C. *et al*., Oncotarget 2016), although they never assessed if these primed populations had differentiated.

Recently, [17]Bar *et al.,* demonstrated that inhibiting the MEK/ERK pathway via PD03 in human parthenogenetic embryonic stem cells lacking paternal alleles led to dysfunction of imprinted genes. However, they did not assess if their cells had differentiated after PD03 treatment (as highlighted in Point 1), or if 5 days of PD03 treatment led to ERK reactivation (as highlighted in Point 2). In *Pbx1-KO* EpiSCs, we see similar dysregulation of imprinted genes (Figure 6), further pointing towards PBX1 regulation of FGF/ERK-mediated DNA methylation in an undifferentiated state with no ERK reactivation. However,

delving further into this space is out of the current scope of the paper as it deviates from the main message of our manuscript. To avoid confusion, we did not include these data in the manuscript.

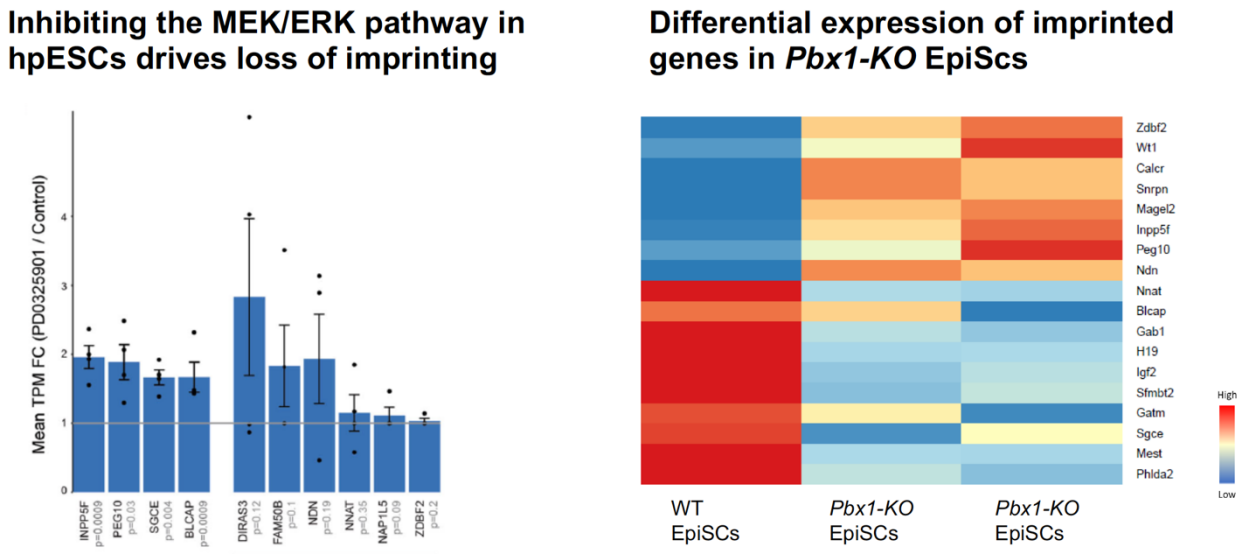


Figure 6. In *Pbx1-KO* EpiSCs, imprinted genes are dysregulated similarly to hpESCs treated with PD03 (ERK inhibitor), reinforcing our finding that PBX1 regulates DNA methylation through the ERK pathway.

B) Treating primed pluripotent stem cells with PD03 alters their undifferentiated state. Interestingly, when added to EpiSCs, PD03 induces spontaneous differentiation towards neuroectoderm or ExM lineages such as primitive erythroid (Figure 7 A). Similarly, during directed differentiation towards these lineages, *Pbx1-KO* EpiSCs also exhibit a bias towards ExM lineages, such as primitive erythroid (Figure 7B) and neuroectoderm (Figure 8B). However, unlike PD03 treatment, *Pbx1-KO* EpiSCs maintain their undifferentiated EpiSC state and only differentiate towards neuroectoderm or ExM upon directed differentiation, displaying a similar fate bias as seen upon PD03 treatment.

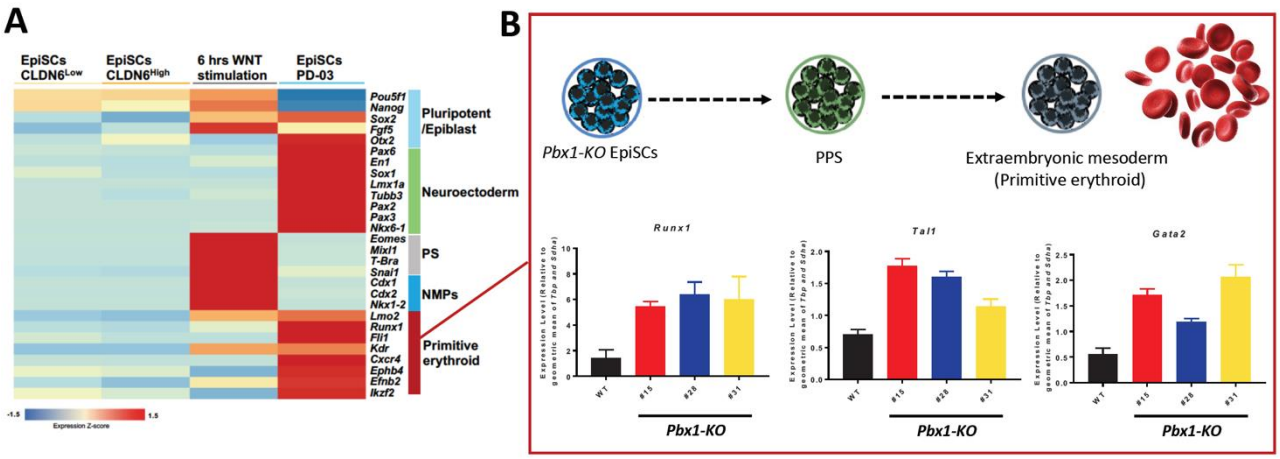


Figure 7. *Pbx1-KO* EpiSCs are biased towards primitive erythroid fate, similar to PD03 treatment in the epiblast. **A**) PD03 treatment of EpisSCs results in the upregulation of primitive erythroid genes. **B)** In the red box, direct differentiation of *Pbx1-KO* towards ExM results in increased expression of primitive erythroid genes, *Runx1, Tal1, Gata2* (Modified from Supp Fig.1h in the manuscript and new data).

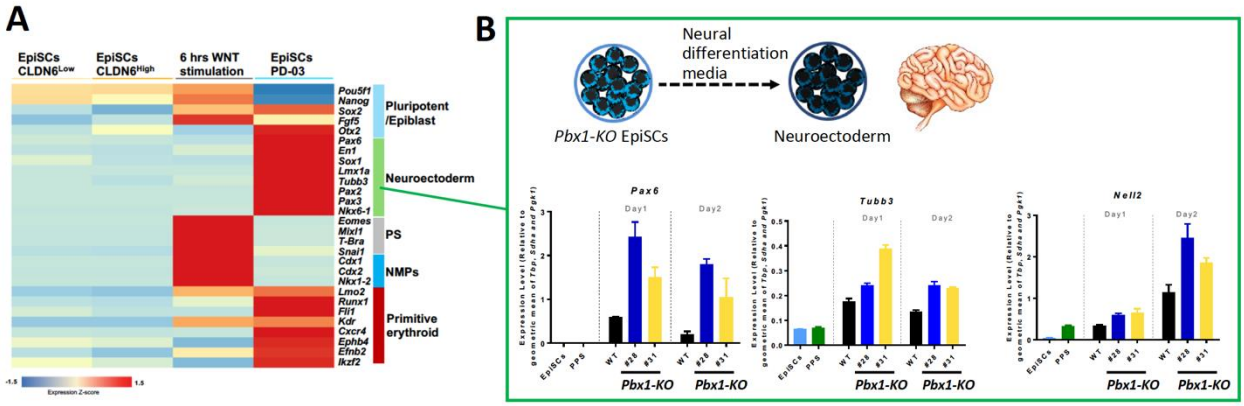


Figure 8. *Pbx1-KO* EpiSCs are biased towards neuroectoderm fate, similar to EpiSCs treated with PD03. **A**) PD03 treatment of EpisSCs results in the upregulation of neuroectodermal genes. **B)** In the green box, direct differentiation of *Pbx1-KO* towards neuroectoderm results in increased expression of neural *genes, Pax6, Tubb3, Nell2* (Modified from Supp Fig.1h in the manuscript and new data).

Furthermore, ERK/FGF signalling is crucial for differentiating pluripotent stem cells towards definitive endoderm (DE) originating from the anterior mesendoderm (AME), and inhibition of FGF/ERK signaling results in reduced differentiation towards DE [20] (Morrison *et al*., Cell Stem Cell 2008). For your perusal, we have added data not included in the manuscript to clarify the common DE differentiation bias observed between ERK inhibition and PBX loss. Differentiation of *Pbx1-KO* EpiSCs toward AME shows reduced expression of DE genes (Figure 9A). Furthermore, FOXA2 expression is also severely perturbed in the DE of *Pbx-KO* embryos (and not in the non-endodermal site: the node) (Figure 9B). *Foxa2* is also differentially methylated in the WT vs *Pbx-KO* EpiSCs (Figure 9C), and regulation of *Foxa2* activity during endoderm development via DNA methylation is well established[21] (Bahar Halper *et al*., JCB 2014).

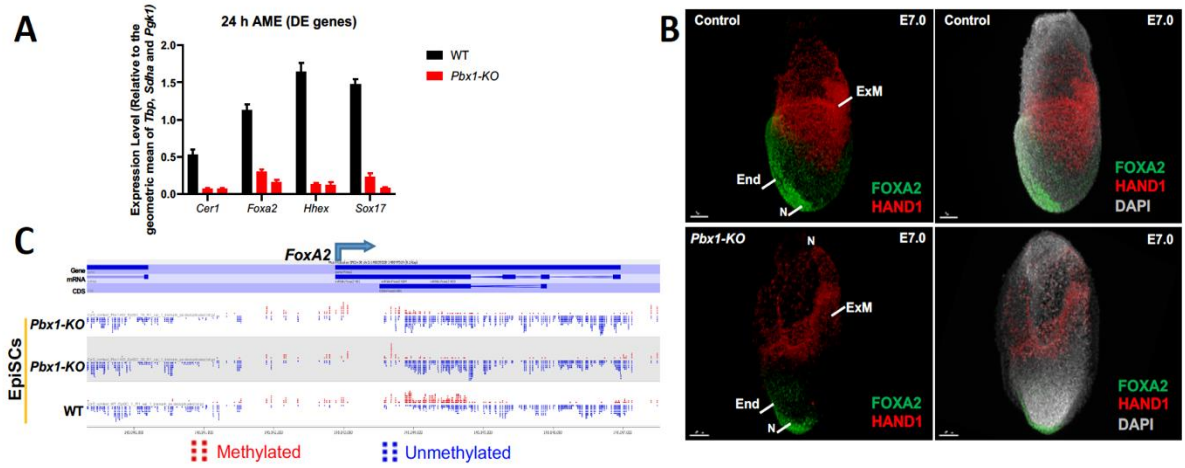


Figure 9. **A**) RT-qPCR analyses of DE markers (*Cer1, Foxa2, Hhex,* and *Sox17*) show reduced DE differentiation in *Pbx1-KO* AME cells. **B**) Confocal maximum intensity projection images of E7.5 control and *Pbx1/2-DKO* embryos probed with FOXA2 (green) and HAND1 (red) antibodies and counterstained with DAPI (grey) reveal reduced expression of HAND1 in ExM and FOXA2 in DE but not in the node (N). **C**) Visualization of WGBS (SeqMonk) indicating differentially methylated regions of the *Foxa2* locus in WT and *Pbx1-KO* EpiSCs shows hypomethylation in the *Pbx1-KO* EpiSCs, while the WT EpiSCs contain methylated CpG regions, validating the previously reported paradoxical nature of DNA methylation regulation of *Foxa2* expression (Bahar Halper *et al*., JCB 2014).

In our manuscript, we also did not focus on the mechanism regulating cardiac differentiation. However, for your perusal, we have added some data to emphasize the similarity between PD03 treatment and *Pbx1-KO*. Classically, cardiac progenitors have been attributed to two main heart fields, termed the first and second heart fields (FHF and SHF). They originate at different time points from the middle primitive streak (MPS), and they will later contribute to the left and right ventricles, respectively.

EpiSCs can be differentiated towards MPS that gives rise to FHF and SHF progenitors. While conventional differentiation protocols and WT cells typically yield modest efficiency in generating SHF progenitors, our data unveil a unique response in *Pbx1-KO* EpiSCs. We found that *Pbx1-KO* EpiSCs fail to differentiate towards FHF cardiac cells, characterized by the expression of FHF markers such as *Hand1*[22] (Barners *et al*., Dev Dyn 2010*) Foxf1, Pitx1*. Conversely, *Pbx1-KO* EpiSCs demonstrate an increased efficiency in differentiating towards SHF cardiac cells, marked by the upregulation of SHF-specific markers including *Tbx1*[23] (Nomaru *et al*., Nature Communications 2021)*, Tbx3, Nkx2-5, Lhx2, Gata4* (Figure 10A). Thus, loss of PBX1 results in a bias towards SHF fate. Similarly, during cardiac differentiation, inhibition of ERK activity via PD03 fine-tunes cardiac lineage segregation towards SHF fate. Our data (Fig. 10B) show that this lineage choice is specifically linked to ERK inhibition (via PD03, red bars), as not adding bFGF to the differentiation medium (-bFGF, light blue bars) does not cause the same pronounced lineage bias. Notably, PD03 treatment at 12h of differentiation leads to loss of *Hand1* (FHF marker) and upregulation of *Tbx1* (SHF marker) (Fig. 10B), while the –bFGF treatment was similar to WT (control, dark blue bars).

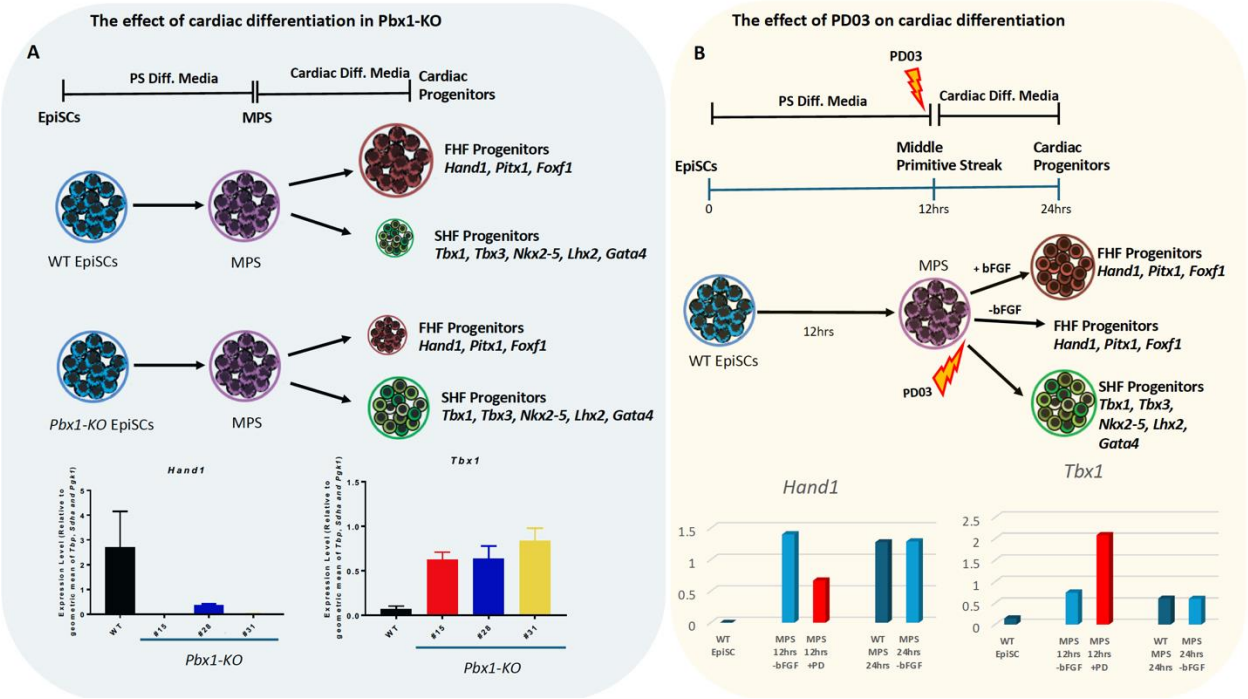


Figure 10. Effect of *Pbx1-KO* on EpiSC differentiation potential and ERK activity regulation during cardiac lineage segregation. **A**) Conventional differentiation protocols and WT cells show modest efficiency in generating Second Heart Field (SHF) progenitors. *Pbx1-KO* EpiSCs exhibit a deficiency in differentiation towards First Heart Field (FHF) cells, marked by low expression of FHF markers (*Hand1, Foxf1,* and *Pitx1*) and an enhanced differentiation towards SHF cardiac cells, characterized by the upregulation of SHF-specific markers including *Tbx1, Isl1, Tbx3, Nkx2-5, Lhx2,* and *Gata4*. **B**) Inhibition of ERK activity via PD03 fine-tunes cardiac lineage segregation. Specifically, PD03 treatment leads to the downregulation of FHF markers (*Hand1,* etc.) and the upregulation of SHF markers (*Tbx1,* etc.).

Furthermore, differentiating EpiSCs without adding bFGF to the MPS differentiation medium does not lead to a SHF-fate bias as observed upon PD03 treatment or in the *Pbx* mutant. Thus, it is the inhibition of ERK, and not just the withdrawal of bFGF from the medium, that induces SHF fate bias. This aligns with our previous observations in response to Reviewer 2 and Reviewer 3 regarding modulation of bFGF leading to possible compensatory mechanism (Figure 3). Thus, our differentiation data emphasizes that PD03 treatment results in an immediate differentiation response that is not sufficiently simulated by bFGF modulation and that our proposed *Pbx1-KO* model remains ideal for investigating the mechanisms influencing lineage priming without inducing differentiation from the EpiSC state.

Altogether, these experiments indicate that EpiSCs with PD03 and *Pbx1-KO* EpiSCs have similar lineage fate outcomes. While we could delve deeper into these differentiation experiments, this approach may divert our focus from the primary objective of this manuscript. Our objective centers on exploring germ-layer priming, a phenomenon only observable at the undifferentiated epiblast state.
However, we could consider incorporating these data as supplementary material should the reviewer determine that they are crucial for strengthening our findings.

2. Use heterozygous mutants or just knockdown components of FGF pathway, to reduce pERK levels, without ablating them.
Upon treating EpiSCs with PD17, an FGFR1 inhibitor, we observed a substantial increase in cell death. In contrast, when we treated EpiSCs with PD03, an MEK/ERK inhibitor, we observed differentiation. These two different effects highlight that it is critical to understand how and where FGF/ERK is modulated[24] (Brewer *et al.,* Genes Dev 2015). Therefore, inhibiting FGF might yield different effects than inhibiting ERK, suggesting that FGF receptor inhibition may not be the most effective approach.
Thus, as the Reviewer mentioned below in the comment, signalling pathways, like transcription factors, have different functions depending on how and where in the pathway, or protein level, they may be regulated. Altogether, diving into these factors is not the focus of our manuscript. However, previously published studies reported that the knockdown of ERK components led to the differentiation of primed pluripotent stem cells[5] (Goke *et al*., Mol. Cell 2013), further highlighting the difficulty in studying lineage priming via such approaches. The main downstream factors of ERK activity, the ETS proteins, have been shown to participate in lineage priming activity according to their expression within different populations[25-27] (Hogart *et al.,* Genome research 2012, Suzuki *et al*., Oncogene 2006, de la Rica *et al*., Genome Biology 2013). Similarly, we showed differential ERK levels, combined with differential expression of ETS family members in the separated populations, providing a system to study lineage bias in undifferentiated primed cells (Fig. 1d , Fig. 4e-i, in the manuscript).

3. If Spry2 is the key regulator, modulate the levels of Spry2 to reduce pERK and see if the phenotype is the same of Pbx1 KO.
Deleting the *Spry2* gene would globally impact ERK activity, potentially prompting cell differentiation and widespread effects[6,7] (Mason *et al.,* Trends in Cell Biol. 2006; Shukla *et al*., Blood 2016). Moreover, given that PBX1 is a repressor of SPRY2, the deletion of *Spry2* will not recapitulate *Pbx1-KO* phenotypic effects, as suggested by Reviewer 2. To establish the connection between PBX and ERK via *Spry2*, we could generate a point mutation in the PBX binding site on the *Spry2* regulatory region, akin to our previous methodology outlined in Mariani *et al*. Nature Communications (2021)[12]. This approach stands unique among existing literature, which typically relies on ChIP-based evidence to claim gene expression regulation[9-11] (Scheibner *et al*., Nature Cell Biology 2021; Metzis *et al*., Cell 2018; Harland *et al*., Nature Cell Biology 2021). However, conducting these experiments is not currently feasible due to our current economic and laboratory constraints. In addition, these experiments will divert our focus from the primary

objective of this manuscript: the demonstration of lineage primed epiblast populations and their subsequent response to different germ-layer differentiation signals.
Furthemore, existing data assessing the overexpression of ERK inhibitors, like DUSPs, showed that DUSP9 overexpression in primed pluripotent stem cells (hESCs), resulted in changes in the DNA methylation states linked to ERK feedback regulation[14] (Choi *et al*., Cell Stem Cell 2017). A phenotype similar to *Pbx1-KO,* where we see a similar upregulation of the ERK feedback inhibitor *Spry2* resulting in differential DNA methylation states. We would also like to highlight, that the overexpression of DUSP9 was the main focus of the paper published in Cell Stem Cell. Thus, adding these experiments would significantly change the focus of our paper.

4. Use validated Erk-associated markers, rather than Claudin, to isolate cells with different levels of pERK.
Claudin-6, a cell surface receptor, emerges as an optimal marker for live cell sorting and subsequent reseeding, facilitating precise testing of lineage differentiation amongst CLDN6 sorted subpopulations. In contrast, ERK-associated markers are intracellular proteins, limiting their utility for live cell sorting. Intracellular FACS sorting necessitates cell permeabilization, which compromises cell viability, hindering the ability to maintain cells alive for lineage preference testing.
However, we performed FACS and Western blot analyses using a pERK antibody, showing pERK reduction in the PBX mutant and differences in CLDN6 sorted populations.
While we did not sort the cells based on an ERK-associated marker, we did compare our CLDN6 sorted populations to *in vivo* spatial transcriptomics published data[28] (Peng *et al*., Dev Cell 2016). As already stated in the manuscript, there is differential expression of ERK-associated markers in anterior and distal posterior epiblast (Figure 11A) that correspond to the CLDN6^{High} and CLDN6^{Low} EpiSCs respectively (Figure 11B). ERK-associated markers, such as ETS family members, have been shown to regulate site-specific DNA methylation in different contexts[25-27] (*de la Rica et al*., Genome Biology 2013, Hogart *et al.,* Genome research 2012, Suzuki *et al*., Oncogene 2006). This would explain the differential methylation states observed in our differently primed, undifferentiated epiblast populations (CLDN6^{High} vs CLDN6^{Low}), where we see differential expression of ETS family members, corroborating *in vivo* observations.

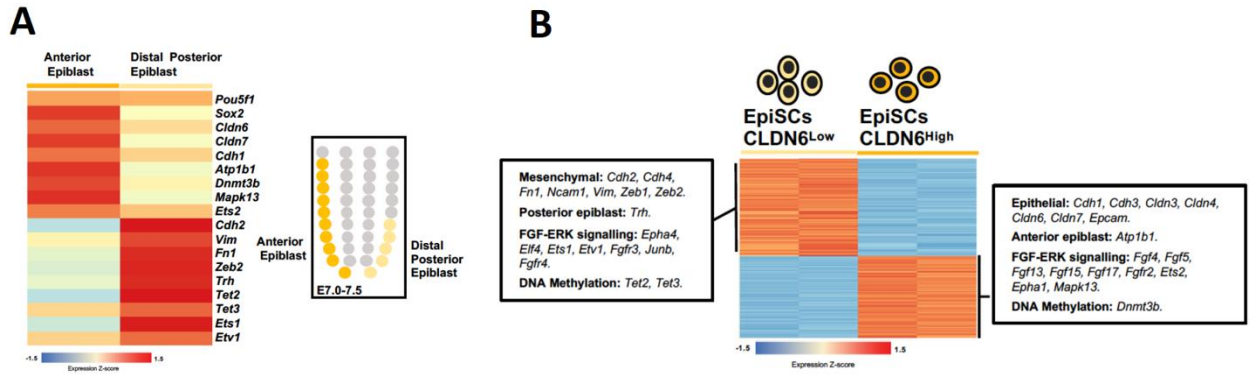


Figure 11. ERK-Associated Marker Expression in Anterior and Distal Posterior Epiblasts. **A**) Heatmap illustrating specific gene expression in the anterior epiblast (dark yellow) and distal posterior epiblast (light yellow), based on FPKM values from E7.0-E7.5 epiblast GEO-sequencing data. Graphical Cornplot, highlighting the positional identity of anterior epiblast (dark yellow) and distal-posterior epiblast (light yellow). **B**) Differential expression of CLDN6^{High} vs. CLDN6^{Low} cells. CLDN6^{Low} cells exhibit higher expression of FGF receptors (*Fgfr3, Fgfr4)* and specific ETS TFs *(Ets1*, Elf4, *Etv1)*. CLDN6^{High} cells are enriched with epithelial markers such as *Cdh1, Cldn6,* and *Cldn7*, along with *Fgf* ligands (*Fgf4, Fgf5,* etc.) and ETS TF *Ets2* (Modified from Supp Fig.1f and Fig1. d in the manuscript).

The authors claim that in Pbx1 KO there are reduced levels of FGF signaling, which, in turn affect epigenetics and differentiation potential. But they now show that blocking FGF actually causes cell death and differentiation, which is very different from the behavior of Pbx1 KO cells. So, the only fair conclusion is that differences in FGF signalling do NOT explain the phenotype of Pbx1 KO cells.

We would like to point out that we do not show that there are reduced levels of FGF signalling. In our manuscript, we state that *Pbx1-KO* EpiSCs have reduced (but not inhibited/blocked) pERK levels, while the FGF ligands are actually increased (Figure 1 and Fig. 4b in the manuscript). We did not claim that PBX1 directly reduces FGF ligand levels; rather, our findings demonstrate reduced pERK in the absence of PBX1. Notably, upregulation of FGF ligands is similarly observed upon PD03 treatment of EpiSCs and in EpiSCs where bFGF is not added to the medium, although in both those cases, the cells begin to differentiate (Figure 3). Furthermore, we would also like to reiterate that different FGF inhibitors have different effects. Indeed, it is a critical aspect where in the signalling cascade FGF or ERK are modulated[24] (Brewer *et al.,* Genes Dev 2015). For example, chemical inhibition via PD17 (FGFR1 inhibitor) and PD03 (MEK/ERK inhibitor) do not necessarily result in the same phenotype, although both technically inhibit FGF signalling. In *Pbx1-KO* EpiSCs, we see a downregulation, but not total elimination of pERK, alongside upregulation of FGF ligands. Thus, there would be differences compared to complete FGFR1 inhibition (via PD17) or complete MEK/ERK inhibition (via PD03). Altogether, our manuscript and additional data highlight PBX1 as a unique model showing ERK feedback regulation resulting in different primed undifferentiated epiblast states, wherein ERK levels are reduced, but not eliminated.

Reviewer #3 (Remarks to the Author):

This response to our prior reviews offered many explanations but few changes based on my and other reviewers' concerns about cell signaling connections. I still do not agree that the manuscript demonstrates an effect through ERK signaling. There are correlative data (signatures associated with ETS factors; correlations between dpERK and claudin), but no causal results that establish changes driven by ERK. Additionally, the authors' responses to my previously raised concerns argue that the Wnt and BMP signaling dependencies are that these connections should simply be known/assumed from literature, and are not directly demonstrated in this study. I am still not comfortable with the authors' conclusive statements in the title, abstract, and summaries of experiments that make claims about these discoveries (e.g. "Erk-driven lineage specification"; "chromatin accessibility and methylome states prime BMP-dependent fate decisions", "DNA methylation… safeguards WNT response"). As stated by me and another reviewer, Pbx perturbations don't just affect Erk but affect many other processes. Also: PI3K/Akt is not part of the MAPK/Erk cascade, as stated in the response to Reviewer 2, but is exactly one of those parallel signaling pathways that could lead to confounding results.
In contrast, I am very convinced by the authors' perturbations to Pbx proteins, sorting of Claudin proteins, and assessment of chromatin state and accessibility. I would be very happy to see that data published without further revisions, but am disappointed that the manuscript can't rest on those demonstrated results but instead has to expand claims to shakier ground

We thank the reviewer for noting our statements in the title, abstract, and summaries. We have changed our title, abstract and summaries in the manuscript accordingly.

We extend our appreciation to the reviewer for their favorable comments regarding our work on PBX proteins, sorting of Claudin proteins, and assessment of chromatin state and accessibility.

As already mentioned in our response to reviewer 2, in our manuscript, we demonstrated that *Pbx1* in the epiblast regulates ERK feedback regulators and downstream effectors, with no transcriptional effects on lineage genes.
We sincerely apologize if the content regarding the causative effect of PBX1 on ERK activity was not as elucidating as intended. We narrowed down the PBX1 regulatory effect on pERK based on RNAseq, GO, ChIPseq, ATACseq analyses, coupled with EMSA showing binding affinity of PBX complexes on *Spry2* enhancer site that is specifically accessible in EpiSCs (ERK dependent), but not ESCs (ERK inhibited).
We conducted a comparative analysis between our manuscript and three seminal studies in the field, focusing on how they address the causal relationship between the binding of transcription factors over the regulatory regions of signaling components. This comparative approach underscores the similarity, if not superiority, of our methodology, thereby reinforcing the significance of our research.

A)[10] Metzis V, Steinhauser S, Pakanavicius E, Gouti M, Stamataki D, Ivanovitch K, Watson T, Rayon T, Mousavy Gharavy SN, Lovell-Badge R, Luscombe NM, Briscoe J. Nervous System Regionalization Entails Axial Allocation before Neural Differentiation. Cell. 2018 Nov 1;175(4):1105-1118.e17. doi: 10.1016/j.cell.2018.09.040.

The study elucidates that pluripotent cells commit to a regional identity before acquiring a neural identity and that this identity is established in the epiblast. Posterior epiblast cells commit to a posterior nervous system (NMP/spinal cord) fate, while anterior epiblast cells commit to an anterior nervous system (neuroectoderm) fate. This posterior vs anterior identity in the epiblast is established by WNT signals and CDX2 transcription factor, which drives chromatin accessibility.
Metzis *et al.* mentioned that CDX2 present in the "WNT+ posterior epiblast" is responsible for posterior neural priming, citing two papers[29,30] (van der Akker *et al.,* Development 2002; Mallo *et al.*, Dev. Biol. 2010). However, it's important to note that neither of these two references mentions the expression of *Cdx2* in the epiblast. Instead, both sources clearly state that *Cdx2* is expressed in the primitive streak and nascent paraxial mesoderm, as initially reported by[31,32] Meyer and Kruss, Development 1993 and Beck *et al.,* Dev. Dyn. 1995. Thus, no one has reported the presence of CDX2 in the epiblast.
More significantly, Metzis *et al.* did not conduct ChIP or any other experiments to support the foundation of WNT regulation, or prove the causal relationship between the binding of transcription factors over the regulatory regions of signaling components. On the contrary, we provide several molecular evidence, including ChIPseq, ATACseq, and EMSA, to elucidate the mechanism describing how PBX1 modulates ERK-*Spry2*. Furthermore, our CLDN6 model is more suitable for epiblast regionalization studies, as a CLDN6 gradient has been observed in the epiblast (Fig. 1 in the submitted manuscript) and others [9](Scheibner *et al*., Nature Cell Biology 2021). Metzis *et al.,* made major claims on regionalization, but there is no evidence backing those claims, while we addressed this aspect via spatial transcriptomics, IF, and compared to previously published bulk spatial transcriptomic data.

B)[9] Scheibner K, Schirge S, Burtscher I, Büttner M, Sterr M, Yang D, Böttcher A, Ansarullah, Irmler M, Beckers J, Cernilogar FM, Schotta G, Theis FJ, Lickert H. Epithelial cell plasticity drives endoderm formation during gastrulation. Nat Cell Biol. 2021 Jul;23(7):692-703. doi: 10.1038/s41556-021-00694-x.
In this paper, Scheibner *et al.,* employed a combination of *in vitro* and *in vivo* techniques, including knock-in reporter systems and high-resolution single-cell transcriptomics, along with time-resolved lineage labeling. They aimed to dissect the morphogenetic processes that differentiate the mesoderm from the endoderm germ layer. Their findings revealed that while the mesoderm formation involves a classical

epithelial-to-mesenchymal transition (EMT) dependent on *Snai1*, the endoderm undergoes a partial EMT process driven by mechanisms of epithelial cell plasticity.
Similar to our approach, Scheibner *et al.*, used RNA-seq and ChIP-seq analyses to link FOXA2 as a direct regulator of WNT activity. However, no EMSA or further causal analyses via point deletion were performed.
The Scheibner *et al.*, study explored the effect of WNT across the context of differentiation rather than priming. Thus, they did not address priming, as it is essential for cells to remain in an undifferentiated state to assess this aspect effectively.
Applying a similar approach and addressing ERK inhibition during differentiation (Figures 7 and 8), we showed that the addition of PD03 in EpiSCs yields differentiation comparable to the cell acquisition output observed during the differentiation of *Pbx1-KO* cells. Although these findings suggest that *Pbx1* mutants and PD03 treatment yield comparable effects on cell differentiation outcomes, they fall outside the scope of our paper, which focuses on elucidating the lineage priming effect in the undifferentiated epiblast state. This is the reason why we did not include them in the manuscript.

C)[11] Harland LTG, Simon CS, Senft AD, Costello I, Greder L, Imaz-Rosshandler I, Göttgens B, Marioni JC, Bikoff EK, Porcher C, de Bruijn MFTR, Robertson EJ. The T-box transcription factor Eomesodermin governs haemogenic competence of yolk sac mesodermal progenitors. Nat Cell Biol. 2021 Jan;23(1):61-74. doi: 10.1038/s41556-020-00611-8.

This paper focused on the important role of EOMES in the epiblast for the generation of Extraembryonic Mesoderm (ExM). However, no comprehensive molecular mechanisms or thorough analyses have been conducted to fully understand the role of EOMES in epiblast cells.
The statement of the priming activity operated by EOMES is based on the following assertion: "*To assess Eomes functional contributions we analyzed E7.5 embryos carrying an epiblast-specific Eomes deletion (Eomes$^{CA/N}$;Sox2.Cre) that disrupts delamination of nascent mesoderm. In contrast to wild-type embryos, Eomes$^{\Delta Epi}$ mutant embryos fail to induce expression of Flk-1 and ER71, genes essential for YS hematopoiesis and vasculogenesis (Extended Data Fig. 2f). Thus, Eomes expression in the epiblast is essential for the generation of ExM*."
Harland *et al.* stated that EOMES expression in the epiblast is crucial for Extraembryonic Mesoderm (ExM) differentiation, implicating the role of EOMES in priming proximal posterior epiblast cells towards ExM lineages. However, the paper does not delve into the precise roles of EOMES within the epiblast, nor does it explore the molecular mechanisms underlying priming.
In our work, we showed that epiblast inactivation of *Pbx1* with the *Sox2^{Cre}* allele in a *Pbx2*-deficient background (*Pbx1/2-KO*) resulted in stronger developmental defects compared to *Pbx1* deletion in PS with the *T-Bra^{Cre}* allele (Supplementary Fig. 5c-e in the submitted manuscript), indicating the critical role of PBX1 in the epiblast. Moreover, we provided stronger evidence that elucidates the link between PBX expression in the epiblast, ERK activity, and subsequent ExM generation, looking into the molecular mechanisms within epiblast cells that regulate ExM specification. By addressing the molecular mechanisms operating within epiblast cells that govern ExM specification, our findings lend greater scientific credibility to our claims.

Also: PI3K/Akt is not part of the MAPK/Erk cascade, as stated in the response to Reviewer 2, but is exactly one of those parallel signaling pathways that could lead to confounding results.

We thank the reviewer for the clarification, we agree that PI3K/AKT is not part of the MAPK/ERK cascade. It may stem from a misunderstanding, but we never mentioned that PI3K/AKT is part of MAPK/ERK

signalling. We said that there was no effect on PI3K/AKT in *Pbx1* mutant. This result is important because it points towards MAPK/ERK signalling and not to the PI3K/AKT. MAPK/ERK is downstream of FGF signalling. PI3K/AKT is also downstream of the FGF signalling pathway, and parallel to ERK signalling. There are indeed other components and effectors downstream of FGF receptor signalling, such as STAT and PLC signalling[24] (Brewer *et al.,* Genes Dev 2015), but addressing each one is out of the scope of the manuscript. For example, most studies focusing on ChIR addition as a WNT agonist as a key requirement in maintaining mouse ES cells[33-35] (Sato *et al*., Nature Medicine 2004; Wray *et al*., Nature Cell Biology 2011; Sokol Development 2011) do not delve into the possible role of GSK-3β inhibition over the AKT pathway, where it is also linked[36] (Suzuki *et al*., Molecular Cell 2013). Curiously, none of these seminal studies in high impact factor papers addressed the role of GSK-3β inhibition on AKT signalling in mouse ES cells: a model cell system that has been heavily studied and utilized. Altogether, as mentioned by Reviewer 3, PI3K/AKT is parallel to ERK signalling and downstream of the FGF signalling cascade. PI3K/AKT is unaffected in *Pbx1-KO* EpiSCs, corroborating our focus on ERK activity. While we do acknowledge that there are other signalling downstream of FGF, such as STAT or PLC, it is out of the scope of our manuscript to address all possible avenues.

We would once again like to appreciate the insightful feedback and suggestions from the reviewers. Below we highlight the key findings presented in our paper for your perusal:

1. Using single-cell RNAseq, FACS and IF, we highlighted the existence of a mix of pluripotent (CLDN6^{High}) and fate-restricted (CLDN6^{Low}) sub-populations within EpiSCs, providing insight into the debated notion that epiblast cells are not pluripotent as a whole, but consist of a mix of pluripotent and multipotent primed cells.
2. We identified an extracellular sorting marker, CLDN6, enabling the sorting of CLDN6^{High} and CLDN6^{Low} EpiSCs, facilitating the investigation of their distinct cellular responses.
3. We demonstrated that CLDN6^{High} and CLDN6^{Low} EpiSC populations exhibit primed states with notable self-renewal capacity and expansion potential *in vitro*.
4. We demonstrated that CLDN6^{Low} EpiSCs respond to WNT and differentiate towards neuromesodermal progenitors (NMPs) and further paraxial mesoderm, but cannot form endoderm or ectoderm.
5. We used single-cell spatial transcriptome, coupled with published bulk spatial transcriptomic data, to provide a prospective localization of CLDN6^{High} and CLDN6^{Low} EpiSCs as anterior and posterior epiblast in the embryos.
6. Our study is the first to demonstrate the existence and maintenance of a self-renewing musculoskeletal progenitor population (NMPs) *in vitro*, with CLDN6^{Low} EpiSCs resembling caudal lateral epiblast which can be held indefinitely in culture and remain fate-restricted towards NMP.
7. We showed that distinct methylome patterns established in the epiblast cells, and not chromatin accessibility, influence regional neural lineage decisions. Our work challenges results from Metzis *et al.,* (2018)[10], who proposed that regional neural identity is set by chromatin accessibility. Moreover, we studied the mechanism of priming in undifferentiated epiblast cells.
8. Our study validates Scheibner *et al.* (2021)[9] findings regarding mesoderm and DE progenitors arising from distinct EMT processes. Additionally, we demonstrate that different epiblast populations give rise to endoderm and mesoderm. This aligns with evolutionary studies (Hashimshony *et al.,* Nature 2015; Ryan *et al.,* Science 2013)[37,38] suggesting a common ancestry for neuroectoderm and endoderm. Specifically, we show that CLDN6^{High} EpiSCs are biased towards neuroectoderm and endoderm. Our work raises an interesting question on how mammals might have adapted mechanisms like DNA methylation to generate tissue diversity.

9. We confirmed previous findings of proximal epiblast cells priming towards the Extraembryonic Mesoderm (ExM) lineage (Harland *et al.,* Nature Cell Biology 2021, Padron-Barthe *et al.,* Blood 2014)[11,39]. Furthermore, we showed that the BMP response within the ExM lineage is predetermined by distinct methylome and chromatin accessibility states in these proximal epiblast cells, providing a molecular mechanism explaining long-standing questions from seminal lineage tracing studies.
10. We demonstrated that ERK signaling influences the DNA methylation landscape in primed EpiSCs, modulating their cell-specific response to growth factors that induce germ-layer differentiation.
11. We introduced a novel cellular model featuring PBX mutants with reduced ERK levels and distinct methylome signatures while maintaining their undifferentiated epiblast state.
12. Our data points towards PBX1 modulation of pERK activity: influencing site-specific DNA methylation in EpiSCs, thereby priming cell signaling responses to differentiation cues both *in vitro* and *in vivo*, as demonstrated by experiments with *Pbx* mutant embryos.
13. Altogether, we characterize two models (CLND6 sorted cells and *Pbx1* mutants) to study the influence of priming in cell fate acquisition, allowing future researchers to further dissect the molecular mechanisms orchestrating the transition from "pluripotency" towards lineage acquisition. Elucidating these mechanisms is key to understanding diseases such as cancer, where similar mechanisms regulate tumorigenesis and migration.

Reference

1. Ficz, G. *et al.* FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency. *Cell Stem Cell* **13**, 351-9 (2013).
2. Lake, D., Correa, S.A. & Muller, J. Negative feedback regulation of the ERK1/2 MAPK pathway. *Cell Mol Life Sci* **73**, 4397-4413 (2016).
3. Samatar, A.A. & Poulikakos, P.I. Targeting RAS-ERK signalling in cancer: promises and challenges. *Nat Rev Drug Discov* **13**, 928-42 (2014).
4. Greber, B. *et al.* Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* **6**, 215-26 (2010).
5. Goke, J., Chan, Y.S., Yan, J., Vingron, M. & Ng, H.H. Genome-wide kinase-chromatin interactions reveal the regulatory network of ERK signaling in human embryonic stem cells. *Mol Cell* **50**, 844-55 (2013).
6. Mason, J.M., Morrison, D.J., Basson, M.A. & Licht, J.D. Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol* **16**, 45-54 (2006).
7. Shukla, A. *et al.* Sprouty 2: a novel attenuator of B-cell receptor and MAPK-Erk signaling in CLL. *Blood* **127**, 2310-21 (2016).
8. Hanafusa, H., Torii, S., Yasunaga, T. & Nishida, E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat Cell Biol* **4**, 850-8 (2002).
9. Scheibner, K. *et al.* Epithelial cell plasticity drives endoderm formation during gastrulation. *Nat Cell Biol* **23**, 692-703 (2021).
10. Metzis, V. *et al.* Nervous System Regionalization Entails Axial Allocation before Neural Differentiation. *Cell* **175**, 1105-1118 e17 (2018).

11. Harland, L.T.G. *et al.* The T-box transcription factor Eomesodermin governs haemogenic competence of yolk sac mesodermal progenitors. *Nat Cell Biol* **23**, 61-74 (2021).
12. Mariani, L. *et al.* A TALE/HOX code unlocks WNT signalling response towards paraxial mesoderm. *Nat Commun* **12**, 5136 (2021).
13. Smith, Z.D. *et al.* Epigenetic restriction of extraembryonic lineages mirrors the somatic transition to cancer. *Nature* **549**, 543-547 (2017).
14. Choi, J. *et al.* Prolonged Mek1/2 suppression impairs the developmental potential of embryonic stem cells. *Nature* **548**, 219-223 (2017).
15. Yagi, M. *et al.* Derivation of ground-state female ES cells maintaining gamete-derived DNA methylation. *Nature* **548**, 224-227 (2017).
16. Di Stefano, B. *et al.* Reduced MEK inhibition preserves genomic stability in naive human embryonic stem cells. *Nat Methods* **15**, 732-740 (2018).
17. Bar, S. *et al.* Identifying regulators of parental imprinting by CRISPR/Cas9 screening in haploid human embryonic stem cells. *Nat Commun* **12**, 6718 (2021).
18. Habibi, E. *et al.* Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell* **13**, 360-9 (2013).
19. Li, C., Liu, B., Zhong, S. & Wang, H. MEK inhibitor PD0325901 and vitamin C synergistically induce hypomethylation of mouse embryonic stem cells. *Oncotarget* **7**, 39730-39739 (2016).
20. Morrison, G.M. *et al.* Anterior definitive endoderm from ESCs reveals a role for FGF signaling. *Cell Stem Cell* **3**, 402-15 (2008).
21. Bahar Halpern, K., Vana, T. & Walker, M.D. Paradoxical role of DNA methylation in activation of FoxA2 gene expression during endoderm development. *J Biol Chem* **289**, 23882-92 (2014).
22. Barnes, R.M., Firulli, B.A., Conway, S.J., Vincentz, J.W. & Firulli, A.B. Analysis of the Hand1 cell lineage reveals novel contributions to cardiovascular, neural crest, extra-embryonic, and lateral mesoderm derivatives. *Dev Dyn* **239**, 3086-97 (2010).
23. Nomaru, H. *et al.* Single cell multi-omic analysis identifies a Tbx1-dependent multilineage primed population in murine cardiopharyngeal mesoderm. *Nat Commun* **12**, 6645 (2021).
24. Brewer, J.R., Molotkov, A., Mazot, P., Hoch, R.V. & Soriano, P. Fgfr1 regulates development through the combinatorial use of signaling proteins. *Genes Dev* **29**, 1863-74 (2015).
25. Hogart, A. *et al.* Genome-wide DNA methylation profiles in hematopoietic stem and progenitor cells reveal overrepresentation of ETS transcription factor binding sites. *Genome Res* **22**, 1407-18 (2012).
26. Suzuki, M. *et al.* Site-specific DNA methylation by a complex of PU.1 and Dnmt3a/b. *Oncogene* **25**, 2477-88 (2006).
27. de la Rica, L. *et al.* PU.1 target genes undergo Tet2-coupled demethylation and DNMT3b-mediated methylation in monocyte-to-osteoclast differentiation. *Genome Biol* **14**, R99 (2013).
28. Peng, G. *et al.* Spatial Transcriptome for the Molecular Annotation of Lineage Fates and Cell Identity in Mid-gastrula Mouse Embryo. *Dev Cell* **36**, 681-97 (2016).
29. van den Akker, E. *et al.* Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* **129**, 2181-93 (2002).
30. Mallo, M., Wellik, D.M. & Deschamps, J. Hox genes and regional patterning of the vertebrate body plan. *Dev Biol* **344**, 7-15 (2010).

31. Meyer, B.I. & Gruss, P. Mouse Cdx-1 expression during gastrulation. *Development* **117**, 191-203 (1993).
32. Beck, F., Erler, T., Russell, A. & James, R. Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev Dyn* **204**, 219-27 (1995).
33. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. & Brivanlou, A.H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* **10**, 55-63 (2004).
34. Wray, J. *et al.* Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. *Nat Cell Biol* **13**, 838-45 (2011).
35. Sokol, S.Y. Maintaining embryonic stem cell pluripotency with Wnt signaling. *Development* **138**, 4341-50 (2011).
36. Suzuki, T. *et al.* Inhibition of AMPK catabolic action by GSK3. *Mol Cell* **50**, 407-19 (2013).
37. Hashimshony, T., Feder, M., Levin, M., Hall, B.K. & Yanai, I. Spatiotemporal transcriptomics reveals the evolutionary history of the endoderm germ layer. *Nature* **519**, 219-22 (2015).
38. Ryan, J.F. *et al.* The genome of the ctenophore Mnemiopsis leidyi and its implications for cell type evolution. *Science* **342**, 1242592 (2013).
39. Padron-Barthe, L. *et al.* Clonal analysis identifies hemogenic endothelium as the source of the blood-endothelial common lineage in the mouse embryo. *Blood* **124**, 2523-32 (2014).

A point-by-point response to the Reviewers

We express our sincere gratitude to the reviewers for their insightful comments and suggestions, which have been instrumental in improving the quality and clarity of our manuscript. Based on the feedback we received upon our first submission, we conducted additional experiments and made significant revisions to address the reviewers' concerns more comprehensively. Changes made to the manuscript are highlighted in red, while our responses to the reviewers' comments are indicated in blue for clarity. These reflections led to the generation of new figures with additional data and the elaboration of a more in-depth discussion. Once again, we thank the reviewers for their valuable input.

To address reviewer 2 and 3 major concerns regarding the link between PBX, CLDN6 and ERK, we collaborated with Prof. Joshua M. Brickman and Dr. Jakub Sedzinski, along with their lab members, at the Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), University of Copenhagen, Denmark. As a result of their contributions, they have been added as co-authors on the manuscript.

Our responses begin with an overview of the key changes and a summary of the results, followed by a point-by-point response to the reviewer.

Reviewer 1 Suggestion

Comment: **Improve clarity regarding the mechanism by which PBX influences cellular responses to differentiation signals.**

Response: To address this request, we created 2 new Figures:

- **Figure 6** to illustrate an inclusive model that connects all key findings of this study.
- **Supplementary Fig. 6** to depict the proposed mechanism in a more detailed and visually clear manner. This figure provides a comprehensive overview, illustrating how PBX in association with ERK signalling, regulates the epigenetic priming of ExM differentiation.

Reviewer 2 and Reviewer 3 – Major Suggestions

Comment: **Perform experiments to treat EpiSCs with an ERK inhibitor at different concentrations**.

Response: As suggested, we treated EpiSCs with an ERK inhibitor (PD03) at varying concentrations. These experiments demonstrated that *Pbx1-KO* EpiSCs exhibit a signalling response similar to $CLDN6^{Low}$ EpiSCs, both characterized by reduced ERK activity.

To highlight these findings, we incorporated new data into the manuscript:

- **Supplementary Fig. 3**: Two new panels were added, illustrating the differentiation potential of PD03-treated EpiSCs.
- **Supplementary Fig. 5**: One additional panel was included to further demonstrate the link between CLDN6, PBX and ERK levels.
- **Supplementary Fig**. 7: New figure added with panels that demonstrate the *Pbx-KO in vivo* and *in vitro* phenotype is similar to $CLDN6^{Low}$ and ERK-inhibited cells in anterior primitive streak tissues.

The findings from these experiments demonstrate an effect through ERK on lineage priming in EpiSCs, showing that dosed ERK inhibition in PD03-treated EpiSCs recapitulated the same differentiation phenotype observed in $CLDN6^{Low}$ and *Pbx1-KO* EpiSCs, establishing a clear link between PBX, CLDN6, and ERK signalling - addressing the reviewers' concerns. These experiments are discussed in more details in the point-by-point response to the reviewer.

Comment: **Address questions regarding the pleiotropic roles of PBX in differentiation**.

Response: We have addressed this concern by adding a new **Supplementary Fig. 7** and a dedicated new section in the manuscript. This section explores PBX's dual role in the epiblast and during differentiation, as well as its HOX-dependent and independent functions:

- **PBX-ERK in the epiblast (precedes differentiation)** – PBX, in coordination with ERK signalling, **primes the epigenetic landscape** before lineage commitment, establishing a permissive state for differentiation. This role occurs independently of HOX genes, which are not expressed in the epiblast.
- **PBX-HOX during WNT induction and PSM differentiation** – PBX functions with HOX genes during WNT-driven presomitic mesoderm (PSM) differentiation, facilitating lineage commitment through chromatin remodeling and transcriptional regulation. PBX contributes to LEF1-mediated WNT signalling, reinforcing PSM identity by guiding LEF1 binding to its target loci.

These additions clarify PBX's pleiotropic roles and provide a more comprehensive view of its dual contributions to cell-context responses during germ layer differentiation.

Comment: Align the manuscript title and focus with the data and conclusions presented.
Response: In response to the reviewers' critique, we revised the manuscript title to better reflect the study's findings and scope. The new title is: "***Cell-context response to germ layer differentiation signals is predetermined by the epigenome in regionalized epiblast populations***."

We hope you find the revised manuscript comprehensive and compelling.

A point-by-point response to the reviewers' comments is provided below. Our comments are highlighted in blue.

Reviewer #1 (Remarks to the Author):
The authors have appropriately addressed most of my concerns. The only thing I would suggest if they could include a more inclusive model to connect all key findings of this study.
We extend our appreciation to the reviewer for their favourable comments regarding our work and their perceptive feedback. Their constructive input has greatly enhanced the clarity of our results and empowered us to highlight the key findings of our study. As suggested, we have modified our previous models, adding two new figures that describe our key findings (Fig. 6 and Supplementary Fig. 6) to the manuscript.

Reviewer #2 (Remarks to the Author):
The authors did not address in a satisfactory way the concerns raised by this reviewer, and by reviewer 3, about the causal role of FGF in the mechanism described.
There are many correlative pieces of evidence pointing at FGF signalling being regulated by Pbx1, potentially via Spry2.
The authors claim that somehow it is not possible to test this link directly, by inhibiting FGF, or, more specifically, by reducing pERK levels, because EpiSCs might die or differentiate when FGF/pERK are reduced.
There are multiple ways to address this point, some already suggested by reviewer 3:

1. Titrate the inhibitor PD03, in order to reduce only partially pERK, at the levels shown in Figure 4c. Such concentration of PD03 should phenocopy Pbx1 KO
2. Use heterozygous mutants or just knockdown components of FGF pathway, to reduce pERK levels, without ablating them.
3. If Spry2 is the key regulator, modulate the levels of Spry2 to reduce pERK and see if the phenotype is the same of Pbx1 KO
4. Use validated Erk-associated markers, rather than Claudin, to isolate cells with different levels of pERK.

I will quote reviewer 3:

'Why not just simply treat EpiSCs with a MEK inhibitor and look for a direct role of ERK signalling … ? And if doing so gives completely different results, then isn't that evidence that the Claudin and Pbx2 results have nothing to do with FGF/Erk signalling at all? '

Although the language is quite direct, I totally agree with the reasoning behind it.

The authors claim that in Pbx1 KO there are reduced levels of FGF signalling, which, in turn affect epigenetics and differentiation potential. But they now show that blocking FGF actually causes cell death and differentiation, which is very different from the behavior of Pbx1 KO cells.
So, the only fair conclusion is that differences in FGF signalling do NOT explain the phenotype of Pbx1 KO cells.

We sincerely thank Reviewer 2 for their insightful feedback and for suggesting the experiments to clarify the link between CLDN6, ERK, and PBX. Their input helped us to in refine our study, and elucidate key aspects of our findings. In response, we conducted additional experiments, which have strengthened our work and provided a deeper understanding of the *Pbx1* phenotype in the epiblast state and its relationship with ERK signalling.
We would like to clarify that while PBX1 regulates ERK levels, FGF signalling is not blocked or eliminated in *Pbx1-KO* EpiSCs; rather, FGF ligands are upregulated. Our data indicate a downregulation of pERK, suggesting a compensatory feedback mechanism within the ERK signalling loop (Fig. 4, Supplementary Fig. 5). In the manuscript, we focus specifically on the connection between PBX and ERK signalling. However, to further illustrate this compensatory feedback mechanism, we provide Figure 1 (attached below), which demonstrates how treatment with the MEK/ERK inhibitor (PD03) in EpiSCs leads to a similar upregulation of *Fgf5* ligand, resembling the effect observed in *Pbx1-KO* EpiSCs (Fig. 4b). Notably, this compensatory response is also observed when FGF is omitted from the EpiSC medium (Figure 1, attached below). We appreciate the reviewer's comments and the opportunity to further refine this aspect.

As suggested by Reviewer 2 in point 1, we treated EpiSCs with different titrations of PD03 (ERK inhibitor) and we included the results in the manuscript.
We treated EpiSCs with different dosages of PD03 (0.1 μM and 0.5 μM) and expanded the cells under these conditions for multiple (5) passages. Prior to differentiation, we stopped treating the cells with PD03, and applied the differentiation cocktail as described in the materials and methods. We did not use 1 μM PD03, as we previously findings showed that higher PD03 concentrations (1 μM) induce cell differentiation (Supplementary Fig. 1g, h).
This PD03-dosage treatment approach was designed to address the following key concerns:

1. Effect of dosed ERK inhibition on lineage priming in EpiSCs.
2. The link between CLDN6, PD03 dosage (ERK dosage) and PBX.

1. **Effect of dosed ERK inhibition in EpiSCs on lineage priming in EpiSCs**.
We treated EpiSCs with different dosages of PD03, and differentiated them into neuroectoderm and APSD (anterior primitive streak derivates: neuromesodermal progenitors (NMPs) and definitive endoderm (DE)).

- **Effect on Neuroectoderm Fate.** Focusing on neuroectodermal fate decisions, dosed (0.1 μM and 0.5 μM) PD03 treatment in EpiSCs resulted in a decrease in neuroectoderm markers (*Pax6* and *Otx2*) upon directed differentiation. Interestingly, the anterior neural (midbrain and forebrain) marker *Otx2* was greatly reduced in neuroectoderm-differentiated PD03-treated EpiSCs. This experiment suggests how ERK signalling could prime anterior neural identity within the epiblast state prior to directed neuroectoderm differentiation. These findings are now included in **Supplementary Fig. 3c**, and discussed in the manuscript.

- **Effect on APSD (Anterior Primitive Streak Derivatives).** We also differentiated untreated and PD03-treated EpiSCs into APSD to examine the impact of ERK inhibition on lineage bias. We saw that treatment with 0.1 μM and 0.5 μM doses of PD03 in EpiSCs, lead to decrease in *Foxa2* expression (DE marker) and an increase in *Tbx6* expression (NMP marker) upon APSD differentiation. This experiment suggests how higher ERK levels prime DE fate bias over NMPs in the epiblast stage, prior to directed APSD differentiation. These findings are now included in **Supplementary Fig. 3d**, and discussed in the manuscript.

2. **The link between CLDN6, PD03 dosage (ERK dosage) and PBX**.
We treated EpiSCs with different dosages of PD03 and we compared them to untreated *Pbx1-KO* EpiSCs. We showed that increasing PD03 dosages, led to a subsequent decrease in *Cldn6* expression, in both WT and *Pbx1-KO* EpiSCs. Untreated *Pbx1-KO* EpiSCs have comparable *Cldn6* levels to 0.5μM PD03 treated EpiSCs. This experiment provides additional evidence linking PBX, ERK and *Cldn6* levels and has been included in **Supplementary Fig. 5j**.

FGF Signalling and *Pbx-KO* Phenotype
To further address Reviewer 2's comment regarding the link between FGF signalling and the *Pbx1-KO* phenotype, we conducted additional experiments (**Supplementary Fig. 7**) where we differentiated WT and *Pbx1-KO* EpiSCs towards APSD fates and analyzed the lineage outcomes. Our results showed that *Pbx1-KO* EpiSCs exhibit reduced expression of definitive endoderm (DE) markers, consistent with the observations from PD03-treated EpiSCs (**Supplementary Fig. 3d**). Furthermore, similar to PD03-treated EpiSCs, *Pbx1-KO* EpiSCs showed an increased formation of NMPs (**Supplementary Fig. 7**), but a reduction in PSM markers. These findings indicate that in the epiblast, PBX-ERK primes initial germ layer specification (including NMPs) via DNA methylation.

This observation aligns with our previous report, where we described an accumulation of NMPs in *Pbx-KO* cells along with a block of *Pbx1-KO* NMPs differentiation into PSM. We previously showed that in the NMPs, PBX interacts with HOX proteins to regulate LEF1-guided WNT-driven presomitic mesoderm (PSM) differentiation (Mariani *et al., Nature Communications,* 2021).
In the revised manuscript, we further elaborate on the pleiotropic role of PBX proteins, highlighting their function in the epiblast (via HOX-independent ERK signalling) and during NMP differentiation (via HOX-dependent LEF1/WNT signalling) (**Supplementary Fig. 7**).
In the revised manuscript, we provide an explanation for the phenotype of NMP accumulation observed in the *Pbx-KO in vivo* and *in vitro* tailbud models, further clarifying PBX's role in regulating lineage commitment.
We thank the reviewer for suggesting these experiments that connected the current work with our previously published report, offering a broader vision into the multifaceted role of PBX in regulating lineage specification.

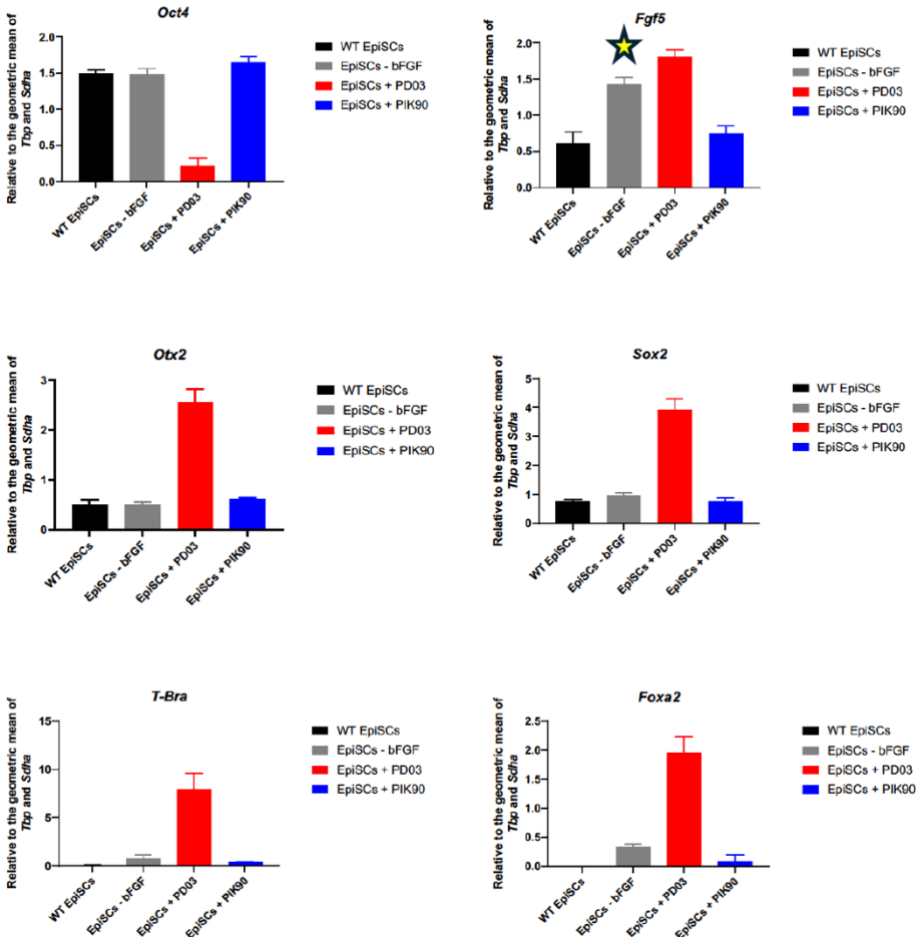


Figure 1. PI3K Inhibition Does Not Replicate ERK Effects in EpiSCs. WT EpiSCs (black bar) and PIK90-treated EpiSCs (blue bar) exhibited similar levels of epiblast (*Oct4* and *Fgf5*) and lineage differentiation markers (Neuroectoderm *Otx2* and *Sox2;* APSD *Tbra* and *Foxa2*). Treatment of high dose of PD03 (1 μM) results in spontaneous differentiation, marked by increased levels of *Otx2*, *Sox2*, *T-Bra*, and *Foxa2*. Thus, PI3K Inhibition Does Not Replicate ERK Effects in EpiSCs. Omitting bFGF (indicated by a star *), as initially suggested by Reviewers 2 and 3, leads to the upregulation of *Fgf5* ligands, with some cells undergoing spontaneous differentiation, as shown by elevated levels of *T-Bra* and *Foxa2*. This result show that ERK inhibition feeds back on FGF signalling in the epiblast state.

Reviewer #3 (Remarks to the Author):

This response to our prior reviews offered many explanations but few changes based on my and other reviewers' concerns about cell signalling connections. I still do not agree that the manuscript demonstrates an effect through ERK signalling. There are correlative data (signatures associated with ETS factors; correlations between dpERK and claudin), but no causal results that establish changes driven by ERK. Additionally, the authors' responses to my previously raised concerns argue that the Wnt and BMP signalling dependencies are that these connections should simply be known/assumed from literature, and are not directly demonstrated in this study. I am still not comfortable with the authors' conclusive statements in the title, abstract, and summaries of experiments that make claims about these discoveries (e.g. "Erk-driven lineage specification"; "chromatin accessibility and methylome states prime BMP-dependent fate decisions", "DNA methylation… safeguards WNT response").

We genuinely appreciate the constructive feedback and suggestions provided by the reviewer. In response to their comments, we have revised the manuscript by toning down the causal claims and refining the title, abstract and overall narrative.
We are also grateful to the reviewer's suggestion to conduct PD03-dosage experiments to further assess causal effects driven by ERK. These new experiments, performed using a well-established ERK inhibitor have provided additional depth and understanding to our findings, helping in connecting CLDN6 sorting and PBX genetic models to ERK signalling.

PD03-treated and *Pbx1-KO* EpiSCs (both with reduced ERK), have comparable CLDN6 levels
To address Reviewer 3's major concerns, regarding the link between CLDN6 and ERK signalling and *Pbx1-KO* models, we performed the suggested PD03-dosage experiments. Since a high dose of PD03 (1 μM) induces cell differentiation (Supplementary Fig. 1 g, h), we treated EpiSCs with lower doses of the MEK/ERK inhibitor (PD03) 0.1 μM and 0.5 μM, and succeed to maintain them under these conditions for multiple passages. Our results showed that EpiSCs treated with 0.5 μM PD03, which have lower ERK, had CLDN6 levels comparable those of *Pbx1-KO* EpiSCs. These findings demonstrate a link between ERK signalling dosage, PBX and CLDN6 levels (**Supplementary Fig. 5j**).

PD03-treated, CLDN6^{Low} and *Pbx1-KO* EpiSCs have similar phenotypic output: Demonstrating an effect on lineage priming through ERK signalling
To further investigate the role of ERK on lineage priming, we treated EpiSCs with different doses of PD03 (0.1 μM and 0.5 μM) and kept them in culture for multiple passages. Before directed differentiation, we withdrew PD03 and differentiated the cells into neuroectoderm and APSD (anterior primitive streak derivatives, including neuromesodermal progenitors (NMPs) and definitive endoderm (DE)).
Since we treat the cells at the epiblast stage, any bias observed in germ layer lineage differentiation is a consequence of ERK-driven priming in the EpiSCs, induced by PD03 inhibition. This would mean that the changes in lineage outcomes are not due to later germ layer differentiation cues, but rather stem from the effect of ERK signalling modulation during the epiblast stage.
Our findings showed that ERK inhibition in EpiSCs resulted in a primed state that recapitulated the differentiation pattern seen in CLDN6^{Low} and *Pbx1-KO* cells:

- PD03-treated EpiSCs formed fewer DE cells upon APSD differentiation, similar to CLDN6^{Low} EpiSCs (Fig. 2) and *Pbx1-KO* EpiSCs (Supplementary Fig. 7).
- PD03-treated EpiSCs formed more NMPs, consistent with previous findings in *Pbx-KO in vivo* and *in vitro* (Mariani *et al., Nature Communications,* 2021) and ERK-low CLDN6^{Low} EpiSCs (Fig. 2).

These results confirm that reduced ERK signalling primes epiblast cells towards different germ layer lineages, supporting our model that ERK helps in determining lineage specification choice. These

experiments are now included in **Supplementary Fig. 3c and Supplementary Fig. 3d**, and discussed in the manuscript.

Role of Signalling in Epiblast Lineage Priming

Since the manuscript focuses on lineage priming in the epiblast state, we used EpiSCs as models, along with cytokine cocktails known to direct EpiSC differentiation into specific fates.

ExM Differentiation: Focus on BMP-responsive lineage genes

We utilized differentiation cocktails with high levels of BMP to direct fate toward ExM, and WNT to guide APSD formation, in line with established findings in the stem cell biology. This approach aligns with a recent publication describing the role of BMP signalling in ExM lineage specification (Hadas *et al.,* Nature, 2024). While our differentiation protocol for ExM also includes WNT, BMP remains the primary driver of ExM differentiation, as it transcriptionally activates key ExM lineage genes (Arnold & Robertson, Nat Rev Mol Cell Biol, 2009). To explore BMP-responsive ExM lineage genes, we focused on *Hand1* (allantois fate) and *Tbx3* (yolk sac fate). The ChIP-seq analyses revealed that neither *Tbx3* nor *Hand1* loci are directly bound by PBX1 in EpiSCs or across differentiation. Our results showed that these genes exhibit distinct DNA methylation and chromatin accessibility patterns in *Pbx1-KO* EpiSCs with low ERK levels, demonstrating that PBX/ERK activity modulate alternative epigenome signatures influencing ExM lineage choices, where BMP is the key lineage inducing signal. To clarify this mechanism, we added a new supplementary Figure illustrating how alternative epigenetic signatures predispose epiblast to ExM differentiation (**Supplementary Fig. 6**).

Additionally, we have refined our discussion to focus specifically on BMP-responsive ExM lineage genes, toning down references to general BMP induction or BMP response as a whole.

APSD Differentiation: Focus on WNT-responsive NMP differentiation, and WNT-independent DE differentiation

Unlike ExM, APSD differentiation do not require BMP. WNT signalling plays a dominant role in inducing early primitive streak differentiation. Sustained WNT signalling is also crucial for NMP formation and presomitic mesoderm (PSM) differentiation (Turner *et al.,* Development, 2014; Garriock *et al.,* Development, 2014). While NMP lineage differentiation towards PSM relies mainly on LEF1-mediated WNT signalling (Mariani *et al.,* Nature Communications, 2021), definitive endoderm (DE) formation is WNT-independent (Scheibner *et al.,* Nature Cell Biology, 2022).

Our findings showed that cells with low ERK levels (CLND6^{Low}, *Pbx1-KO* and PD03-treated EpiSCs) preferentially differentiate to NMPs when exposed to WNT. Focusing on CLDN6-sorted population, we showed that LEF1-mediated WNT response is primarily on CLDN6^{Low} differentiated cells (NMPs) (Supplementary Fig. 4a). These low ERK populations exhibit reduced efficiency in generating DE, with reduced expression of *Foxa2* (Fig. 2a,b; Supplementary Fig. 3d; Supplementary Fig. d). Altogether, our work focuses on demonstrating the effect of ERK signalling on lineage priming in the epiblast, and how these differently primed epiblast cells respond to APSD differentiation medium to give rise to NMPs or DE. Using cellular models (CLDN6-sorting, *Pbx1-KO* and PD03-treated EpiSCs) and molecular approaches, (ATACseq, ChIPseq and WGBS), we find that the methylome signatures of the epiblast cells plays a critical role in predetermining their response to APSD differentiation signals, with NMP being WNT-dependent (Supplementary Fig. 4a) and DE being WNT-independent (Scheibner *et al.,* Nature Cell Biology, 2022). Overall, we have toned down our claims in the manuscript to reflect these changes.

We appreciate Reviewer 3's feedback and acknowledge the key importance of BMP and WNT roles in differentiation. However, a detailed dissection of these pathways is beyond the scope of this paper. Our study is focused on how epiblast cells respond to germ layer lineage inducing signalling cues, generating ExM when treated with BMP and APSD when treated with WNT. We understand the point of Reviewer 3, and in response to their feedback, we have adjusted our claims and revised the manuscript to emphasize the epiblast cellular response rather than a detailed dissection of BMP and WNT roles during differentiation.

As stated by me and another reviewer, Pbx perturbations don't just affect Erk but affect many other processes. Also: PI3K/Akt is not part of the MAPK/Erk cascade, as stated in the response to Reviewer 2, but is exactly one of those parallel signalling pathways that could lead to confounding results.

PI3K Inhibition Does Not Replicate *Pbx1- KO* or ERK-Dependent Phenotype

We thank the reviewer for the clarification, we agree that PI3K/AKT is not part of the MAPK/ERK cascade. It may stem from a misunderstanding, but we never mentioned that PI3K/AKT is part of MAPK/ERK signalling. We apologize for the lack of clarity in the manuscript. NGS data analysis revealed there was no effect on PI3K/AKT in *Pbx1-KO* mutant (RNAseq data included in paper). This observation was confirmed by the immunoblot of PI3K in WT vs *Pbx1-KO* EpiSCs (Figure 2C, below). Furthermore, GO enrichment analysis from the RNAseq, ChIPseq and ATACseq, along with EMSA, consistently indicate that MAPK/ERK signalling is the primary pathway in which PBX is involved in the epiblast, with no significant enrichment of alternative signalling pathways.

It is important to note that while both MAPK/ERK and PI3K/AKT are downstream of FGF signalling, they operate in parallel. To further address Reviewer 3's concerns on the role of PI3K signalling in the epiblast state, we treated EpiSCs with PIK90 (PI3K inhibitor). Unlike the effects observed upon PD03-treatment, CLDN6-sorting, or in *Pbx1-KO* EpiSCs, PIK90-treated EpiSCs behaved similarly to WT EpiSCs (Figure 1, above, Answer to Reviewer 2). WT EpiSCs (black bar) and PIK90-treated EpiSCs (blue bar) exhibited similar levels of epiblast and lineage differentiation markers. Thus, PI3K inhibition does not replicate the ERK inhibition or *Pbx-KO* phenotype in EpiSCs. While informative, we did not add this figure to the manuscript to maintain the message and focus on ERK signalling in a simpler manner. However, if Reviewer 3 feels it would add value to the manuscript, we are happy to include the figure and discussion in the manuscript.

In contrast, I am very convinced by the authors' perturbations to Pbx proteins, sorting of Claudin proteins, and assessment of chromatin state and accessibility. I would be very happy to see that data published without further revisions, but am disappointed that the manuscript can't rest on those demonstrated results but instead has to expand claims to shakier ground.

We sincerely thank Reviewer 3 for their positive assessment on our extensive data and work on *Pbx*, *Cldn6* and the underlying molecular mechanisms. Their recognition of our efforts is greatly appreciated and reinforces the significance of our findings.

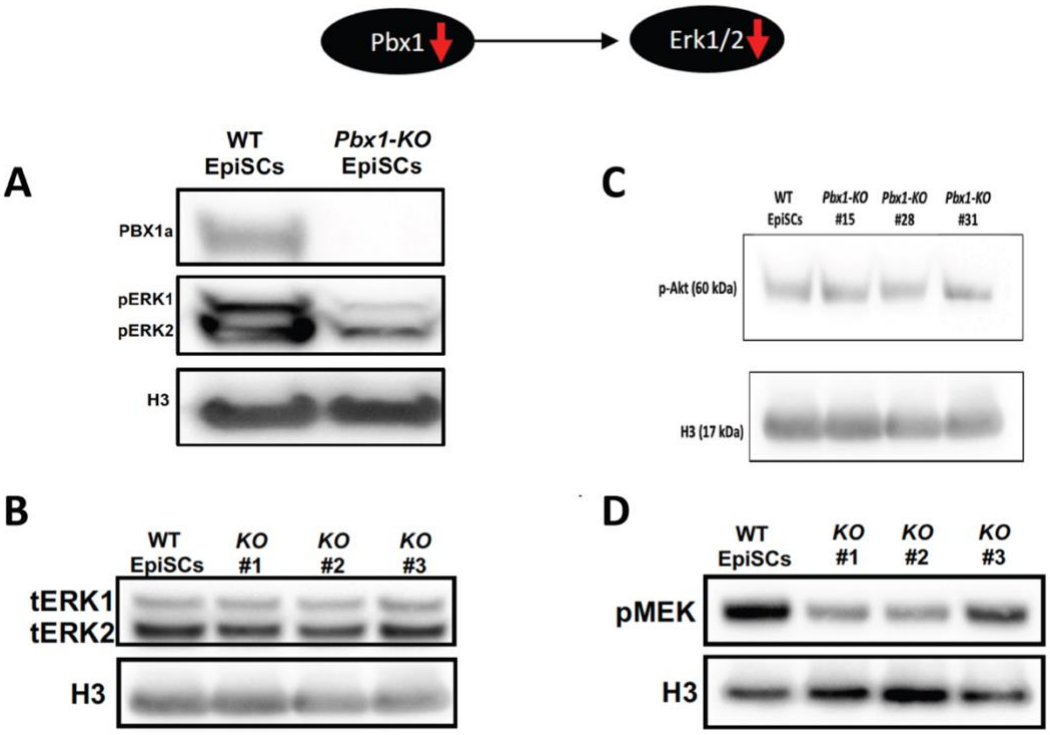


Figure 2. Loss of *Pbx1* results in reduced pERK activity without altering tERK and pAKT levels. (Modified from Fig 4c, and Supp Fig 5h-I in the manuscript). **A**) Western blot analysis confirms the absence of PBX1 and reduction of pERK1/2 in *Pbx1-KO* EpiSCs. Western blot analyses showing. **B**) unchanged levels of total ERK1/2 proteins and pAKT **C**), and reduced levels of pMEK **D**) in three *Pbx1-KO* EpiSC cell lines compared with WT.