Mechanisms underlying WNT-mediated priming of human embryonic stem cells

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MS TITLE: Mechanism Underlying Wnt-Mediated Acquisition of Cellular Memory in Human Gastruloids

AUTHORS: Anna Yoney, Lu Bai, Ali Brivanlou, and Eric Siggia

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The referees provide constructive suggestions for clarifications and improvements to strengthen the study. In my view, there are three major issues that should be addressed. First, all three referees wanted further evidence to support the conclusions from the Smad2 binding assays. Second, the referees would also appreciate further evidence to support the claim that Eomes substitutes for WNT signaling. Finally, more clarity on the quantitation and statistics of several of the experiments is needed.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this paper, Yoney et al., investigate the mechanism underlying Wnt and Activin signaling pathways cooperation in the induction of ME genes in hESCs. The authors claim that Wnt-induced EOMES is necessary to establish the Wnt-mediated acquisition of cellular memory, necessary for the cooperation with Activin:SMAD2/3. The analysis of the cooperation between these two pathways have been extensively studied in hESCs and several molecular mechanisms have been elucidated. The idea that EOMES is the effector of WNT priming is somehow novel.
However, in order to support this claim, a substantial increase in the amount of experiments is needed. In the current manuscript, this claim largely relies on the experiments in Figure 7C and 7D, where authors overexpress EOMES and examine the expression of ME genes in the absence of WNT treatment. They analyze 6 genes by qPCR (ME and DE markers) and found that overexpressing EOMES is sufficient to increase their expression in ACT-treated cells. However, more studies are needed to support that the EOMES drives ME or DE lineage differentiation in the absence of WNT. Furthermore, authors claim in the title that they found a new mechanism in human gastruloids, but no gastruloid analyses are shown in this study. There are also issues related to the experimental approach, such as lack of statistical analysis in most of the figures. Also, there are quantifications of immunostaining signals but the images are not shown (not in the main or supplemental figures).

Comments for the author

Major points:

- Increase the number of biological replicates (most figures have 1 or two replicates) for key findings in main figures and add appropriate statistical analysis.

-There is insufficient data to demonstrate that EOMES can substitute WNT in the induction of ME fate in hESCs. More functional analyses are needed to proof this claim. For example, performing a RNAseq analysis in EOMES overexpressing cells (and compared to WNT-treated cells) will help to support the claim. Phenotyping characterization of EOMES-expressing cells by immunostaining or by assessing differentiation capacity toward mesoderm derivatives may also help to support this claim.

- Authors should either eliminate the word gastruloid in the title or include gastruloid analysis in the paper.

-Figure 1D: The authors quantify intensity of IF signals and plot it in Histograms. This quantification method seems unnecessary complicated, and it makes difficult to assess the differences in the expression of some of the markers analyzed. For instance, for the expression of EOMES, what is the conclusion between Wnt+SB->E7+SB->ACT and WNT->ACT? The authors claim that adding Activin at later stages still sufficient for Wnt to prime the genes, so Wnt+SB->E7+SB->ACT and WNT->ACT should have similar levels of EOMES. However, this type of representation is not clear to make this comparison; I was not able to discern whether there is equal, more, or less EOMES expression in the +SB versus -SB condition. Moreover, there are not statistical analysis or error bars. Besides only the quantification is shown without a representative image, which makes even more difficult to assess these results. Including the corresponding images and updating the quantification graph will help to interpret the data.

-Figure2: The ChIP-qPCR analysis of Smad2 shows very low enrichment of the specific signal in the positive regions versus negative control region. Moreover it shows high variability between the 2 technical replicates that are plotted.

Then, it is difficult to assess whether the Smad2 binding was equal or different in Activin versus Wnt+Activin condition. I would recommend to increase the number of replicates to show more robust results.

-Figure5B-C: There may be a potential effect of the IWR1 for some of the genes in the 0h treatment, but the effect is very mild and the lack of statistics makes even more difficult to assess the effect of the IWR1 treatment. Furthermore, I think there is a confusion with the description of this figure in the text :
"Application of this drug together with Wnt completely abolishes ME differentiation (Yoney et al., 2018). When endo-IWR1 was added immediately after Wnt treatment (0h) together with Activin, the induction of some ME genes was reduced. This effect was much more attenuated when added at later time points (4h 8h, and 12h; Figure 5B, C). Importantly, marker genes for definitive endoderm (DE), including SOX17, GATA4, and FOXA2, were also induced in the presence of endo-IWR1, indicating that hESCs can differentiate into more mature endoderm after the elimination of β-Catenin." Thus, what is the conclusion about the role of IWR1? Does it increase endoderm genes?

-Figure 7C: It is difficult to assess the mild differences in the expression of key marker genes when only two replicates are shown and the WNT/ACT point (that is used as control to compare the expression levels) is missing in some of the graphs and timepoints. WNT/ACT points should be included in all timepoints and more replicates are needed.

-Figure 7D: The effect of IWR1 shown in Figure 5 is so weak that is difficult to assess whether EOMES is able to compensate for its effect in Figure 7D. I would recommend to add more replicates, add statistics, and I would combine the Figure 5B and 7D graphs to facilitate comparisons.

Minor points:

Figure 1C: it would be nice to add the name of some relevant genes close to the heatmap.

Figure S1B/S1D: Can the authors explain why the EOMES, BRA and GSC markers do not colocalize in the ME cells? I would assume that these markers partially colocalize in the ME cells.

Figure3A: The authors say: "There are quantitative changes in the ATAC signals over specific TF binding sites (one example is shown in Figure 3A for MIXL1)" , but what is the specific TF site on MIXL1 that authors refer to?

Figure S4B: It would be nice to include an image of EOMES to show the right localization of the overexpressed protein.

Also, I am not sure about the difference between Figure 7C and S4D. Is it on the length of treatment?

Figure S4D: What is the difference between TT-EOMES and EOMES? Did authors use different primers to amplify exogenous and endogenous EOMES mRNA? If so, why TT-EOMES is induced by ACT (with no Dox)?

In line 273: Authors say that BRA is not essential for ME differentiation.
However, BRA KO mice die after gastrulation due to mesoderm defects. I would recommend to eliminate/modify this sentence.

Reviewer 2

Advance summary and potential significance to field

Yoney et al explore a long-held question in developmental biology – how do the WNT and Activin/Nodal (ACT) signalling pathways promote the generation of ME from pluripotent cells? In agreement with their previous work, they demonstrate that the generation of ME-like fates in vitro relies on exposure of human ESCs first to WNT signalling, followed by Activin, in order to promote

the induction of genes such as Eomes, Bra and Gsc. They demonstrate that this transition does not appear to proceed by either signal alone, or in the reverse order. They provide evidence that a defined period of WNT exposure is required to generate ME, and that this involves the activation of Eomes, a well-established regulator of ME differentiation. Eomes can promote ME differentiation, although the mechanism remains unclear.

Comments for the author

Overall, this study aims to address an important question in developmental biology – how do extrinsic signals, namely, WNT and Activin (ACT), promote the differentiation of pluripotent cells to a ME identity? The paper is well structured and written. However, there are some areas that require clarification, to help interpret the data that is presented.
(1) The mRNA-seq data attempts to provide a global overview of the genes that are induced following WNT, and those that define ME identity. This sets up the entire paper and the analysis strategy used. The authors could improve readability and interpretation of these findings by annotating the gene sets that define each cluster on the heatmap. In particular, cluster 3 in Fig1B shows a set of genes that are increased in WNT/ACT vs WNT or ACT alone conditions.
This cluster appears to include two different subclusters – a set of genes which are expressed higher in WNT/ACT (relative to all other conditions) as well as a second set that appears to represent a unique set of genes that are only expressed following WNT/ACT conditions (bottom half of cluster 3). What genes and pathways/processes are enriched in each of these two subclusters, and to what extent do the expression signatures presented relate to previously published mRNA-seq datasets, such as Tsankov et al 2015? The later would provide support that the conclusions from the re-analysis of previously published ChIP-seq data are not driven by differences in the culture conditions (where there are notable differences in ESC culture with mTeSR and the differentiation strategy used).
(2) The authors show that SB treatment with WNT appears to generate a more homogenous population of Eomes and Bra expressing cells in the population albeit at a lower level of expression (Figure 1D). Thus, a low level of activin appears present in the culture, and influences the expression profiles observed.
It is therefore difficult to strictly conclude that WNT/beta-catenin acts alone upstream of activin to specify ME fate (line 102-104) as the data collected from qPCR and mRNA-seq analyses do not include SB treatment.
(3) The ATAC-seq data generated in this study suggests that the transition to an ME-like identity does not appear to result in any global changes in chromatin accessibility. A potential explanation for this is that the culture conditions may generate a mixture of cell types following WNT/ACT treatment. This may explain why only select regions appear to show changes in accessibility, as the exit from pluripotency may be difficult to control, leading to a range of cell states being captured in the population. The authors should use single cell methods, such as IF or flow cytometry, to quantify the proportion of cells in the culture that express ME markers, in response to WNT/ACT treatment.
(4) I am not sure it is possible to conclude that “enhancement likely results from SMAD2 binding and subsequent opening of the local chromatin” (line 215).
The differences presented as quantiles in Figure 1E seem to represent the peaks called from the ChIP-seq data, (as the global view in 1D appears similar). Do these regions represent all SMAD2 binding sites from each ChIP-seq dataset or a different subset? What is the genomic distance represented by each quantile? How the engagement of SMAD2 directly relates to the accessibility profiles observed at these sites, is not clear. Footprinting methods may enable predictions of which factors are engaged with the chromatin over the different conditions.
(5) The treatment with endo-IWR1 is an important addition to the study, to conclude that Eomes induction does not depend on WNT/beta-catenin. However, it is not yet clear in these experiments if the endo-IWR1 treatment is effective in promoting the degradation of beta-catenin, the proportion of cells affected, and the time scale in which this occurs. This needs to be presented to interpret the data from the TT-Eomes experiments, and to be able to conclude that WNT signalling is not required. Please provide IF images and quantifications over the time course to demonstrate the timing at which nuclear beta-catenin is lost following endo-IWR1 treatment.

Minor comments:

Figure 1D. The x axis for Eomes is extremely different to BRA and SOX2 – could the authors please verify if this is correct. It is also difficult to interpret from these plots what level is considered background staining versus a low level of expression.
Please indicate the minimum and maximum range used to display BigWig tracks in Figure 2A, Figure 3A.
Figure 6B – it was not clear to me why the 20kb range was specifically set to examine ATAC-seq peaks of WNT-primed genes.
Please define what is meant by "enhanced" ATAC peaks
Line 270. "EOMES is activated by Wnt alone and becomes highly expressed with Wnt priming followed by Activin". The induction of Eomes is very low in WNT alone conditions in Figure 1B qPCR, relative to the WNT/ACT condition. If Eomes can promote its own expression, why is there very little Eomes detected after 24hrs of WNT treatment?
Line 309. "These results strongly suggest that EOMES is the effector of Wnt priming…" The data support that EOMES is 'an' effector, but are not able to rule out other factors. Please amend this statement.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Yoney and colleagues addresses the important question of the signal integration of the two major pathways, canonical Wnt and Nodal/TGFbeta for induction of mammalian gastrulation. They apply hESC differentiation and base the current study on previous work that demonstrated that signalling activities of Wnt and TGFbeta can be temporally separated so that a primary Wnt-treatment is sufficient to provide competence/prime cells to differentiate to Mesendoderm cells following a secondary TGFbeta stimulation.
The authors demonstrate that rather than direct interactions at the level of Wnt- and TGFbeta-signalling pathways, the effect of Wnt-priming is mediated by the induction of Eomes that together with Smad2 induces ME gene expression.

This key message represents a very interesting and compelling finding that contributes to resolve the initial steps of Mesendoderm differentiation during vertebrate gastrulation. The expression data that demonstrate the stepwise, successive induction of differentiation by Wnt and Activin are convincing. However, the molecular analyses that should demonstrate (the negative result) that there are no direct interactions of Wnt- and TGFbeta signalling pathways on the level of chromatin leave significant doubts and questions.
The key finding of a stepwise induction of Eomes expression which then acts in concert with Smad2 to activate ME gene expression was not analysed in the current manuscript even though the ChIP and ATAC data files are available and this key positive finding could be described in more depth.
At several places within the manuscript important statements are not substantiated by data as described in more detail below (major and minor concerns). This might simply reflect a lack of the associated data analysis files in this manuscript since the experimental data are available and relevant analyses could be easily included.

In conclusion, I find this a highly relevant and important study with an appealing conclusion. However, the key conclusions have to be based on more careful data analyses and data representation. I suggest to include additional analyses into a majorly revised manuscript that allows to draw the relevant conclusions made by the authors.

Comments for the author

Major concerns:
1. From Figures 2 and 3 the authors conclude that Wnt-signals don't alter Smad2 binding and activities directly. They perform a very global analysis of Smad2 binding in ESCs and ME cells from different published data sets to conclude that Smad2 binding is mostly unchanged between both states. However, this broad analysis is not very convincing and requires some more depth to draw this conclusion. Especially, since one of the papers from which data were used (Kim et al 2011),

describes major differences in SMAD2 binding between ESCs and endoderm. Why do the authors use data sets from different publications if the work by Tsankov et al. contains data for both ESCs and ME? I suggest to compare these two datasets to see if there are changes at relevant sited of Mesendoderm target genes? The authors should plot SMAD2 binding in vicinity of genes whose expression changes mostly during Mesendoderm differentiation (most highly induced in Wnt/Act conditions, or overlaps of beta-Catenin and Smad2 binding) and not perform a global, genome-wide analysis. Most likely the authors will find differences in Smad2-binding at genes with biological significance, including Eomes. So, I suggest that the authors perform some additional analysis to further enforce their findings, that I find highly surprising.

2. The key finding is the regulation of Eomes by Wnt which primes cells to respond to Smad2 using Eomes as a co-factor. However, the aspect of Eomes-regulation by Wnt is not addressed in the manuscript, despite availability of relevant data of ChIP-seq (Smad2, beta-Catenin) and ATAC-data. Please provide the analysis that shows the regulation of Eomes by beta-Catenin, which is further enhanced by Smad2. What is the effect of Wnt-stimulation that primes the Eomes locus to respond to Smad2? This regulation should look considerably different to the regulation of following mesendoderm genes and could thus serve to validate the analyses in Figures 2 and 3.

3. The manuscript uses the term “mesendoderm” very generally but it isn’t defined what mesendoderm refers to precisely. The authors should please define this lineage more clearly (by markers), especially since they claim that the mesendoderm lineage does not rely on Brachyury but on Eomes functions (lane 273 -275). However, both T-box factors seem to compensate for each other, and also Eomes is dispensable for the generation of mesendoderm cell types from mESCs in vitro since EomesKO ESCs can express markers like Mixl1 and Lhx1 (Tosic et al. 2019).
So, I suggest to clearly describe the lineage decision they are referring to and indicate relevant markers for this lineage also in the Figures, e.g. the heatmap of Figure 1C. Generally, throughout the manuscript only very few markers were applied but quite general statements are made about transcriptional outputs. Here, gene tables should be provided, such as for (Figure 1) 189 genes changed after 24 hrs of Wnt, 661 up and 383 down after Wnt/Act treatment, 113 Smad target genes …

Minor Comments:
Figure 1:
A, B) Please label the scheme of hESC treatment (Fig. 1A) and place the scheme and the data in the same order (Fig. 1B, C). This current presentation is unnecessarily confusing.
C) Heatmaps without any labelling are little informative. The authors should please indicate some representative genes in the clusters and annotate the clusters. At least the most induced genes of cluster 3 should be shown with some more detailed information.
In the results section (lanes 110 ff) groups of regulated genes are described. These should be provided in a table and possibly also some examples indicated in the heatmap.
It is indicated that this experiment was conducted as n=2. How could they calculate p-values by only 2 replicates?
Lane113: Please indicate mentioned genes, including Eomes, Bra and Nodal in the heatmap.
D) IF of nuclear staining seems like an unnecessary complicated way of measuring gene expression of TFs.
Why not simply using qPCRs as performed in Figure 1B. This would also allow for time-resolved measurements which would be more informative, such as in resolving why Sox2 remains highly expressed in Wnt+SB-E7+SB-Act conditions in Figure 1D-
Why are a.u. once given in 1,2,3… and then as 1000, 2000, 3000?
Figure 2:
A) As described above the analysis is not convincing to show that there are no differences in SMAD2-binding between conditions. Especially, since there are already some differences visible in the few examples shown (e.g. Cer1), however, at relatively low resolution. Other available datasets (Tsankov et al) should be analysed from experiments that are more comparable.
Please indicate the scale of genomic regions and counts at peak heights.
In the text (lane 168 ff) expression changes of these genes are described but not shown.
B) This data representation of SMAD2 peaks is very difficult to interpret. Does the R-value of R = 0,81 indicate similarity or differences? Selecting and analysing the regions that are associated to relevant ME genes might resolve larger differences that are not visible in the current analysis.
D) The few examples of (not clearly indicated) regions of some ME genes are not very convincing if the goal of these experiments is to show no changes in SMAD2 binding.
Figure 3 (also discussed as major concern)

This is a very global analysis of ATAC at beta-Catenin and SMAD2 bound regions, and the interpretation seems biased. The minor differences at beta-Catenin bound regions are considered significant, while changes of ATAC at SMAD2 bound regions following Wnt-stimulation are considered non-significant.
However, chromatin at SMAD2 bound regions is generally more accessible and (biological) significant changes might be masked in this analysis where SMAD2 bound regions were chosen that are present in ESCs.
This might also explain the counter-intuitive result that SMAD2-binding should be inversely correlated with chromatin accessibility (Figure 3D).
A) Please show relevant sites of the Eomes locus as reference for a gene that should show some change in locus control. What are the effects of beta-Catenin and/or SMAD2 on Eomes expression?
In the text it is described that there are quantitative changes on ATAC signals over specific TF binding sites (Lane 196f).
Where is this found? Which genes are associated to these sites? Is this also found at the Eomes locus? The given example at the Mixl1 locus is not very striking.
B, D) Please indicate how many peaks are analyzed, and indicate in the Figure legend the conditions of the experiments where the Beta-Catenin binding sites are taken from.
C) Described changes of ATAC signals in response to different treatment regimes are hardly visible. For the ATAC-peaks that are most strongly bound by beta-Catenin please show some examples and indicate which group of genes they belong to. Please indicate associated genes and show additional examples of changes in ATAC peaks for relevant target genes. The given example of changes in Mixl1 ATAC is hardly visible and there can be considerable doubts that this type of change in ATAC peaks is really relevant and biological significant, even if there is a statistical significance. The indicated peak at the Mixl1 locus (Figure 3A strongest peak in WNT) doesn't follow the general pattern of ATAC peaks (Figure 3C, strongest in WNT/ACT).
So why did the authors choose this peak over others?
Please show the Eomes locus to demonstrate the Wnt-priming effect, if there is one.
D) The most obvious pattern in all ATAC tracks is an inverse correlation of Smad2-binding with ATAC signals.
What does this mean?
C,D) Lanes 210 ff in the text states that ATAC signals over Smad2 sites remain identical in E7 and WNT conditions is rather surprising and requires further analyses. Are the Smad2 sites analysed identical in both conditions ESCs and ME cells? It is very likely that the sites are NOT identical but gain similar ATAC-signal strength. Please directly compare identical sites.
Lane 214: The corresponding Figures are Figure 3D, E (not C, D!)
E) Please indicate a quantification of overall ATAC-signals in 5 conditions (Fig. 3B, D). The changes observed in the analysis of Figure 3E are not obvious in Fig. 3D.
B,D) What are the numbers of peaks analysed?
Figure S3:
What is the value of this information/graph? Is this small fraction of genes meaningful?
Figure 4:
C) Again (compare comment Figure 1), it is unclear, why IF was used to measure gene expression which most likely is inferior to quantitative measurement of gene expression in comparison to qPCR.
Figure 5:
C) This part of the Figure is not discussed in the text.
Figure 6:
C) It is unclear to me why this graph indicates the probability of Wnt/Act enhanced peaks. Doesn't the analysis correlate the presence of Wnt/Act enhanced peaks to Wnt-sensitive/insensitive genes? So, this "probability" rather reflects a calculated value and could be labelled accordingly.
C, D) What is the overlap of ATAC peaks in Figure 6B and the EOMES motif, or Eomes-binding? How often is overlap found/not found?
Which other TFs are found? Please provide full list of TF motif analysis.

First revision

Author response to reviewers' comments

We would like to thank all three reviewers for thoughtful criticisms and suggestions. We have addressed each of the reviewers' concerns and comments, which we believe have significantly enhanced the clarity of our manuscript. The following is a detailed point-by-point reply (blue text) to each comment (black) from the reviewers. We underlined all the sentences describing the changes made to the manuscript. In the manuscript, the changes were highlighted in red.

Reviewer 1 Comments for the Author:

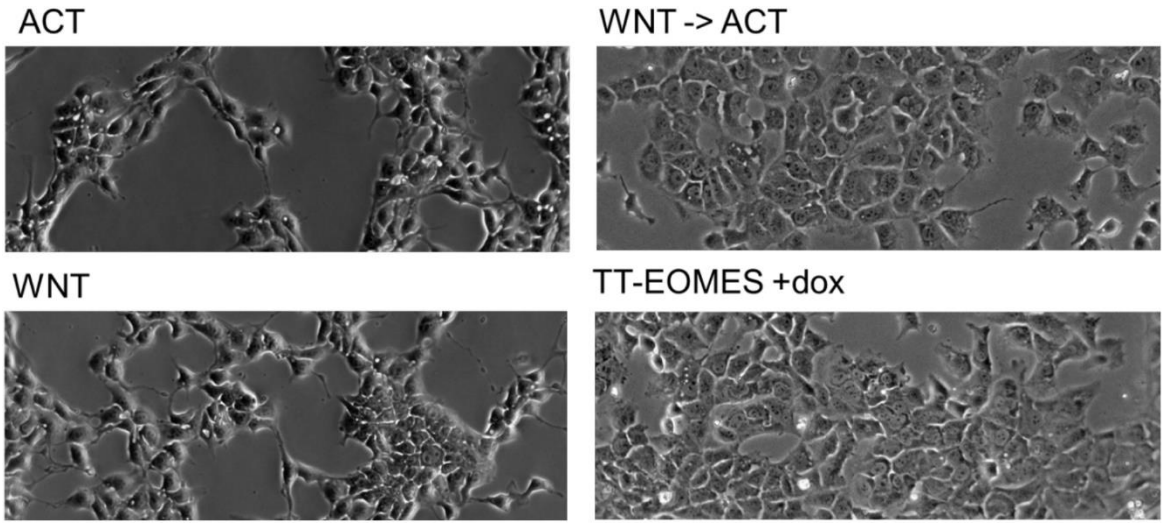
Major points:

- Increase the number of biological replicates (most figures have 1 or two replicates) for key findings in main figures and add appropriate statistical analysis.

We have added additional replicates, data, and statistics where possible given the time constraints and resources.

-There is insufficient data to demonstrate that EOMES can substitute WNT in the induction of ME fate in hESCs. More functional analyses are needed to proof this claim. For example, performing a RNAseq analysis in EOMES overexpressing cells (and compared to WNT-treated cells) will help to support the claim. Phenotyping characterization of EOMES-expressing cells by immunostaining or by assessing differentiation capacity toward mesoderm derivatives may also help to support this claim.

Cells that express TT-EOMES show similar "flattening" morphology that we saw in WNT/ACT condition (see below and added to Figure S7). The key ME developmental genes that we tested (endogenous *EOMES*, *GSC*, *SOX17*, *FOXA2*, *GATA6*), except *BRA*, are all upregulated 10^2 – 10^4 fold, comparable to the WNT/ACT condition (Figure 7C). We agree with the reviewer that RNAseq measurement will be more convincing, but unfortunately we could not find someone to perform this experiment since both Anna and Lu left the original lab.



- Authors should either eliminate the word gastruloid in the title or include gastruloid analysis in the paper.

We have eliminated the word from the title.

-Figure 1D: The authors quantify intensity of IF signals and plot it in Histograms. This quantification method seems unnecessary complicated, and it makes difficult to assess the differences in the expression of some of the markers analyzed. For instance, for the expression of EOMES, what is the conclusion between Wnt+SB->E7+SB->ACT and WNT->ACT? The authors claim that adding Activin at

later stages still sufficient for Wnt to prime the genes, so Wnt+SB->E7+SB->ACT and WNT->ACT should have similar levels of EOMES. However, this type of representation is not clear to make this comparison; I was not able to discern whether there is equal, more, or less EOMES expression in the +SB versus –SB condition. Moreover, there are not statistical analysis or error bars. Besides, only the quantification is shown without a representative image, which makes even more difficult to assess these results. Including the corresponding images and updating the quantification graph will help to interpret the data.

We have previously shown that adding SB during Wnt priming has little effect on the ability of subsequent Activin to induce ME markers (Yoney et al., 2018). In this work, we carried out this experiment again and reached the same conclusion (see Figure 1D). To further test the memory of WNT priming, we added an additional day between the WNT and ACT treatment with or without SB (WNT/-/ACT and WNT+SB/SB/ACT), and the cells still show downregulation of SOX2 and upregulation of BRA, albeit to a quantitatively lesser extent. We interpret these results to mean 1) the WNT effect decays over time, and the cells are not stably committed to the "WNT-primed" state, and 2) WNT priming effect is still available for at least a day after WNT washout.

We added representative images in Figure 1D, included violin plots in place of the histograms, and added an additional replicate in what is now Figure S3. We also added more explanation of this result in the main text on page 8-9.

Some lots of EOMES antibody had higher non-specific staining, so to correct for this the nuclear fluorescence signal was normalized to the cytoplasmic signal in the EOMES data that was included in the original submission. We removed the EOMES data from this experiment to avoid confusion since it is the only data in the original submission that was analyzed in that way. Instead, we included another experiment in which the EOMES signal did not need to be normalized and differences are obvious in the unprocessed images (see Figure S3).

-Figure2: The ChIP-qPCR analysis of Smad2 shows very low enrichment of the specific signal in the positive regions versus negative control region. Moreover, it shows high variability between the 2 technical replicates that are plotted. Then, it is difficult to assess whether the Smad2 binding was equal or different in Activin versus Wnt+Activin condition. I would recommend to increase the number of replicates to show more robust results.

Two- to six-fold enrichment in ChIP signal in Figure 2D is on the low side, but still within the typical range of ChIP signals. The two replicates presented are biological replicates, and we added the individual data onto the plot. We apologize for the mistake in the original figure legend, which stated that the replicates are technical replicates. We corrected the figure legend.

-Figure5B-C: There may be a potential effect of the IWR1 for some of the genes in the 0h treatment, but the effect is very mild and the lack of statistics makes even more difficult to assess the effect of the IWR1 treatment. Furthermore, I think there is a confusion with the description of this figure in the text: "Application of this drug together with Wnt completely abolishes ME differentiation (Yoney et al., 2018). When endo-IWR1 was added immediately after Wnt treatment (0h) together with Activin, the induction of some ME genes was reduced. This effect was much more attenuated when added at later time points (4h, 8h, and 12h; Figure 5B, C). Importantly, marker genes for definitive endoderm (DE), including SOX17, GATA4, and FOXA2, were also induced in the presence of endo-IWR1, indicating that hESCs can differentiate into more mature endoderm after the elimination of β-Catenin." Thus, what is the conclusion about the role of IWR1? Does it increase endoderm genes?

When applied together with Wnt, endo-IWR1 completely abolishes the induction of EOMES, BRA, and GSC at the protein level as determined by immunofluorescence analysis, which we previously published (Yoney et al. *eLife*, Figure 7A). We included the same measurement by RT-PCR to demonstrate that this drug strongly inhibits ME gene expression at the RNA level as well. With respect to expression of the pluripotency markers *NANOG* and *SOX2*, which are significantly down-regulated in the WNT/ACT condition (Figure 1B), we also added statistics to show that IWR1-endo added with WNT significantly blocks this down-regulation consistent with it blocking differentiation. These data are now shown in Figure 5.

The main point of Figure 5 is to test the idea that, after WNT priming, although WNT is washed away, some β-catenin may remain in the nucleus and potentially co-activate ME genes with SMAD2. When the drug was added immediately after WNT treatment with Activin (0h), the expression of ME and DE genes were indeed reduced compared to the WNT/ACT positive controls. However, when the drug was added a few hours into the Activin treatment, it no longer has any effect. The conclusion is that a short overlap between β-CATENIN and SMAD2 indeed promotes ME differentiation, but such overlap beyond a few hours is not necessary (presumably because EOMES has been fully activated by then). We clarified these points in the manuscript (page 13-14).

-Figure 7C: It is difficult to assess the mild differences in the expression of key marker genes when only two replicates are shown and the WNT/ACT point (that is used as control to compare the expression levels) is missing in some of the graphs and timepoints. WNT/ACT points should be included in all timepoints and more replicates are needed.

We respectfully disagree with the reviewer that there are mild differences in expression since the axes are on log 10 scale. Both biological replicates show that by 24 and 48 hours after Activin, marker gene expression increases 2 – 4 orders of magnitude relative to pluripotency conditions (100 – 10,000 fold). We believe that data provided support the main conclusion of the figure that sufficient induction of exogenous EOMES, i.e. with 6 h dox > 0.05 µg/mL, induces endogenous maker genes to same levels as WNT/ACT.

-Figure 7D: The effect of IWR1 shown in Figure 5 is so weak that is difficult to assess whether EOMES is able to compensate for its effect in Figure 7D. I would recommend to add more replicates, add statistics, and I would combine the Figure 5B and 7D graphs to facilitate comparisons.

Endo-IWR1 has a strong inhibitory effect on ME differentiation when added together with Wnt (now presented in Figure 5). However, when added throughout the whole course of EOMES overexpression with Activin treatment, the drug has no effect (Figure 7D). This result shows that Wnt effects can be completely bypassed with EOMES overexpression, i.e. that the effects we see from dox expression of EOMES are not mediated by Wnt.

Minor points:

Figure 1C: it would be nice to add the name of some relevant genes close to the heatmap.

A few developmental genes are now labeled on the side of the heatmap. We also included a new supplementary table that lists all the genes included in each cluster.

Figure S1B/S1D: Can the authors explain why the EOMES, BRA and GSC markers do not colocalize in the ME cells? I would assume that these markers partially colocalize in the ME cells.

ME cells express all markers, but BRA is not highly correlated with EOMES and GSC expression meaning that some cells have high BRA and low EOMES/GSC. These cells appear mostly red in the merged images (Figure S1B and S2B). We included scatter plots in Figure S1C and S2C to show the level of correlation between different markers. The cells with low BRA likely reflect a transition in gene expression, because BRA is turned off as cells differentiate from ME to DE. This is consistent with previously published observations:

D'Amour, K., Agulnick, A., Eliazer, S. *et al*. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* **23,** 1534–1541 (2005). https://doi.org/10.1038/nbt1163

McLean, A.B. *et al*. Activin A Efficiently Specifies Definitive Endoderm from Human Embryonic Stem Cells Only When Phosphatidylinositol 3-Kinase Signaling Is Suppressed. STEM CELLS, 25: 29-38 (2007). https://doi.org/10.1634/stemcells.2006-0219
This point is now discussed in the text on page 5 and 13-14 and these references have been included.

Figure3A: The authors say: “There are quantitative changes in the ATAC signals over specific TF binding sites (one example is shown in Figure 3A for MIXL1)”, but what is the specific TF site on

MIXL1 that authors refer to?

Based on ChIP data, the region with ATAC-seq signal increase upstream *MIXL1* overlaps with SMAD2 and EOMES binding sites, but not with that of ß-Catenin. The enhanced ATAC peak near *LEFTY1* and *LEFTY2* overlap with ß-catenin and SMAD2 (see plot below). Note that the ATAC enhancement near *LEFTY1* and *LEFTY2* are comparable in E7/ACT and WNT/ACT. We added this information to the main text on page 11 and included the plot below in Figure S5C.

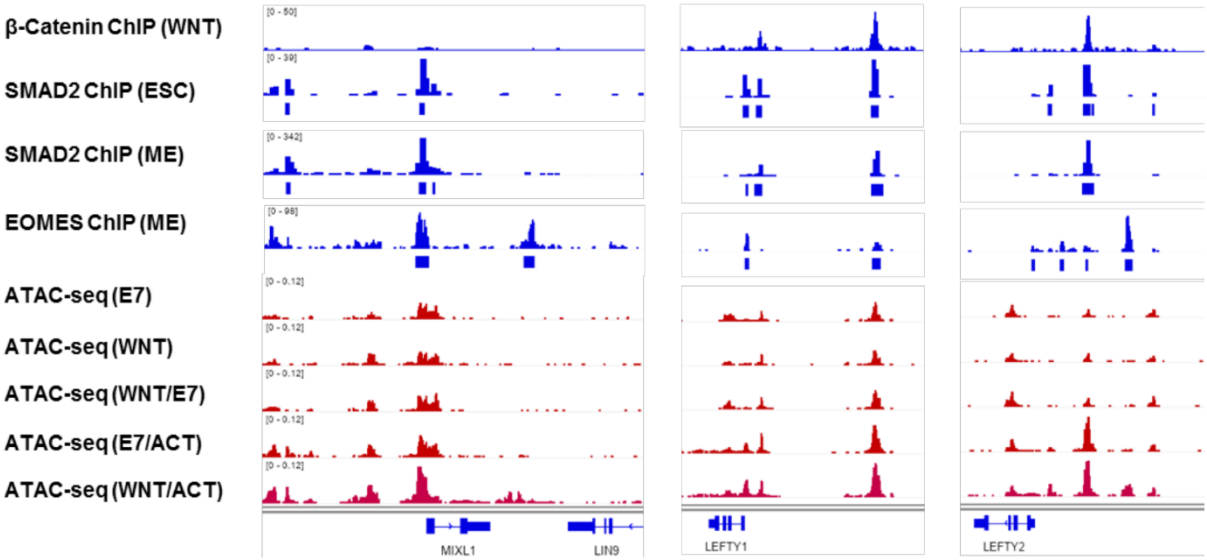


Figure S4B: It would be nice to include an image of EOMES to show the right localization of the overexpressed protein.

We have included images in the figure which is now Figure S8B.

Also, I am not sure about the difference between Figure 7C and S4D. Is it on the length of treatment?

In Figure 7C, we applied the dox with Activin simultaneously, while in Figure S4D (data now in S7C), we applied the dox alone. In the latter condition, as reported before, we found that *EOMES* expression by itself induces *MESP1*, which drives the cells towards cardiac mesoderm. In contrast, *EOMES* expression in the presence of Activin blocks this path and allows the cells to differentiate into mesendoderm. Figure S8A is used to justify the protocol we used in Figure 7A. This explanation is added to the manuscript on page 16.

Figure S4D: What is the difference between TT-EOMES and EOMES? Did authors use different primers to amplify exogenous and endogenous EOMES mRNA? If so, why TT-EOMES is induced by ACT (with no Dox)?

The reviewer is correct that we used different primers to amplify the endogenous and exogenous EOMES, which differ in their 3' UTR. We have now included a table of primers to clarify this (Table S3). To avoid confusion and provide more accurate data throughout the figures, we also removed measurements of exogenous EOMES from the unmodified parental line, in which the transcript does not exist, and any PCR amplification is non-specific, which we verified by a melting curve following RT-PCR amplification. Accordingly, we renormalized TT-EOMES expression to -dox conditions in the modified line. TT-EOMES has some background expression level in the absence of dox as we do see amplification by RT-PCR. However, the leaky transcription is not enough to differentiate the cells, and we are able to propagate this line as a pluripotent hESC line.

In line 273: Authors say that BRA is not essential for ME differentiation. However, BRA KO mice die after gastrulation due to mesoderm defects. I would recommend to eliminate/modify this sentence.

We thank the reviewer for noting this important point. We have modified the text on page 15.

Reviewer 2 Advance Summary and Potential Significance to Field:

Overall, this study aims to address an important question in developmental biology – how do extrinsic signals, namely, WNT and Activin (ACT), promote the differentiation of pluripotent cells to a ME identity? The paper is well structured and written. However, there are some areas that require clarification, to help interpret the data that is presented.

We thank the reviewer for the positive comment.

(1) The mRNA-seq data attempts to provide a global overview of the genes that are induced following WNT, and those that define ME identity. This sets up the entire paper and the analysis strategy used. The authors could improve readability and interpretation of these findings by annotating the gene sets that define each cluster on the heatmap. In particular, cluster 3 in Fig1B shows a set of genes that are increased in WNT/ACT vs WNT or ACT alone conditions. This cluster appears to include two different subclusters – a set of genes which are expressed higher in WNT/ACT (relative to all other conditions) as well as a second set that appears to represent a unique set of genes that are only expressed following WNT/ACT conditions (bottom half of cluster 3). What genes and pathways/processes are enriched in each of these two subclusters, and to what extent do the expression signatures presented relate to previously published mRNA-seq datasets, such as Tsankov et al 2015? The later would provide support that the conclusions from the re-analysis of previously published ChIP-seq data are not driven by differences in the culture conditions (where there are notable differences in ESC culture with mTeSR and the differentiation strategy used).

We used a number of clusters (8 in total) such that the final clusters do not change based on the randomized starting conditions for each run of the algorithm. For easier description, we combined three of them into cluster 3 (genes more activated by WNT/ACT than other three conditions), and another three into cluster 4 (repressed genes). We added these details to the Methods section on page 24-25. A few key developmental genes are now labeled in Figure 1C. We also included all the genes that belong to each cluster (or sub-cluster) in a supplementary table (Table S1).

We looked into the RNA-seq data in Tsankov et al 2015. Unfortunately, they only listed one excel file for the reads in mesendoderm cells with no comparison to ES cells. Direct comparison between two RNAseq read counts can be tricky, and indeed, there is only a moderate correlation between their data and ours in WNT/ACT condition (Pearson correlation coefficient r = 0.44). Correlation between their data and our genome-wide RNAseq data in other conditions yielded similar results (E7: 0.42, WNT: 0.43, WNT/E7: 0.42, and E7/ACT: 0.43). However, when we specifically examined genes that are activated by the combination of WNT and ACT, which include key genes for ME differentiation, they are much more correlated with the Tsankov et al dataset in WNT/ACT condition (r = 0.64) than all the other conditions (E7: 0.20, WNT: 0.25, WNT/E7: 0.21, and E7/ACT: 0.29). Therefore, we conclude that the change of gene expression in our experiments are consistent with that in Tsankov et al 2015.

(2) The authors show that SB treatment with WNT appears to generate a more homogenous population of Eomes and Bra expressing cells in the population, albeit at a lower level of expression (Figure 1D). Thus, a low level of activin appears present in the culture, and influences the expression profiles observed. It is therefore difficult to strictly conclude that WNT/beta-catenin acts alone, upstream of activin to specify ME fate (line 102-104) as the data collected from qPCR and mRNA-seq analyses do not include SB treatment.

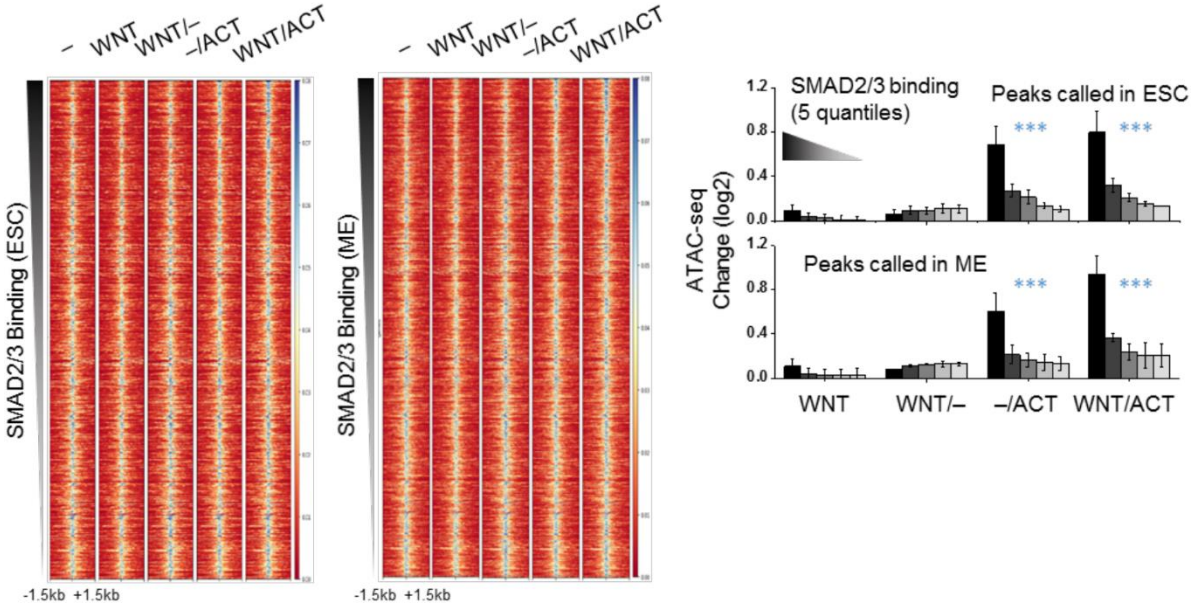
We thank the reviewer for pointing out these differences. We based the conclusion in lines 102-104 on the fact that reversing the order of the two signals (ACT/WNT vs WNT/ACT) does not produce ME and the qualitative fact that ME markers are still induced when SB is used with Wnt. However, because we do not use SB in the other experiments in this paper and may have missed other differences in the two protocols at the level of RNA, we have modified the conclusion to remove the word up-stream: "Taken together, these results demonstrate that Wnt priming through β-Catenin is required to switch the output of Activin signaling from pluripotency maintenance to ME speciation." (page 6).

(3) The ATAC-seq data generated in this study suggests that the transition to an ME-like identity does not appear to result in any global changes in chromatin accessibility. A potential explanation for this is that the culture conditions may generate a mixture of cell types following WNT/ACT treatment. This may explain why only select regions appear to show changes in accessibility, as the exit from pluripotency may be difficult to control, leading to a range of cell states being captured in the population. The authors should use single cell methods, such as IF or flow cytometry, to quantify the proportion of cells in the culture that express ME markers, in response to WNT/ACT treatment.

In a typical experiment, we observed a high number of cells with ME marker expression. From three independent replicates of the WNT/ACT condition we observed 94%, 99%, and 88% of cells with at least one of the ME makers (BRA, EOMES, or GSC) expressed at a level greater than 3 standard deviations above the average background level in pluripotency (-/ACT). Therefore, we do not expect that our bulk measurements would obscure quantitative changes in the ATAC- signal. We added this information to the main text on page 5.

(4) I am not sure it is possible to conclude that "enhancement likely results from SMAD2 binding and subsequent opening of the local chromatin" (line 215). The differences presented as quantiles in Figure 1E seem to represent the peaks called from the ChIP-seq data, (as the global view in 1D appears similar). Do these regions represent all SMAD2 binding sites from each ChIP-seq dataset or a different subset? What is the genomic distance represented by each quantile? How the engagement of SMAD2 directly relates to the accessibility profiles observed at these sites, is not clear. Footprinting methods may enable predictions of which factors are engaged with the chromatin over the different conditions.

We assume that the reviewer is talking about Figure 3D and 3E here. The regions in Figure 3D represent Smad2 binding sites from the ESCs (all binding sites are included in the plot). We also used the sites derived from the mesendoderm dataset and got very similar results (see below). This is expected because the Smad2 binding sites are similar in these two cell types (Figure 2). This information is now included as supplementary Figure S5D. In Figure 3D, each heatmap spans ± 1.5 kb, which is now labeled in the plot.



We agree with the reviewer that the plot above only shows the correlation between Smad2 binding and ATAC peak increase, not the causal relation. Nevertheless, since Smad2 enters the nucleus and binds to chromatin during the Activin treatment, it is a likely scenario that SMAD2 binding leads to further chromatin opening. Elucidation of the precise chromatin opening mechanism, which may involve nucleosome remodelers and/or histone modifiers, is beyond the scope of the current manuscript.

(5) The treatment with endo-IWR1 is an important addition to the study, to conclude that Eomes induction does not depend on WNT/beta-catenin. However, it is not yet clear in these experiments if the endo-IWR1 treatment is effective in promoting the degradation of beta-catenin, the proportion of cells affected, and the time scale in which this occurs. This needs to be presented to interpret the data from the TT-Eomes experiments, and to be able to conclude that WNT signalling is not required. Please provide IF images and quantifications over the time course to demonstrate the timing at which nuclear beta-catenin is lost following endo-IWR1 treatment.

When applied together with Wnt, endo-IWR1 completely abolishes the induction of EOMES, BRA, and GSC at the protein level in all cells as determined by immunofluorescence analysis, which we previously published (Yoney et al. *eLife*, Figure 7A). We included the same measurement by RT-PCR to demonstrate that this drug strongly inhibits gene expression at the RNA level as well. These data are now shown in Figure 5. The fact that endo-IWR1 can inhibit differentiation when applied simultaneously with Wnt, indicates that endo-IWR1 acts on sufficiently fast time scales to block any endogenous Wnt signaling in the TT-Eomes experiments. Additionally, reduction in the Wnt target gene, *Axin2*, was observed within 2 h of IWR1 treatment in cancer cells (Chen et al. 2009, Figure 7H).

Minor comments:
Figure 1D. The x axis for Eomes is extremely different to BRA and SOX2 – could the authors please verify if this is correct. It is also difficult to interpret from these plots what level is considered background staining versus a low level of expression.

We apologize for the confusion with regards to the axis labeling. Some batches of Eomes antibody had higher non-specific staining, so to correct for this, the nuclear fluorescence signal was normalized to the cytoplasmic signal. We removed the EOMES data from this experiment to avoid confusion since it is the only data in the original submission that was analyzed in that way. Instead, we included another experiment in which the EOMES signal did not need to be normalized and differences are obvious in the unprocessed images (see Figure S3).

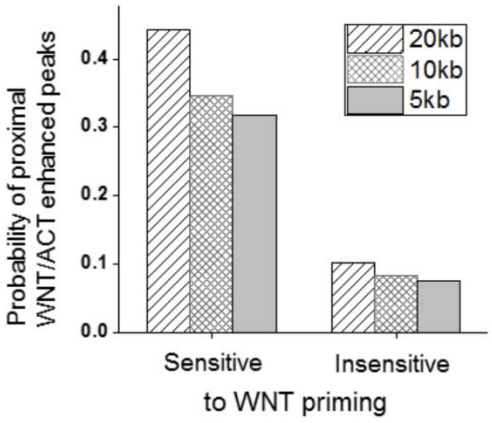
For ME markers, we consider background staining to be the levels in the pluripotency condition (-/ACT). This may not represent truly zero expression, because the mRNA levels in the -/ACT condition are detectable but very low: EOMES the count is ~30 in -/ACT conditions vs ~10,000 in WNT/ACT condition and for BRA (or *TBXT*) ~8 in -/ACT vs ~1,600 in WNT/ACT (Table S1). Regardless of whether background staining represents zero or low protein levels, it does not change our conclusion that cells retain memory of Wnt priming during gap between Wnt and Activin presentation.

Please indicate the minimum and maximum range used to display BigWig tracks in Figure 2A, Figure 3A.

These are now added to the plot.

Figure 6B – it was not clear to me why the 20kb range was specifically set to examine ATAC-seq peaks of WNT-primed genes.

We chose 20kb because some known enhancer elements are over 10kb away from the gene (e.g. the beta-catenin site 12 kb upstream the *MIXL1* gene). We also tried 10kb and 5kb as the cutoff and got similar results (see below).



Please define what is meant by “enhanced” ATAC peaks.

First, we identified ATAC peaks using MACS2 peak calling for each sample and combined these peaks using MergeBED. We then used MulticovBed to count the reads within each peak (peak areas) for each sample. Finally, peak areas were compared using DESeq2 to identify the ones that show significant differences (P<0.01) among samples. This explanation is in Methods section on page 25.

Line 270. “EOMES is activated by Wnt alone and becomes highly expressed with Wnt priming followed by Activin”. The induction of Eomes is very low in WNT alone conditions in Figure 1B qPCR, relative to the WNT/ACT condition. If Eomes can promote its own expression, why is there very little Eomes detected after 24hrs of WNT treatment?

Compared to E7, EOMES mRNA level is increased by ~200 fold in Wnt-only condition, and ~1500 fold in Wnt/Activin condition, so Wnt can significantly induce EOMES expression. We changed the axes to log scale to make this increase more visible. It is possible that the EOMES positive feedback (i.e. that EOMES activates its own expression) is already working in Wnt but cannot reach high level without SMAD2. Alternatively, EOMES concentration needs to reach a critical level to jumpstart the feedback, and it is not high enough in the Wnt condition. Another possibility is that EOMES translation is repressed in the Wnt condition and that following Activin/Smad2 activation EOMES is released from such repression. We added these sentences to the discussion (page 18-19).

Line 309. “These results strongly suggest that EOMES is the effector of Wnt priming…” The data support that EOMES is ‘an’ effector, but are not able to rule out other factors. Please amend this statement.

We changed the sentence accordingly (page 17).

Reviewer 3 Advance Summary and Potential Significance to Field:

In conclusion, I find this a highly relevant and important study with an appealing conclusion. However, the key conclusions have to be based on more careful data analyses and data representation. I suggest to include additional analyses into a majorly revised manuscript that allows to draw the relevant conclusions made by the authors.

We thank the reviewer for the positive comment.

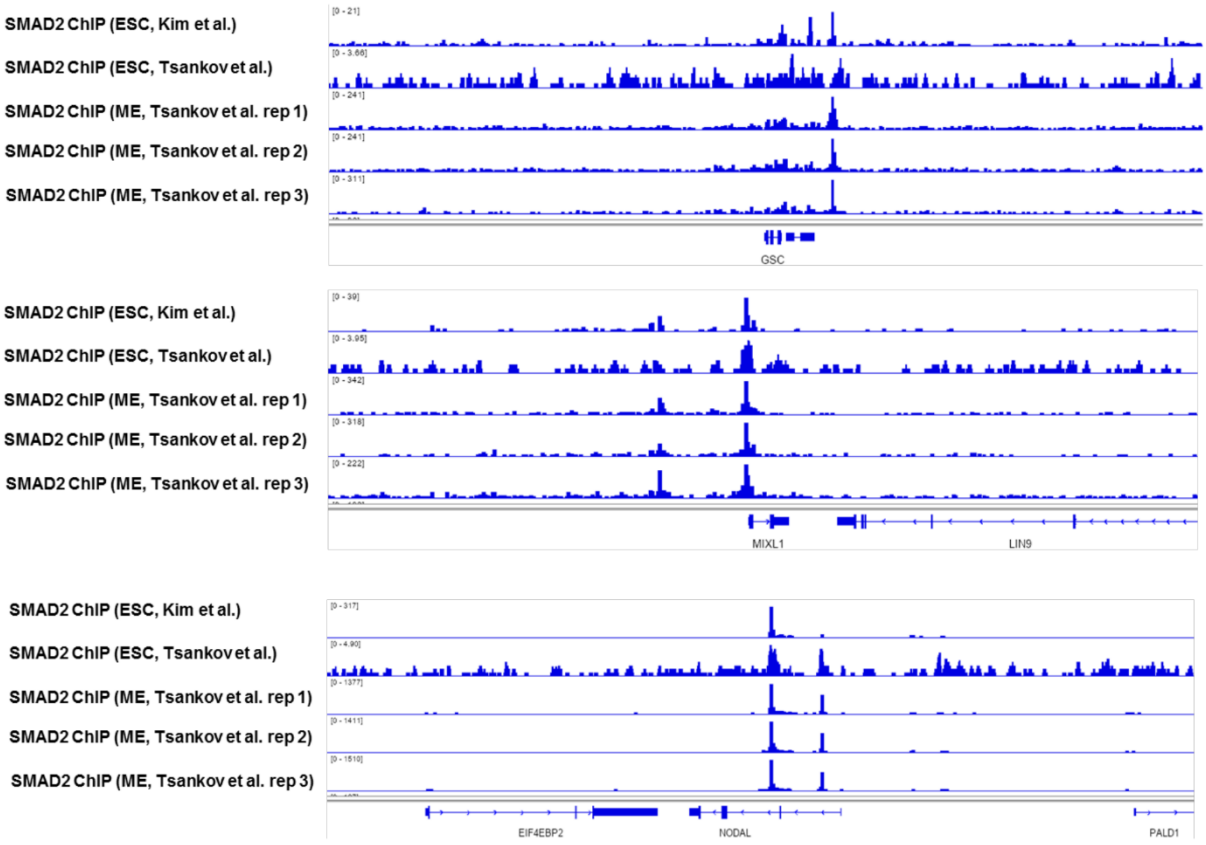
Major concerns:

1. From Figures 2 and 3 the authors conclude that Wnt-signals don’t alter Smad2 binding and activities directly. They perform a very global analysis of Smad2 binding in ESCs and ME cells from different published data sets to conclude that Smad2 binding is mostly unchanged between both states. However, this broad analysis is not very convincing and requires some more depth to draw this conclusion. Especially, since one of the papers from which data were used (Kim et al 2011), describes major differences in SMAD2 binding between ESCs and endoderm. Why do the authors use

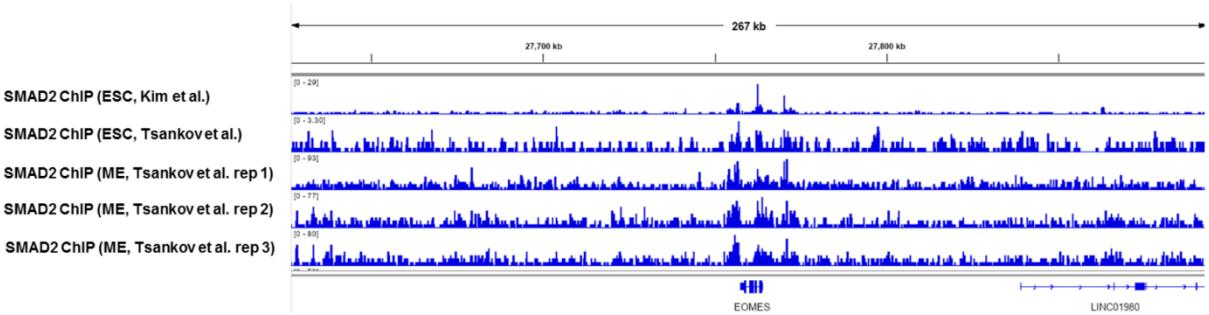
data sets from different publications if the work by Tsankov et al. contains data for both ESCs and ME? I suggest to compare these two datasets to see if there are changes at relevant sited of Mesendoderm target genes? The authors should plot SMAD2 binding in vicinity of genes whose expression changes mostly during Mesendoderm differentiation (most highly induced in Wnt/Act conditions, or overlaps of beta-Catenin and Smad2 binding) and not perform a global, genome-wide analysis. Most likely the authors will find differences in Smad2-binding at genes with biological significance, including Eomes. So, I suggest that the authors perform some additional analysis to further enforce their findings, that I find highly surprising.

We agree with the reviewer that it will be ideal to use the ESC and mesendoderm ChIP from the same publication. Unfortunately, the Smad2 ChIP in ESC from Tsankov et al. has poor signal-to-noise ratio and cannot reliably provide the peak information. However, at high loci with high Smad2 ChIP signal, this dataset also shows Smad2 peaks (see below). We know that ChIP data tend to be noisy. The fact that ChIP from two different labs show similar patterns supports the notion that Smad2 binding is similar in these two cases.

Kim et al. compared the Smad2 binding in hESC with endoderm cells that have been treated with Activin for five days. Importantly, primitive streak and mesodermal markers including BRA and FGF4 are *no longer* expressed in their endoderm cells. This is very different from our Wnt/Activin condition where both genes are highly expressed, consistent with a mesendoderm identity. Based on literature, Smad2 binding in ESCs relies on FoxH1, Oct4, and Nanog, all of which remain highly expressed in our mesendoderm condition; in the endoderm cells from Kim et al., Oct4 and Nanog are significantly down-regulated, which likely allows Smad2 to target other loci and explains the differences in our findings. These points are clarified in discussion on page 20.



In Figure 2, we compared the Smad2 peaks either on the global scale (2B), or in the vicinity of WNT-dependent or independent genes (2C). The correlation between ESC and ME ChIP are comparable in all of these cases. Below we showed the Smad2 ChIP data near the EOMES gene in a >200kb segment. In all of these cases, we see three weak peaks, and the pattern remains unchanged from ESC to ME.



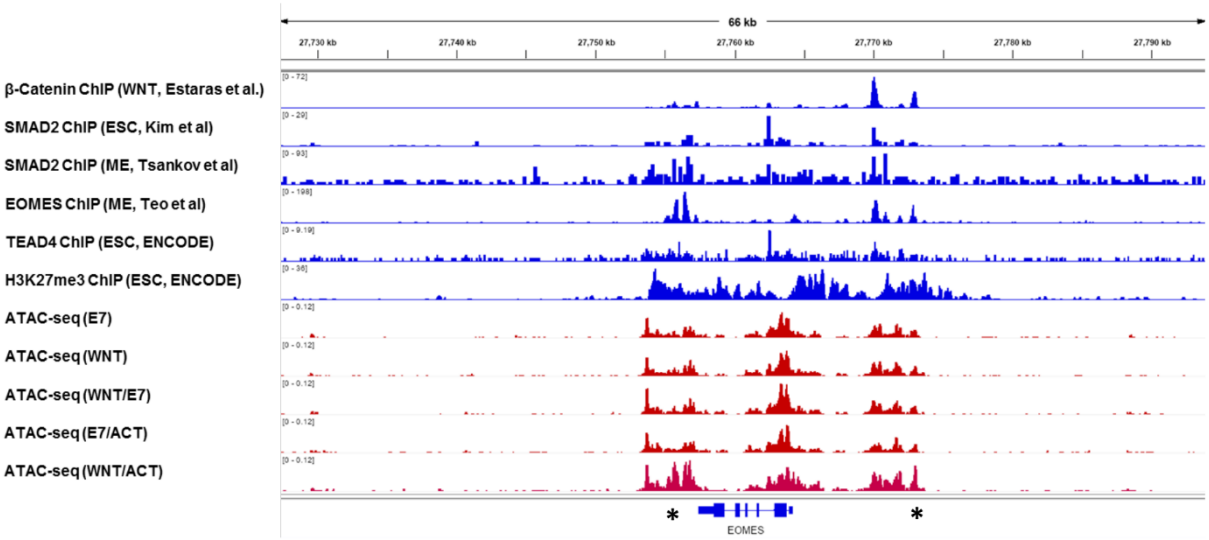
2. The key finding is the regulation of Eomes by Wnt which primes cells to respond to Smad2 using Eomes as a co-factor. However, the aspect of Eomes-regulation by Wnt is not addressed in the manuscript, despite availability of relevant data of ChIP-seq (Smad2, beta-Catenin) and ATAC-data. Please provide the analysis that shows the regulation of Eomes by beta-Catenin, which is further enhanced by Smad2. What is the effect of Wnt-stimulation that primes the Eomes locus to respond to Smad2? This regulation should look considerably different to the regulation of following mesendoderm genes and could thus serve to validate the analyses in Figures 2 and 3.

Compared to the basal level in E7, EOMES mRNA level is essentially unchanged in the Activin- only condition, increased by ~200 fold in Wnt-only condition, and ~1500 fold in Wnt/Activin condition. Therefore, EOMES is induced by β-catenin, and can be further induced by Smad2 after Wnt priming.

ChIP data show that β-catenin, Smad2/3, and EOMES itself, all bind to the vicinity of the EOMES gene (see below). ATAC-seq patterns are similar throughout different conditions, and in particular, there is virtually no change between E7 and WNT-primed conditions. There are two ATAC peaks that are significantly enhanced in the WNT/ACT condition (labeled with *), which may be due to EOMES binding as they overlap with EOMES peaks. Based on our expression data and published ChIP data, we interpret EOMES regulation as follows:

1) In the Activin-only condition, Smad2 binds closely to the EOMES gene without significant activation. This could be due to the fact that EOMES in ESCs are covered by the repressive histone mark, H3K27me3. Alternatively, weak TEAD4 ChIP signals were also detected across this region, and TAZ/YAP/TEAD was shown to repress SMAD2 activation.
2) In Wnt-only condition, β-catenin binds upstream the EOMES gene, and that activates EOMES to a moderate level. At this point, EOMES may start to bind its own promoter / enhancer, but unable to activate itself to a very high level without SMAD2; alternatively, concentration of EOMES may not be sufficient to engage in the positive-feedback loop at this stage.
3) In Wnt/Activin condition, a brief overlap between β-catenin and Smad2 further induces EOMES expression, which in turn activates and maintains its own expression, and no longer requires β-catenin.

These explanations are included in the discussion on page 18-19.



3. The manuscript uses the term "mesendoderm" very generally but it isn't defined what mesendoderm refers to precisely. The authors should please define this lineage more clearly (by markers), especially since they claim that the mesendoderm lineage does not rely on Brachyury but on Eomes functions (lane 273 -275). However, both T-box factors seem to compensate for each other, and also Eomes is dispensable for the generation of mesendoderm cell types from mESCs in vitro since EomesKO ESCs can express markers like Mixl1 and Lhx1 (Tosic et al. 2019). So, I suggest to clearly describe the lineage decision they are referring to and indicate relevant markers for this lineage also in the Figures, e.g. the heatmap of Figure 1C. Generally, throughout the manuscript only very few markers were applied but quite general statements are made about transcriptional outputs. Here, gene tables should be provided, such as for (Figure 1) 189 genes changed after 24 hrs of Wnt, 661 up and 383 down after Wnt/Act treatment, 113 Smad target genes …

We have annotated Figure 1C and added an additional table with genes belonging to each cluster (Table S1).
We are referring to the anterior mesendoderm lineage which expresses EOMES and GSC, and goes on to give rise to DE, which additionally expresses SOX17 and FOXA2. SOX17, FOXA2, and LHX1 are expressed in BraKO ESCs but not EomesKO ESCs (Tosic et al.). We also observe expression of LHX1 and MIXL1 in the WNT/ACT condition (Table S1), which are expressed in the primitive streak. We do not observe expression of key markers of cardiac, paraxial, or lateral plate mesoderm, including *MESP1/2*, *TBX6*, *MSGN1/2*, *HAND1*, or *ISL1*.
We have added additional description of the lineage and marker genes on page 5 and 7.

Minor Comments:

Figure 1:

A, B) Please label the scheme of hESC treatment (Fig. 1A) and place the scheme and the data in the same order (Fig. 1B, C). This current presentation is unnecessarily confusing.
C) Heatmaps without any labelling are little informative. The authors should please indicate some representative genes in the clusters and annotate the clusters. At least the most induced genes of cluster 3 should be shown with some more detailed information. In the results section (lanes 110 ff) groups of regulated genes are described. These should be provided in a table and possibly also some examples indicated in the heatmap.

Lane113: Please indicate mentioned genes, including Eomes, Bra and Nodal in the heatmap.

Figure 1A is now labeled, and the data in Figures 1B and C have been arranged in the same order. Key genes in cluster 3 are now labeled on the side of the heatmap. We also included a new supplementary table that lists all the genes included in each cluster (Table S1).

It is indicated that this experiment was conducted as n=2. How could they calculate p-values by only 2 replicates?

RT-PCR data in Figure 1B include three biological replicates (individual data points are included in the plot). For the RNA-seq data, p-value was calculated by the DESeq2 program, which can apply to sample size = 2.

D) IF of nuclear staining seems like an unnecessary complicated way of measuring gene expression of TFs. Why not simply using qPCRs as performed in Figure 1B. This would also allow for time-resolved measurements which would be more informative, such as in resolving why Sox2 remains highly expressed in Wnt+SB-E7+SB-Act conditions in Figure 1D- Why are a.u. once iven in 1,2,3… and then as 1000, 2000, 3000?

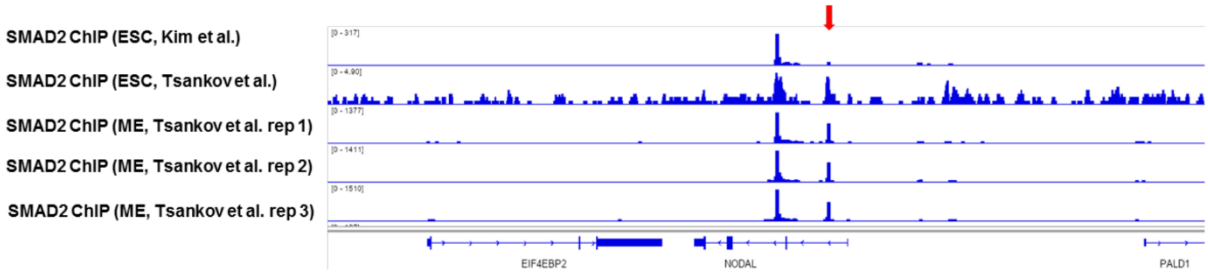
With respect to our standard 2-day protocol, we made similar conclusions whether we used RT- PCR (Figure 1B) or IF (Figure S1 this manuscript and Yoney et al. 2018) to assay gene expression changes. An advantage of using IF, especially in 1D, is that we can determine how the extra day effects the response in *individual cells*. Thus, in the case of Sox2 we can determine that it remains expressed at intermediate levels in all cells, whereas by RT-PCR we would not be able to distinguish this scenario from a scenario with a bi-modal distribution, i.e. in which Sox2 remains highly expressed as in the -/ACT condition in some cells and a portion of cells down-regulate Sox2 to the level of WNT/ACT.

Overall, we interpret the extra day of E7 as delaying the differentiation relative to the condition in which Wnt is immediately followed by Activin, due to a slow but incomplete loss of Wnt memory. Consequently, Sox2 is only partially down-regulated and ME markers only partially up-regulated. Nevertheless, cells must retain some effect of Wnt priming in order to be able to activate ME genes above the background levels in pluripotency (-/ACT).

We apologize for the confusion with regards to the axis labeling. Some batches of Eomes antibody had higher non-specific staining, so to correct for this, the nuclear fluorescence signal was normalized to the cytoplasmic signal. We removed the EOMES data from this experiment to avoid confusion since it is the only data in the original submission that was analyzed in that way. Instead, we included another experiment in which the EOMES signal did not need to be normalized and differences are obvious in the unprocessed images (see Figure S3).

Figure 2:
A) As described above the analysis is not convincing to show that there are no differences in SMAD2-binding between conditions. Especially, since there are already some differences visible in the few examples shown (e.g. Cer1), however, at relatively low resolution. Other available datasets (Tsankov et al) should be analysed from experiments that are more comparable.

We explained above why we did not use the ESC ChIP data from Tsankov et al. Even with this low signal-to-noise dataset, at high peaks, we can still see similarities between ESC and ME cells. Some differences between the Tsankov et al. and Kim et al. datasets (e.g. like the Smad2 peak near *NODAL* marked below) is likely due to noise, because the same peak was present in the Tsankov et al. ESC SMAD2 ChIP-seq data.



Please indicate the scale of genomic regions and counts at peak heights.

The peak heights are labeled in the plot as a range in brackets. The genomic regions for each panel is ~20 kb (added in the figure caption).

In the text (lane 168 ff) expression changes of these genes are described but not shown.

The mRNA change of these genes measured by the RNA-seq are now included in the supplementary Figure S4.

B) This data representation of SMAD2 peaks is very difficult to interpret. Does the R-value of R = 0,81 indicate similarity or differences? Selecting and analyzing the regions that are associated to relevant ME genes might resolve larger differences that are not visible in the current analysis.

D) The few examples of (not clearly indicated) regions of some ME genes are not very convincing if the goal of these experiments is to show no changes in SMAD2 binding.

While Figure 2B shows the global analysis of ChIP peaks, Figure 2C focuses on ChIP peaks proximal to WNT-priming dependent genes identified in our analysis in Figure 1. The correlation in this subgroup (R = 0.86) is compatible with the global correlation (R = 0.81). ChIP data tend to be noisy, and even among the three replica of the ME ChIP data, the average correlation is only ~0.71. We therefore conclude that Smad2 binding is not sensitive to WNT priming. This explanation is added to page 10.

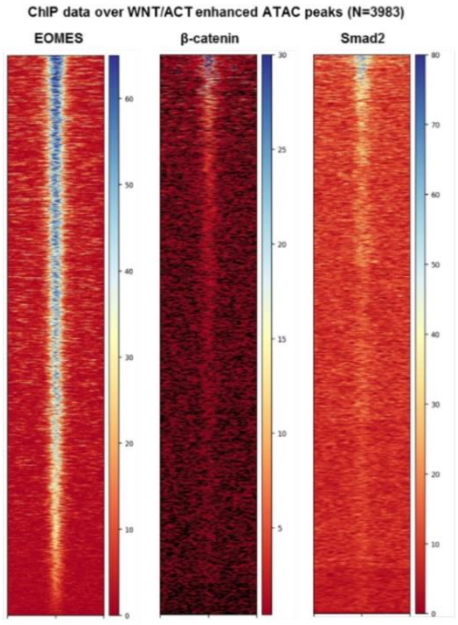
Figure 3 (also discussed as major concern) This is a very global analysis of ATAC at beta-Catenin and SMAD2 bound regions, and the interpretation seems biased. The minor differences at beta- Catenin bound regions are considered significant, while changes of ATAC at SMAD2 bound regions following Wnt-stimulation are considered non-significant. However, chromatin at SMAD2 bound regions is generally more accessible and (biological) significant changes might be masked in this analysis where SMAD2 bound regions were chosen that are present in ESCs. This might also explain the counter-intuitive result that SMAD2-binding should be inversely correlated with chromatin accessibility (Figure 3D).

ß-catenin does not have DNA binding domain, and it targets DNA through LEF1/TCF, which associates with DNA without WNT signaling. This could explain why ß-catenin sites are already highly accessible in the E7 basal condition, and why ß-catenin binding only mildly enhances this accessibility. Following Wnt-stimulation, the ATAC signal change over the top 20% of the ß- catenin sites (0.18 ± 0.02) is higher than that in the top 20% of the Smad2 sites (0.09 ± 0.05).
Nevertheless, the ATAC increase over the top 20% Smad2 sites after WNT treatment is still statistically significant ($P < 10^{-3}$). We suspect that this is due to the fact that many Smad2 binding sites are in close proximity to that of ß-catenin. To test this idea, we split all the Smad2 binding sites in ESCs into ß-catenin-proximal (within 500bp of a ß-catenin ChIP peak) or ß-catenin-distal. The ATAC signal only increased for the ß-catenin-proximal Smad2 peaks (0.09 ± 0.03), but not the distal ones (0.01 ± 0.03). We applied the same analysis to Smad2 binding sites in MEs, and reached similar conclusion: ATAC increase for the ß-catenin-proximal Smad2 peaks (0.15 ± 0.06), for distal ones (0.03 ± 0.06). Therefore, we conclude that the slight increase of ATAC signal increase over Smad2 sites after WNT treatment is caused by ß-catenin binding in nearby regions. Neither ß-catenin binding nor this slight ATAC signal increase significantly affects Smad2 binding (because Smad2 binding pattern is similar with or without WNT priming).
The other comments about Smad2 binding site choice and inverse correlation between ATAC and ChIP data are addressed below.

A) Please show relevant sites of the Eomes locus as reference for a gene that should show some change in locus control. What are the effects of beta-Catenin and/or SMAD2 on Eomes expression? In the text it is described that there are quantitative changes on ATAC signals over specific TF binding sites (Lane 196f). Where is this found? Which genes are associated to these sites? Is this also found at the Eomes locus? The given example at the Mixl1 locus is not very striking.

The tracks of ChIP-seq and ATAC-seq data near *EOMES* are shown above. The overall ATAC- seq pattern is similar among all the conditions, with two enhanced ATAC-seq peaks under the Wnt / Activin condition. The increased ATAC-seq peaks overlap with EOMES binding sites, which is consistent with the idea that EOMES binding in ME cells lead to chromatin opening.

Enhanced ATAC-seq peaks in Wnt / Activin at a genome-wide scale is analyzed in Figure 5. These peaks tend to overlap with EOMES binding sites, but not so much with ß-catenin and Smad2 sites (see plot on the right). These data are consistent with the idea that highly expressed EOMES in Wnt / Activin can associate with chromatin and cause further opening. In Figure 5C, we also show that these peaks more frequently occur in WNT-priming sensitive genes than the insensitive ones. The figure on the right is included as Figure S6C.



B, D) Please indicate how many peaks are analyzed, and indicate in the Figure legend the conditions of the experiments where the Beta-Catenin binding sites are taken from.

17809 ß-catenin and 4032 Smad2 binding sites are included in B and D. ß-catenin sites were measured in ESCs after Wnt treatment (added to the figure legend).

C) Described changes of ATAC signals in response to different treatment regimes are hardly visible. For the ATAC-peaks that are most strongly bound by beta-Catenin please show some examples and indicate which group of genes they belong to. Please indicate associated genes and show additional examples of changes in ATAC peaks for relevant target genes. The given example of changes in Mixl1 ATAC is hardly visible and there can be considerable doubts that this type of change in ATAC peaks is really relevant and biological significant, even if there is a statistical significance. The indicated peak at the Mixl1 locus (Figure 3A, strongest peak in WNT) doesn't follow the general pattern of ATAC peaks (Figure 3C, strongest in WNT/ACT). So why did the authors choose this peak over others?

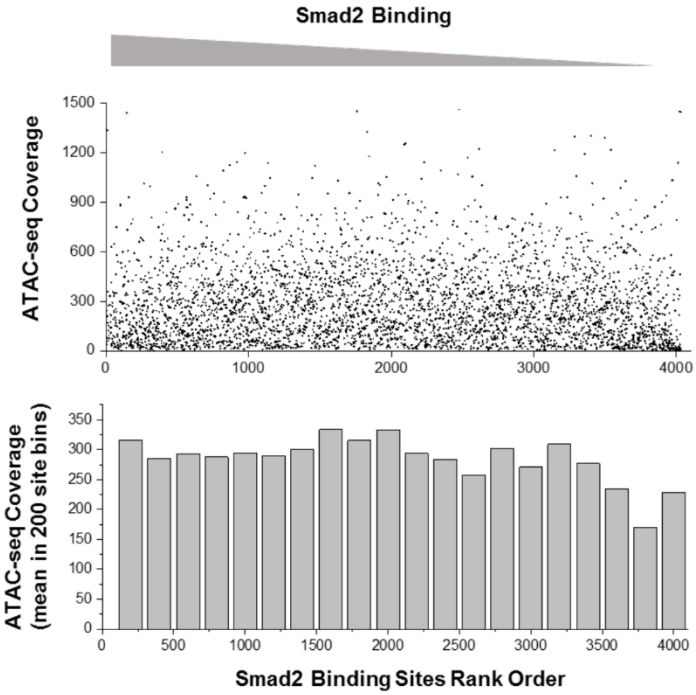
Please show the Eomes locus to demonstrate the Wnt-priming effect, if there is one.

In Figure 3 B & D, if we focus on the top portion of the graph (strongest binding sites), we can see ATAC-seq signal increase in some conditions. For ß-catenin, the most obvious condition is WNT, and for Smad2, is -/ACT and WNT/ACT. Overall, these data indicate that in conditions where ß- catenin or Smad2 are imported into the nucleus, the average ATAC signal over their binding sites increases.

There is one ATAC peak near the *MIXL1* gene that are significantly increased in the WNT/ACT condition (marked by *). This peak is one example of the "WNT-priming" sensitive peak we analyzed in Figure 6. We added another example in Figure 3A (near the *LEFTY1* and *LEFTY2* region), where two peaks (marked by *, both associated with Smad2) show significant increase in both the -/ACT and WNT/ACT conditions.

D) The most obvious pattern in all ATAC tracks is an inverse correlation of Smad2-binding with ATAC signals. What does this mean?

To check if ATAC signal is indeed inverse correlated with Smad2 binding, we used the centers of the sorted Smad2 peaks (in ESC, like in Figure 3D), took 400bp regions around them (+/- 200 bp), and calculated the ATAC-seq reads in these regions (multicovbed). There is no clear inverse correlation in these data. The figure below plots the ATAC-seq coverage at each Smad2 site (sorted from 1 to 4032 as strongest to weakest), or by binning every 200 sites.



C,D) Lanes 210 ff in the text states that ATAC signals over Smad2 sites remain identical in E7 and WNT conditions is rather surprising and requires further analyses. Are the Smad2 sites analysed identical in both conditions ESCs and ME cells? It is very likely that the sites are NOT identical but gain similar ATAC-signal strength. Please directly compare identical sites.

The comparison is over identical Smad2 binding sites, either identified in ES (Figure 3 D & E, upper panel) or in ME (Figure S2 C & Figure 3E, lower panel).

Lane 214: The corresponding Figures are Figure 3D, E (not C, D!)

This is corrected in the text on page 12.

E) Please indicate a quantification of overall ATAC-signals in 5 conditions (Fig. 3B, D). The changes observed in the analysis of Figure 3E are not obvious in Fig. 3D.
F)
We agree with the reviewer that the ATAC signal increases shown in Figure 3 are not huge effects, but if we zoom into the top part of Figure 3B and Figure 3D (see below), the effects are visible (ATAC signal increases over ß-catenin sites are most visible in WNT condition, and for Smad2, the increases occur in -/ACT and WNT/ACT conditions). Figure 3C and Figure 3E provide the quantification of the ATAC signals over ß-catenin and Smad2 sites.

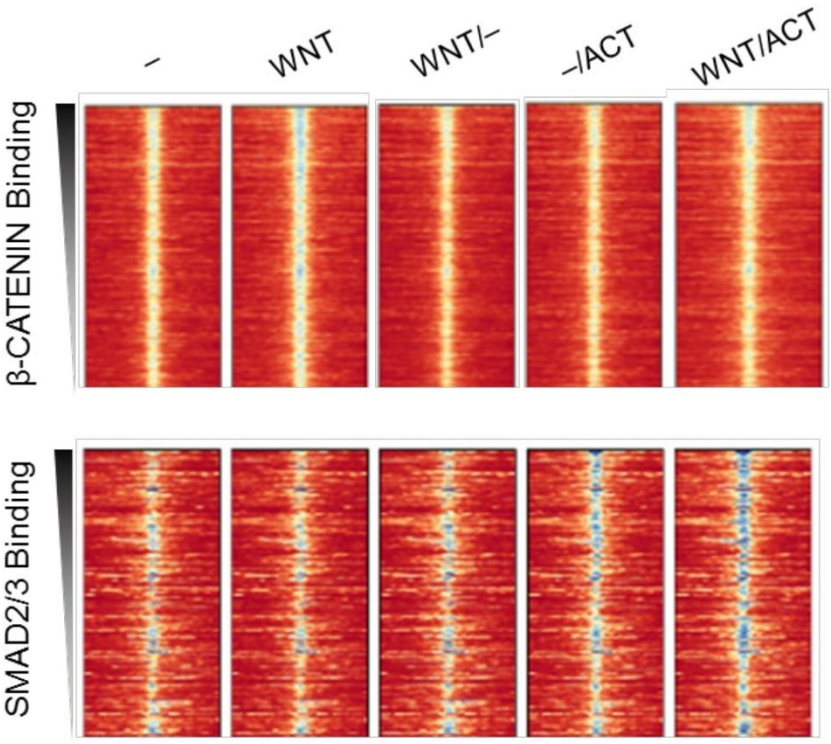


Figure S3:

What is the value of this information/graph? Is this small fraction of genes meaningful?

In this plot (now it is Figure S6), we are investigating if β-catenin or EOMES can be a co-activator of Smad2 to drive the expression of Wnt-priming sensitive genes. If that's true, we expect to see β-catenin or EOMES to co-bind with Smad2 in the vicinity of these genes. Indeed, in both cases, there is higher probability for the co-binding to happen near the Wnt sensitive genes vs the insensitive ones.

Figure 4:

C) Again (compare comment Figure 1), it is unclear, why IF was used to measure gene expression which most likely is inferior to quantitative measurement of gene expression in comparison to qPCR.

We hypothesize that expression of protein is stronger evidence that cells have committed to differentiation.

Figure 5:

C) This part of the Figure is not discussed in the text.

This is corrected in the text on page 13-14. Also Figure 5C is now Figure 5D.

Figure 6:
C) It is unclear to me why this graph indicates the probability of Wnt/Act enhanced peaks. Doesn't the analysis correlate the presence of Wnt/Act enhanced peaks to Wnt-sensitive/insensitive genes? So, this "probability" rather reflects a calculated value and could be labelled accordingly.

The reviewer is correct that this plot reflects a positive correlation between Wnt/Act enhanced peaks to Wnt-sensitive genes. To make this more clear, we labeled the y axis as "Fraction of genes with proximal WNT/ACT enhanced peaks".

C, D) What is the overlap of ATAC peaks in Figure 6B and the EOMES motif, or Eomes-binding? How often is overlap found/not found?

Which other TFs are found? Please provide full list of TF motif analysis.

As shown above (reply for Figure 3), the WNT/ACT enhanced peaks have large overlap with EOMES binding. The overlap depends on the EOMES ChIP threshold, but using cutoff q value 0.01, the overlap is ~80%. The AMES analysis also detected EOMES motif in ~80% of the enhanced peak sequences. This information is added to page 15.

AME result for enriched peaks in WNT/ACT enhanced peaks near WNT-sensitive genes are included as Table S2. Note that EOMES is by far the most enriched motif.

Second decision letter

MS ID#: DEVELOP/2021/200335

MS TITLE: Mechanisms Underlying WNT-mediated Priming of Human Embryonic Stem Cells

AUTHORS: Anna Yoney, Lu Bai, Ali Brivanlou, and Eric Siggia

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

First, thank you for clarifying the issue with Figure 1E.

As you will see, the referees express interest in your work, but continue to have some concerns. The referees note that there are only two replicates for some of the experiments. I agree that ideally increasing the replicates would strengthen these data, nevertheless I accept that the data that is presented supports the conclusions and that you show the individual datapoints. The referees also question the use of Kim et al and Tsankov et al datasets to examine SMAD binding. I understand that these type of data can be noisy, but the signal could also be indicative of less specific binding in ES cells. Clearly stating this potential caveat of the analysis in the Discussion would alert readers to this. It might also be appropriate to show both sets of ES cell data so that a reader can more easily assess the data. Overall, the evidence supports that conclusion that EOMES is the effector of WNT signaling for the development of mesendoderm and this will be of interest to the field.

If you are able to revise the manuscript, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the original paper, Yoney et al., investigate the mechanism underlying Wnt and Activin signaling pathways cooperation in the induction of ME genes in hESCs. The authors claim that Wnt-induced EOMES is necessary to establish the Wnt-mediated acquisition of cellular memory, necessary for the cooperation with Activin:SMAD2/3. The analysis of the cooperation between these two pathways have been extensively studied in hESCs and several molecular mechanisms have been elucidated. The idea that EOMES is the effector of WNT priming is somehow novel. An extensive revision was required to help to support authors' claims. The review required reanalysis of some data presented, adding replicates to key data and more experiments to characterize the Eomes expressing cells.

Comments for the author

Authors partially respond to the criticisms by reanalyzing some of the data presented in the first version. For instance, they included violin plots which help with the interpretation of the IF analysis. They also include explanations to clarify some of the results. However, they did not add additional experiments or performed a third replicate to support critical data (e.g Figure 5 and 7). Overall, although I appreciate the efforts of the authors addressing the criticisms, I believe that more robust data should be shown to support the main claim of the paper.

Reviewer 2

Advance summary and potential significance to field

Yoney et al explore a long-held question – how do the WNT and Activin/Nodal (ACT) signalling pathways promote the generation of ME from pluripotent cells? In agreement with their previous work, they demonstrate that the generation of ME-like fates in vitro relies on exposure of human ESCs first to WNT signalling followed by Activin, in order to promote the induction of genes such as Eomes Bra and Gsc. They demonstrate that this transition does not appear to proceed by either signal alone, or in the reverse order. They provide evidence that a defined period of WNT exposure is required to generate ME, and that this involves the activation of Eomes, a well-established regulator of ME differentiation.
Eomes can promote ME differentiation, although the mechanism remains unclear.

Comments for the author

I have some minor comments aimed to improve readability and interpretation of the data.

Line 134 - 136: Please revise the labels used to describe the clusters here, as they are not consistent with what is presented in the heatmap and differ to the description used in the supplementary table. For example, cluster 1 is labelled "activin only" but is also induced in WNT/ACT conditions looking at the heatmap in Fig 1C. Cluster 2 on the heatmap appears to be similar in all conditions except -/ACT, so it doesn't appear to reflect genes induced in "WNT only" conditions.

Line 137. It's not clear what gene list this refers to. Please provide the data as a supplementary item and reference this in the text.
Line 141. These data have also not been presented in the manuscript. Please include this and reference the item in the text.
Line 159. I believe these data are also not included in the current version of the manuscript. Please include the data and refer to the item in the text here and at line 173, where the authors are concluding that they have been able to "unbiasedly identify a group of Activin/SMAD2 target genes whose transcriptional responses are affected by WNT priming"

Line 189. "Despite the extended interval between WNT and Activin stimulation Activin can still induce ME markers and down-regulate SOX2 at the protein level albeit to a lesser extent (Figure 1D, E..)". The IF image of SOX2 in Figure 1D appears consistent with this conclusion, yet, at odds

with the quantification presented in Figure 1E. Unlike the IF, there appears to be very little difference between the last two conditions, WNT+SB/ACT and WNT+SB/+SB/ACT.
Figure 2A. The authors have included the range on the y axis in this revised version. This demonstrates that the two conditions have been plotted on different scales. This also applies to Figure S5. Please revise this and instead present the coverage using consistent ranges, or explain why this hasn't been applied. In addition, information regarding how the data have been normalised should be included in the methods section. Please include where relevant, if input samples were used in the any of the ChIP-seq analyses.
Line 212. As above, please clarify which gene list this refers to.
Line 241 "Furthermore, a vast majority of genes contain ATAC-seq peaks near their TSS and within transcript regions, regardless of their transcriptional status" - please provide a reference to the data that demonstrates this.

Reviewer 3

Advance summary and potential significance to field

Yoney and colleagues revised their manuscript now entitled “Mechanisms Underlying WNT-mediated Priming of Human Embryonic Stem Cells” and provide extensive comments as response to the three reviewers. The reviewers overlapped in several aspects of their judgement of this manuscript to say that major improvements would be required to substantiate the claims made. However, despite the efforts, the revised manuscript and the figures do not reflect the criticism that was raised and surprisingly text and figures were largely left unchanged.

Comments for the author

While the manuscript presents evidence that Wnt-signals promote ME formation by generating a permissive state for high level Eomes expression by Nodal/Activin/Smad2 signalling, the (global) analysis of chromatin-binding and -accessibility does not resolve any molecular detail or mechanism to explain their findings.
Two major criticisms were a) the chromatin binding analysis of SMAD2 in different conditions, and b) the lack of analysis of Eomes-regulation by successive canonical Wnt (b-Catenin) and Nodal (Smad2). Both aspects were not sufficiently addressed despite being central to the manuscript.
a) To claim that SMAD2 binding is unchanged between hESC and ME cells isn’t convincing from presented data. The analysis remains superficial and lacks the depth to make this claim. I can’t follow the argumentation about “noisy” ChIP-data as mentioned by the authors. From presented data in the manuscript and in the rebuttal letter, it is obvious that SMAD2-binding is NOT identical between ESC and ME cells (Nodal has 2 peaks in ME, 1 in ESC; Cer1 has 2 peaks in ESC and 1 in ME, Gsc-peak pattern look different etc, Fig. 2A). How were the data normalized, since the indicated scales are very different (Fig. 2A), but the analysis of Fig. 2B that should show high correlation of SMAD2 ChIP peak area between ESC and ME seems very similar. This is still not explained sufficiently (which genes, which areas, data of these plots would be required) and can’t be followed. From Fig. 2A we see very different scales in peak heights so how was this correlation normalized/calculated? To use data from same sets of experiments, we requested that the authors should please stick to data from Tsankov et al. The authors conclude from these data that they have a poor signal-to-noise ratio. However, these results could also be interpreted very differently, so that SMAD2-binding at the indicated ME sites is simply much less in ESCs. It would be helpful to see a heatmap of global SMAD2 binding of the Tsankov ESC SMAD Chip-seq to assess if it is overall noise or just at the sites shown.
b) The mechanism of Wnt-priming leading to Eomes expression is discussed but no data and no reference to existing data (also from mouse ESC differentiation) is presented in the manuscript that explains regulation on the level of chromatin. The presented models thus could be seen as rather speculative (Fig. 8).

Some additional issues:
1. New violine plots were added in exchange for previous Figures as Fig. 1E (expression of BRA, SOX2).
The plots for the last two conditions are identical, which seems surprising for quantification of fluorescent images.

2. The analysis of Fig. 2 B, c is not clear. What are these genes, how were these data generated? How were ChIP data normalized for this analysis?
3. The merged images of Fig.S1B and Fig. S2B now look quite different to the previous versions. Please clarify.
4. The abstract still contains gastruloids as term, however the experiments were not done as gastruloids, at least from my understanding. Please clarify.
5. The plots of Fig. S6 remain unclear even after the explanation given by the authors. Could the authors please provide heat maps of these genes and plot for the same regions ß-CATENIN, EOMES and SMAD2 binding. Including summary curves? Also, in lines 341-342 they claim that EOMES binds near SMAD2 in the vicinity of Wnt-primed genes (are these the same as Wnt-dependent genes?), but this is not shown there is no significant difference. And what is the value of 0.6 for fraction of genes?
6. Scale bars next to heatmaps are not described.

In summary, we do not see that the revised version of the manuscript has improved the analyses to support the major claims made by the authors. This is mostly due to a rather superficial analyses of (published) ChIP data and/or data quality. Thus, we can't suggest this manuscript for publication in Development at this stage.

Second revision

Author response to reviewers' comments

We would like to thank all three reviewers for thoughtful criticisms and suggestions. We have addressed reviewers' concerns and comments, which we believe have significantly enhanced the clarity of our manuscript. The following is a detailed point-by-point reply (blue text) to each comment (black) from the reviewers. We underlined all the sentences describing the changes made to the manuscript. In the manuscript, the changes were highlighted in red.

Reviewer 1 Comments for the Author: Authors partially respond to the criticisms by reanalyzing some of the data presented in the first version. For instance, they included violin plots which help with the interpretation of the IF analysis. They also include explanations to clarify some of the results. However, they did not add additional experiments or performed a third replicate to support critical data (e.g Figure 5 and 7). Overall, although I appreciate the efforts of the authors addressing the criticisms, I believe that more robust data should be shown to support the main claim of the paper.

We thank the reviewer for the positive response.

Reviewer 2 Comments for the Author: I have some minor comments aimed to improve readability and interpretation of the data.

We thank the reviewer for thoroughly reading of the manuscript.

Line 134 - 136: Please revise the labels used to describe the clusters here, as they are not consistent with what is presented in the heatmap and differ to the description used in the supplementary table. For example, cluster 1 is labelled "activin only" but is also induced in WNT/ACT conditions looking at the heatmap in Fig 1C. Cluster 2 on the heatmap appears to be similar in all conditions except -/ACT, so it doesn't appear to reflect genes induced in "WNT only" conditions.

We revised the cluster descriptions so that they are consistent with the heatmap and what is presented in the supplementary table. The clusters are now described as follows:

K-means clustering of these genes revealed four major clusters. Cluster 1 includes genes for which the expressions show comparable increases in -/ACT and WNT/ACT, but less in WNT and WNT/-, indicating that these genes are induced by activin (n = 349). Cluster 2 represents genes induced by WNT (n = 206). Cluster 3 includes genes that show higher induction in WNT/ACT than WNT or ACT

alone (n = 222). Cluster 4 represents genes that are repressed by the WNT and / or ACT condition (n = 435).

Line 137. It's not clear what gene list this refers to. Please provide the data as a supplementary item and reference this in the text.

We now include a new tab in the Supplementary Table 1 listing the fold change and P-value of the 189 genes affected by WNT.

Line 141. These data have also not been presented in the manuscript. Please include this and reference the item in the text.

This line refers to the following sentence: “The expression of pluripotency makers NANOG, OCT4, SOX2, and KLF4, either remained constant or was upregulated in response to WNT or Activin, confirming the lack of differentiation with either signal alone.” We now added the RNAseq data of these genes as Figure S1D.

Line 159. I believe these data are also not included in the current version of the manuscript. Please include the data and refer to the item in the text here, and at line 173, where the authors are concluding that they have been able to "unbiasedly identify a group of Activin/SMAD2 target genes whose transcriptional responses are affected by WNT priming".

We found 113 Smad2 targets that are induced by WNT/ACT (higher expression in WNT/ACT than WNT or ACT alone; P-value cutoff 0.05). The identity of these genes, as well as their mean RNAseq counts are now listed in a new supplementary table, Table S2. We also included the list of Smad2 target genes that are *not* sensitive to WNT priming in the same excel file.

Line 189. "Despite the extended interval between WNT and Activin stimulation, Activin can still induce ME markers and down-regulate SOX2 at the protein level, albeit to a lesser extent (Figure 1D, E..)". The IF image of SOX2 in Figure 1D appears consistent with this conclusion, yet, at odds with the quantification presented in Figure 1E. Unlike the IF, there appears to be very little difference between the last two conditions, WNT+SB/ACT and WNT+SB/+SB/ACT.

We apologize for the mistake, which led to the duplicated data being included in Figure 1E. The data for WNT+SB/ACT was copied twice and the actual data for WNT+SB/SB/ACT was not included in violin plots in Figure 1E for BRA and SOX2. We have corrected the figure. As shown in our original submission when the data was presented in histogram form the conditions were not identical, so this mistake arose during the revisions to Figure 1.

Figure 2A. The authors have included the range on the y axis in this revised version. This demonstrates that the two conditions have been plotted on different scales. This also applies to Figure S5. Please revise this and instead present the coverage using consistent ranges, or explain why this hasn't been applied. In addition, information regarding how the data have been normalised should be included in the methods section. Please include where relevant,if input samples were used in the any of the ChIP-seq analyses.

The two bigwig files were generated using different scaling methods. Now we replotted them using the same scaling method (RPKM) and also with higher spatial resolution (10bp) (see revised Figure 2A). Note that the scaling method does not affect peak locations or peak areas (which were based on bam files) and does not change any conclusions. Input samples were not used in the ChIP-seq analyses.

Line 212. As above, please clarify which gene list this refers to.

The identify of these genes, as well as their mean RNAseq counts are now listed in a new supplementary table, Table S2.

Line 241 "Furthermore, a vast majority of genes contain ATAC-seq peaks near their TSS and within transcript regions, regardless of their transcriptional status" - please provide a reference to the

data that demonstrates this.

All our ATACseq data are uploaded to NCBI's Gene Expression Omnibus. GEO access number: GSE176222. This number is added to the main text (page 11) and to the "Data and software availability" section and to the main text.

Reviewer 3 Comments for the Author:

While the manuscript presents evidence that Wnt-signals promote ME formation by generating a permissive state for high level Eomes expression by Nodal/Activin/Smad2 signalling, the (global) analysis of chromatin-binding and -accessibility does not resolve any molecular detail or mechanism to explain their findings.

Two major criticisms were a) the chromatin binding analysis of SMAD2 in different conditions, and b) the lack of analysis of Eomes-regulation by successive canonical Wnt (b-Catenin) and Nodal (Smad2). Both aspects were not sufficiently addressed despite being central to the manuscript.

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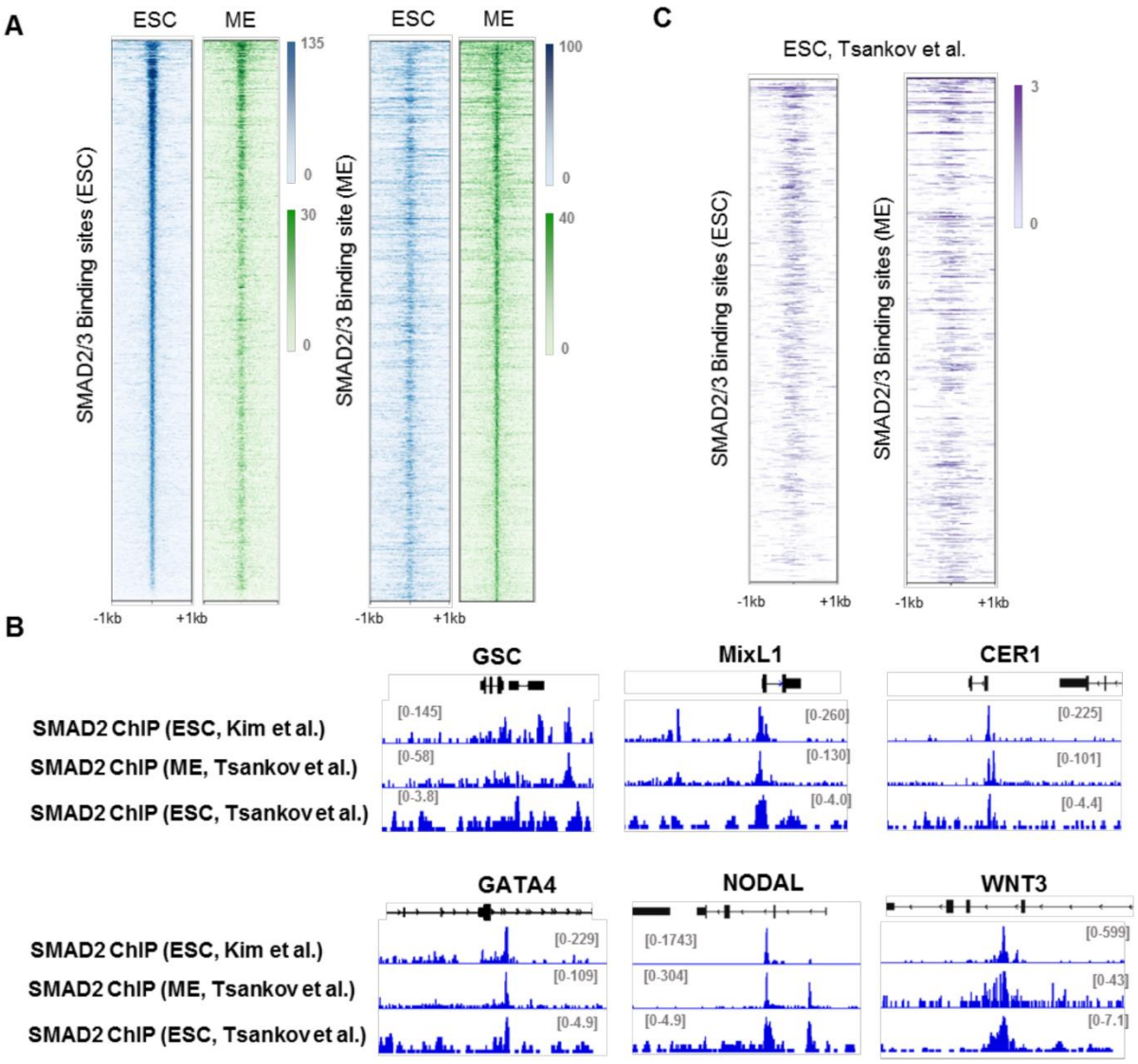
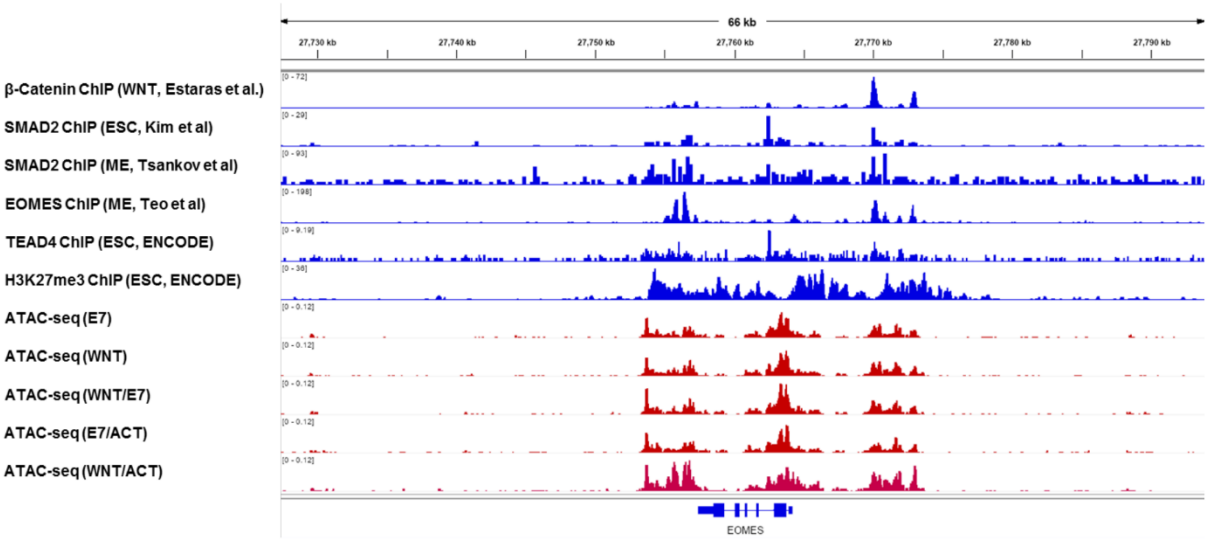
1) SMAD2/3 ChIP-seq peaks from Kim et al (ESC) and Tsankov et al (ME) have an overall correlation of 0.8, while the average correlation among the three replica of the ME ChIP data is only ~0.71. Therefore, given the reproducibility of the ChIP-seq, the data from Kim et al and Tsankov et al have no statistical difference. To further demonstrate the similarity of these two ChIP-seq datasets, we used more standard heatmaps to show the correlation between the ChIP- seq peaks (panel A below). We now included this panel as Figure 2B.

2) Even with the ESC data from Tsankov et al., some peaks are still visible, and they are consistent with the peaks measured by the same group in ME condition. Panel B below shows the same example tracks in Figure 2A, but this time, we added the ESC data from Tsankov et al. (GSM1505747). Except for the peaks over *GSC* (the lowest peaks among these examples), ChIP peaks over all other regions in Tsankov et al ESC data are visible, and their patterns and locations agree with the ME data. In particular, for Nodal, where Reviewer #3 pointed out that Kim et al. data look different from the Tsankov et al. ME data, the patterns in Tsankov et al. ESC data turn out to be more similar to ME. In addition, heatmaps of this Tsankov et al. ESC data show weak enrichment of SMAD2 at most annotated SMAD2 binding sites derived from both ESC or ME cells (panel C below). We believe this evidence supports the similarity of the Smad2 binding in these two cell types. As suggested by the editor and reviewer, we now included Panel B & C below as Figure S4B & C. Panel A is added to Figure 2.



b) The mechanism of Wnt-priming leading to Eomes expression is discussed but no data and no reference to existing data (also from mouse ESC differentiation) is presented in the manuscript that explains regulation on the level of chromatin. The presented models thus could be seen as rather speculative (Fig. 8).

Relevant ChIP data and ATACseq measurements on EOMES locus was included in the previous rebuttal. We are not clear on what "regulation on the level of chromatin" the reviewer is referring to. There is no apparent change in the ATAC peaks near EOMES gene in WNT vs E7 condition (see below), indicating that WNT priming does not directly change the chromatin accessibility over this region. The model in Figure 8 right now is speculative. To get to the exact mechanism, we need to individually manipulate β-catenin, SMAD2, and EOMES concentration and/or perform promoter/enhancer bashing, which are beyond the scope of the current manuscript.



Some additional issues:

1. New violine plots were added in exchange for previous Figures as Fig. 1E (expression of BRA, SOX2). The plots for the last two conditions are identical, which seems surprising for quantification of fluorescent images.

We apologize for the mistake, which led to the duplicated data being included in Figure 1E. The data for WNT+SB/ACT was copied twice and the actual data for WNT+SB/SB/ACT was not included in violin plots in Figure 1E for BRA and SOX2. We have corrected the figure. As shown in our original submission when the data was presented in histogram form the conditions were not identical, so this mistake arose during the revisions to Figure 1.

2. The analysis of Fig. 2 B,c is not clear. What are these genes, how were these data generated? How were ChIP data normalized for this analysis?

To be consistent, we changed the label to “Peaks close to WNT-primed genes” and “Peaks close to genes not primed by WNT”. The list of these genes, as well as their RNA-seq counts in all five conditions, are now included in the Supplementary Table 2. How they were obtained is described on page 7-8 of the main text. Figure 2C (now 2D) includes all Smad2 peaks identified near these genes (gene +/- 10kb range as stated in the legend).

As explained above, we updated Figure 2A so that the tracks have the same scaling method. The peak areas were calculated using “multicovbed” using the alignment bam files and the bed files of Smad2 peaks without further normalization. This is now stated in the methods.

3. The merged images of Fig.S1B and Fig. S2B now look quite different to the previous versions. Please clarify.

The linear contrast adjustment is arbitrary and meant to facilitate visualization and to best reflect the quantified data. To regenerate this figure to have both the merged and individual channels the raw images had to be processed again, so there may be some differences in contrast adjustment. These differences do not affect the plots since quantification is done on the raw images.

4. The abstract still contains gastruloids as term, however the experiments were not done as gastruloids, at least from my understanding. Please clarify.

We removed the term from the abstract.

5. The plots of Fig. S6 remain unclear even after the explanation given by the authors. Could the authors please provide heat maps of these genes and plot for the same regions ß-CATENIN, EOMES

and SMAD2 binding. Including summary curves? Also, in lines 341-342 they claim that EOMES binds near SMAD2 in the vicinity of Wnt-primed genes (are these the same as Wnt- dependent genes?), but this is not shown, there is no significant difference. And what is the value of 0.6 for fraction of genes?

For Figure S6A & D, we collected WNT-priming sensitive vs insensitive genes (now listed in Supplementary Table 2), and for each gene, we asked if we can find at least one β-Catenin or EOMES ChIP-seq peak within TSS ± 5kb. 0.6 means 60% of the genes contain a β-Catenin or EOMES ChIP peak within this range. For SMAD2 co-localization analysis in Figure S6 B & E, we first collected SMAD2/3 ChIP peaks within TSS ± 5kb for each gene (genes with no SMAD2 binding site were ignored) and asked what fraction of these Smad2 peaks are <500 bp away from a β-Catenin or EOMES ChIP-seq peak (co-localized). 0.6 in this case means 60% of Smad2 peaks are co-localized with β-Catenin or EOMES.

Even though both WNT-priming sensitive and insensitive genes tend to have proximal EOMES binding sites, for the WNT-priming sensitive ones, EOMES sites are more likely to co-localize with SMAD2, so the original claim in the manuscript is correct.

6. Scale bars next to heatmaps are not described.

Figure 1C, the scale bar represents colors corresponding to log2 fold change of the RNAseq counts of the four conditions relative to E7. Figure 2C, the scale bar represents colors corresponding to ChIP intensity of Smad2. Figure 3B, the scale bar represents colors corresponding to ATAC-seq intensity (same for all the ATACseq heatmaps). These descriptions are added to the corresponding figure legends.

Third decision letter

MS ID#: DEVELOP/2021/200335

MS TITLE: Mechanisms Underlying WNT-mediated Priming of Human Embryonic Stem Cells

AUTHORS: Anna Yoney, Lu Bai, Ali Brivanlou, and Eric Siggia
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Referee reports on this version are appended below. Referee 1 continues to have concerns about the strength and interpretation of the data, but having taken account of the input from all three reviewers and your responses to the criticisms, I am satisfied that the conclusions of the study are supported by the data and that you have discussed caveats and limitations.

Reviewer 1

Advance summary and potential significance to field

I still think that the data presented in this manuscript are not robust and do not support the main conclusions claimed by the authors.

Comments for the author

Authors did not address the concerns raised on the number of replicates in key experiments. Also, authors did not add additional experiments to support the main claim of the paper; which is that EOMES is sufficient to replace Wnt signal in ME differentiation. Overall, I think that the data presented in this manuscript do not support the conclusions and it is too preliminary to be published in Development.

Reviewer 2

Advance summary and potential significance to field

In previous studies, the authors established that a defined temporal sequence of signals (WNT, followed by activin) promotes the exit from pluripotency and differentiation towards a mesendoderm (ME) fate in a model using human ESCs. Here the authors extend this previous finding by providing evidence from mRNA-seq ATAC-seq and some reanalysis of existing published ChIP-seq data, together with genetic and biochemical pertubations, that a defined period of WNT signalling is required to induce Eomes, a well-established regulator of ME fate. Eomes induction, followed by activin exposure, is sufficient in this context to promote ME differentiation, without further manipulation of WNT signalling.

Comments for the author

Thank you to the authors for making the revisions. I have no further comments.