Peer Review File

Manuscript Title: Reconstruction and deconstruction of human somitogenesis in vitro

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In this work, Miao, Pourquie and colleagues present two 3D models of human somitogenesis: somitoids and segmentoids. Whereas somitoids only recapitulate the temporal component of somitogenesis, segmentoids recapitulate both space and time. The authors then use the modularity and accessibility of the models to demonstrate that an active cell sorting based on differential expression levels of Mesp2 underlies the establishment of anterior and posterior somite compartments. The authors conduct some beautiful experiments to dissect the (in)dependence of distinct patterning modules involved in somitogenesis. In general, the work elegantly demonstrates the power of in vitro systems. However, I have some significant concerns regarding:

i) The initial characterisation of the in vitro systems, which is not fully up to the standards in the field.
ii) The lack of mechanistic understanding what drives the cell sorting.
iii) The claim that the sorting mechanisms identified appear to be conserved in vivo.

I will detail these concerns, as well as some other points, below.

Major points:

1. In the meantime, the Budjan et al. "somitoids" manuscript the authors added to the submission has been published in eLife (DOI 10.7554/eLife.68925). The authors should therefore: i) include this publication in the introduction of the submitted work; ii) discuss the differences and commonalities between the published somitoids and the somitoid system presented in the submitted work; iii) demonstrate and discuss the additional value of the new somitoids system.

2. The somitoid and segmentoid system should be much better characterised; proper quantifications should be performed:

i) The authors should provide a thorough morphometric analysis for both the whole structure as well as the substructures (similar to the one performed in Veenvliet et al, Science 2020 for trunk-like-structures). E.g. area, perimeter, circularity, somite size, somite number, etc

ii) These morphometric data should be compared to the embryo

iii) The authors should report reproducibility metrics: they e.g. state that "the posterior end

sometimes appears bifurcated". Which % of the segmentoids develops a single axis? Which % forms a double axis? What is the elongation efficiency? What is the somite formation efficiency?

iv) From the (live) imaging data it seems that, at least in some cases, the segmentoids produce multiple somites with each cycle of the segmentation clock (similar to the TLS-CL reported by Veenvliet et al., Science 2020). Is this indeed the case? In what percentage of the structures does this happen? How many somites are formed with each cycle? How does this evolve over time?

v) For immunostainings, the authors should report the penetrance of the shown staining (n structures with similar staining pattern / n structures analysed)

vi) Instead of (or complementary to) quantifications of the individual structures shown for AP somite polarity (eg 4c, S7c) the authors should demonstrate reproducibility for a high(er) number of structures, showing intra-/inter-experimental variability, provide a statistical testing for e.g. CTRL vs ROCKi, etc

3. Despite some beautiful experiments, it remains unclear what drives the self-sorting. I feel there is a missed opportunity here to provide mechanistic insights with the unique experimental systems the authors have at hand. The experiments the authors performed should already provide hints: is there differential expression of morphogenetic regulators (adhesion, actin-cytoskeleton, …) in Mesp2-high vs Mesp2-low cells? The authors might also be able to leverage the scRNA-seq data, by e.g. performing "in silico sorting" of aPSM cells and/or check for genes that covary with Mesp2 expression levels. They should then check if these differences are also observed in vivo (see point below).

4. The authors claim in vivo relevance, but the experimental evidence for this (HCR on in fixed embryos) is extremely limited (and in fact does not show any cell sorting since the data are not time-resolved). The authors should generate a Mesp2 reporter line similar to the human one and culture embryos ex vivo to demonstrate that cell sorting takes place in vivo. This would also e.g. allow for the sorting of Mesp2-high vs Mesp2-low cells to show that differential expression of morphogenetic regulators (see point 3) is conserved in vivo.

5. To me, it still remains unclear why the segmentation clock would be needed to achieve AP patterning within single somites. I understand how the segmentation clock will be important to allocate pre-somitic tissue (segmental stripes), but as I pointed out above it appears that in many cases more somites than expected (i.e. >2) are formed during the same cycle. Why do these somites (generated in parallel) nevertheless establish A and P domains (and not form separate "full A" and "full P" somites as in the somitoids)?

6. I have multiple concerns and suggestions regarding the scRNA-seq analysis:

i) in both segmentoids and somitoids the authors report the identification of NMPs. However, are these cells truly bipotent. Their progeny surely suggests the cells are mono-fated, at least at earlier timepoints (also see comment below)? The authors should carefully analyse if these cells are true NMPs (e.g. compare to in vivo transcriptional signatures), or if they are already fate-biased (similar

to what was reported for the “NMPs” in chemically modulated TLS (TLS-CL) in Veenvliet et al., Science 2020.

ii) Are the authors sure the 24h clusters are NMPs? Based on marker expression (Pou5f1, Sox2, T) these could also be epiblast(-like) cells. There clearly seem to be three clusters of NMPs in 3e (left, 24h; middle, 48h; right, 72/96h). The authors should carefully check the transcriptional differences between these clusters, and how these compare to the in vivo signatures.

iii) The PAGA graphs in Fig. 3g are an extreme oversimplification of the data, and in fact do not match well with the scvelo data in Fig. S5b (also see comments below)

iv) The proportions quantified in Fig. S5a do not match the data shown in S5b: at 24h the proportions bar graph only reports NMPs, whereas in b it is clear there are also PSM cells. Same for the neural cells at 72h, that are there in the velo map, but not in the proportion bar graphs.

v) Fig S5b: at 24h the velocity errors point from the PSM to the NMP cluster? This contradicts the known in vivo trajectory.

vi) Fig S5b indeed shows that the NMPs at 24h and 48h are mono-fated. So are these cells really NMPs?

vii) FigS5b: at 98h, no single velo arrow points from the NMPs to the PSM, which contradicts known in vivo biology and the notion that the NMPs at this timepoints give rise to somites. Also, arrows from the neural cluster point to the NMPs.

viii) The authors claim that “the cell types generated in the two systems are similar”. The authors should provide a more detailed analysis to provide support for this claim: A) direct comparison of cell clusters (conserved and differential markers); B) comparative analysis of proportions of cell types in both systems (including somitic cell states A vs P, D vs V, …)

ix) The authors claim the presence of anterior (Tbx18+) and posterior (Uncx+) somitic cells in the merged data-set. It is unclear to me why the authors performed this analysis on the merged instead of the separate data-sets (segmentoids vs somitoids). Also, given the total number of somitic cells, the fraction of cells expressing (one of) the markers appears very low, especially in somitoids. The authors should quantify this, and explain why so few somitic cells express A or P markers. Also, could the low fraction of somitic cells expressing A or P markers reflect somites being in an immature state? Of course I acknowledge that the authors show in Fig 1 that a large fraction of the somites expresses Uncx; why this discrepancy between the imaging and scRNA-seq data? Finally, A and P identities being dispersed suggests the sub clustering is driven by a different distinction of somitic cell states, which the authors should check (see point below)

x) Do the data provide evidence for dorsal and ventral transcriptional cell identities? Dermomyotome vs sclerotome signatures? Migratory precursors, syndetome, endotome? How do the somitoids and segmentoids compare with regards to these signatures?

Minor points:

1. The authors should make clear that the stable expression of mCherry in the anterior part of the more mature somites is observed owing to the use of an H2B reporter. This is not explained clearly in the manuscript, and for non-specialists the discrepancy between the in vivo expression of MESP2 and the reporter expression will be extremely confusing.

2. For the somitoids protocol the authors transfer the spheroids to laminin coated substrates according to the main text, but in the Methods they describe the coating as laminin or gelatin. Same for the somitoid dissociation-aggregation experiments. The authors should quantify and discuss if there are differences between the use of laminin and gelatin. Given that laminin is much more expensive than gelatin, one wonders why laminin would be used at all if gelatin also works?

3. The authors describe that the somite-like rosettes in somitoids have a laminin-rich basal lamina. Were these somitoids generated using gelatin or laminin as a substrate? In case of the latter, is the laminin detected at the basal lamina native or exogenous?

4. Could the authors speculate why the expression of the HES7 reporter ceases after ~72h in somitoids?

5. For the somitoids dissociation-reaggregation experiments, dissociations were performed at several timepoints. From the methods section it seems that the subsequent culture conditions were always the same, independent of the timepoint of dissociation. Is this correct? The authors should clarify this in the Methods.

6. The authors should perform dissociation-reaggregation experiments with the segmentoids at similar timepoints as the somitoids. One would expect that if the already formed somites are dissociated and reaggregated individual somites would not display AP polarity (as in the somitoids). On the other hand, when dissociating and reaggregating cells from structures prior to somite formation, one might expect that the somites still display AP polarity (as opposed to somitoids).

7. The authors suggest that “rosette formation is an actomyosin dependent self-organising property of cells differentiated to the somite stage and does not depend on a prior prepatmtern established by the clock and wavefront system”. These findings should be discussed in the light of similar findings reported in Dias et al., 2013 (“Somites without a clock”) and Veenvliet et al, 2020 (TLS modulated with CHIR and LDN that produce multiple self-organised somites with each cycle).

8. Did the authors titrate the % of Matrigel to demonstrate the concentration range permissive for segmentoid formation?

9. For somitoids, laminin or gelatin substrate is permissive for morphological somite formation. Did the authors test if for segmentoids Matrigel can be substituted with laminin and/or gelatin supplementation? If not, could the authors speculate why segmentoids, but not somitoids, need Matrigel for morphological somite formation?

10. The authors show Hox collinearity in the segmentoids. Did the authors perform this analysis (Fig S6a) on all the cells, or a subset (NMP, PSM?). The latter would be more informative (is the Hox code indeed layed out in the axial progenitors as is the case in vivo?). Also, what is the Hox code of the somites produced in the somitoids? Are these representing the somites at a single or at multiple axial levels? This is not clear from the analysis performed in Fig S6b, but instead requires in situ / immuno labelling experiments.

Referee #2 (Remarks to the Author):

In order to elucidate the mechanism of human somite formation, the authors reproduced human somite formation in vitro by constructing two different types of 3D culture systems using iPS cells. One is termed Somitoid, in which oscillatory expression of the segmental clock gene Hes7 was observed, followed by initiation of Mesp2 expression, and formation of Pax3-positive somite-like rosettes. By using this system, the authors found that the rosettes are formed independently of Hes7 and Mesp2, but depend on ROCK and myosin II. In Somitoids, Mesp2-expressing cells with an anterior identity and Uncx4-expressing cells with a posterior identity aggregated exclusively. A line of evidence strongly support that the sorting of Mesp2-high cells forms a mutually exclusive pattern of rosettes. Next, the authors examined the mechanism of antero-posterior (A-P) polarity formation using Segmentoid, another iPS-derived organoid that recapitulates the special pattern of somites, including the A-P patterning within a somite. In Segmentoids, Hes7 was oscillated in the region corresponding to the PSM, and the expression of Mesp2 was localized to the anterior region within each somite. In contrast, in Hes7-deficient Segmentoid, Mesp2- and Uncx4-expressing cells did not form a clear nested A-P pattern. These results suggest that the segmentation clock is not essential for the specification of the anterior and posterior identities, but for the formation of their striped spatial patterns. Finally, the authors noted that Mesp2 mRNA expression is detected in salt and paper in mouse and chicken PSM, suggesting that cell sorting actually occurs even in in vivo somitogenesis. Based on these results, they concluded that cell sorting plays an important role in the formation of A-P polarity within a somite.

This paper presents two important results for elucidating the mechanism of somite segmentation. One is that the authors successfully reproduced human somite formation using two different types of 3D in vitro systems. Another one is that the authors proposed a new model of vertebrate somite formation that involves two steps: "cell fate determination, which is independent of the segmental clock," and "cell rearrangement along the anterior-posterior polarity by cell sorting”.

The experiments were carefully designed, and the results obtained with their in vitro systems were accurately described and rationally interpreted. Especially, I did not find any serious problem in the description of the generation and characterization of the organoids. As for the formation of A-P polarity of somite, the result that A-P polarity is formed without clock propagation is very surprising and will have a great impact on our understanding of somite patterning mechanisms. On the other hand, I think there is still room for improvement to make the conclusions more certain.

1. Fig. 4a: Judging from this data, it seems that the wave of oscillatory expression of Hes7 was not traveled from posterior to anterior in the PSM. I would like the authors to clarify this point in the

text and discuss the possibility that the lack of wave propagation emphasizes the effect of cell sorting in A-P patterning of somites. I am also curious how the segmented clock is reflected in the A-P patterning without forward propagation of clock oscillations, and would like to ask the authors for a possible explanation.

2. Fig. 4g: In order to clarify the role of Hes7 in A-P patterning, I suggest that the authors analyze the abnormalities of A-P patterning in the Segmentoid system of the Hes7 mutant in more detail. For example, the time-course analysis shown in Figure 4H of wild-type segmentation may also be useful for that of the Hes7 mutant.

3. Fig. 4j right: In this cell tracing analysis, the authors should increase the number of Mesp2-high cells and also examine Mesp2-low cells to verify whether the sorted patterns of Mesp-high and -low cells are significantly different.

4. Fig. 4k, l, It would be better to trace the movement of Mesp2-high cells in vivo as well. If this is difficult, how about showing the staining patterns of Mesp2-positive cells shifted to the anterior side using embryos at different phases of the segmentation cycle?

Specific point
1. Extended data Fig.2: “No MESP expression” shown at the top of this figure is not correct. My understanding is that Mesp2 is still being expressed, but perhaps its devo production has stopped during this time.

Referee #3 (Remarks to the Author):

In this manuscript, Miao et al describe two novel systems to study human somitogenesis. In the first, termed somitoids, iPSC spheroids were induced to PSM fates and then plated on laminin. Over the next 48-72 hours, epithelial structures resembling somites emerge. The authors show that this process involves 3-4 oscillations of the segmentation clock (as read out by a HES7 reporter), followed by upregulation of MESP2 and then PAX3. Interestingly, the culture does not form structures with AP polarity but rather separate rosettes with the characteristics of anterior and posterior segment halves. This separation is achieved through cell sorting, is still present in HES7-/- mutants, and is blocked by Rock-Inhibitor. In the second model, termed segmentoids, iPSCs are first differentiated towards PSM fates, then aggregated and placed in 3D culture in Matrigel. These segmentoids then elongated and create clusters of somite like objects at one end, which is identified as the anterior side. In contrast to the somitoid model, the segments in these structures have AP polarity. It is argued that the cell sorting mechanism which creates separate structures in somitoids, also creates this AP pattern in segmentoids. Overall, these are interesting models which will be very useful for studying human somitogenesis, and the experiments are rigorously performed and clearly presented. However, there are some technical issues with the manuscript, and I am not convinced that the data support that the cell sorting mechanism is operating in segmentoids, and by extension, in vivo. The authors should also address a recently published paper which creates similar structures also called ‘somitoids’. These concerns are listed below:

1. With regard to the cell sorting mechanism, some of the authors’ data argue against it being the primary mechanism in segmentoids. Rock inhibitor, which should disrupt this sorting, does disrupt the separation of anterior and posterior structures in somitoids but does not disrupt AP patterning in segmentoids. Further, disruption of the segmentation clock through HES7 knockout does not affect the separation of anterior and posterior structures in somitoids, but does abrogate AP patterning of segments in segmentoids. Together, these data seem to argue that while cell sorting separates the two types of structures in somitoids and the segmentation clock is dispensable for this, the progression of the segmentation clock is essential for delineating the A-P pattern in segmentoids, and in this case cell sorting is dispensable (although looking at the images in Figure S7, it may sharpen boundaries).
2. If the cell sorting mechanism were the primary mechanism in segmentoids and in vivo, as cell sorting is a random process, it hard to see what would guarantee that the “anterior” fated cells actually end up in the anterior. This information would seem to need to come from an external source such as the clock. Can the authors’ model explain not only the separation of anterior and posterior but the correct polarity in each segment?
3. Even in the somitoids, it is not completely convincing that cell sorting is the only mechanism involved in separating anterior and posterior. There are other ways that a salt-and-pepper pattern could be resolved, such as cells switching fate. Could these also play a role here? From the data in S2g, it is unclear whether the fraction of MESP2+ cells is constant over time as the sorting model would predict. Could the authors quantify this?
4. Looking at the data in movies 2, 4, it appears that MESP2 expression is biased towards the center of the somitoid while UNCX is biased towards the edge. This should be quantified. It also seems to undercut the notion that the anterior and posterior rosettes are randomly distributed resulting from cell sorting.

5. For the in vivo data in 4k,l, it is unclear where the boundary of the forming segment that is expressing MESP2 is. Is MESP2 expressed in the entirely segment in a salt and peppter pattern or is it biased towards the anterior portion? If the sorting model is correct, one would not expect a bias in MESP2 at the outset. Could the authors indicate the segment boundary in the image?
6. In Fig 4c, it seems from the images, that each stripe of MESP2/UNCX contains several epithelial structures that can be seen in the DAPI image. Could the authors provide a merged image or some other way to see the relationship between the stripes of MESP2/UNCX and these epithelial structures? Even better would be to show images of MESP2 or UNCX merged with N-Cad or other markers which would delineate these structures.
7. In the scRNA seq data in 3f,I why does such a small percentage of the cells labeled "somite" express MESP2, UNCX, or TBX18? Is this an issue of dropouts in the data or is it something biological?
8. The data in S6 are not so clear in supporting the colinear activation of HOX genes. Could the authors plot this in a more informative way, maybe with time on the x axis and the expression of different HOX genes on the y axis so that the sequence of expression can be better visualized?
9. In line 192, the SOX2 expressing cells are referred to as "neural tube-like structures". I have a hard time seeing this from the images. This should be better explained and higher resolutions images provided.
10. In Fig 2k,n, I found the color of the diagrams confusing. Throughout the figure, MESP2 cells are marked in magenta, but here the magenta seems to indicate the whole population collected at 72h whether MESP2 positive or not. I would suggest using a different color.
11. The authors should cite and discuss Budjan et al eLife 2022 which creates different structures termed somitoids. The senior author here is also a coauthor of that manuscript and it has been included as a related manuscript here but is not discussed.

Referee #4 (Remarks to the Author):

Using human iPS, the authors established two culture systems to produce “Somitoids” and “Segmentoids”, which reproduced parts of somitogenesis in human. The major target of this article was to reveal mechanism of how anterior and posterior sub-compartment of single somite is established. Using reporter genes to mark specific events in somitogenesis, the authors clearly showed that Somitoids reproduced early temporal events for initiating somitogenesis without special organization. However, they found Somitoids are composed of two distinct cell populations (MESP2-high and MESP2-low) and those are segregated by sorting mechanism. To reveal whether this sorting mechanism is also involved in the A/P compartmentalization in vivo, the authors examined MESP2-reporter expression in Segmentoid and proposed that this would be the case.

This article proved that most of events observed in somitogenesis are reproduced in Segmentoids and the reproduction system is useful for the further study. However, most of parts were confirmation of events observed in mice. One new finding is the possible involvement of sorting in the A/P compartmentalization in vivo. However, the evidence is rather weak and no molecular cue underling the sorting mechanism was addressed at all even though they performed single cell transcriptome analyses. In addition, there are several questions should be addressed.

Specific comments

1. Title: I could not get why the authors use the words “Reconstruction and deconstruction” in the title. I could not find any explanation in the text. What does deconstruction mean?

2. Abstract: the following sentence is vague.
Line 35-37; we demonstrate a large degree of independence of the various patterning modules involved in somitogenesis including the segmentation clock, somite epithelialization and AP polarity patterning.

3. Line 51-52; The periodicity of somite formation involves a molecular oscillator called the segmentation clock. This oscillator controls the rhythmic activation of Notch, Wnt and FGF pathways which manifest as traveling waves of target gene expression in the posterior PSM.
What is the oscillator?

4. Line 86-87; HES7 signals initiated from the peripheral region of the spreading organoid and propagated as concentric waves toward the center.
Cells proliferated actively since the size of organoid enlarged. The initiation of HES7 signal from peripheral region is interesting. What kind of cells located in the periphery?
Is there any counting mechanism (of cell cycle) will be responsible for the Hes7 initiation? What is the upstream of Hes7?

5. Line 90-91; onset of MESP2 immediately follows the arrest of HES7 oscillations.
What is the other condition for the onset of MESP2. Is there any indication from single cell transcriptome analyses? Is there any meaning for about 4 cycles of HES7? It was shown that MESP2 can be activated in the absence of HES7 protein, indicating that arrest of Hes7 oscillation is not

required for MESP2 activation.
DAPT treatment prevented expression of UNCX and MESP2 expression. How about HES7 oscillation? When do the authors treat with DASP? They should indicate the time of drug treatment in each Figure legends.

6. Line139-141; need more explanation with reference. What kind of mechanism is predicted and what can be addressed by the reporter system here.
The reporter construction is not clearly shown here and in the reference. Is this MESP2 knockin reporter that allow MESP2 protein translation with t2a-H2B-mcherry? Or is this a transgene driven by MESP2 promoter-enhancer ?
I would like to know what is reported by this reporter. Does it report transcription activity with extended stable mcherry protein expression (this is Trajectory of MESP2 expression?)

7. 72 h Somitoid cells showed sorting ability but 96h cells lost the ability even mcherry signal intensity is different. This is very interesting but the molecular mechanism is not addressed at al. This result indicates that molecule responsible for cell sorting is transiently expressed at 72 h and disappear after 96 h. What kind of molecule would be candidate?

8. Segmentoid formation is also very interesting especially for the elongation with AP polarity. What is the mechanism to determine the budding point and the direction. Is there any cellular bias accounting for the initial symmetry breaking?

9. There are several segmental structures in Segmentoid. One has single row of segments, but others have grape-like segments. Is there any common features between rosettes formed in somitoid and somite-like structure in Segmentoid.

10. The authors suggested a sorting mechanism between MESP2-high and low cells is responsible for the establishment of A/P polarity of a somite. It is likely but the direct evidence is weak. Because in vitro culture is possible, some single cell tracking data should be presented.

11. Line 262-265; the segmentation clock is not required for the expression of AP identity genes in individual cells, but its output conferring rhythmicity to MESP2 induction and segment determination appears to play an important role in the spatial organization of stripes of anterior and posterior identity in the forming somites.

This part is difficult to understand if sorting mechanism is only based on the MESP2 expression. What is the function of rhythmicity? The authors speculate A/P patterning is generated by sorting mechanism depending on the level of MESP2 expression. In the absence of segmentation clock, MESP2 expression is uniformly induced in any cells that prevent cell sorting (?). This may be explained by nematic order parameter (in extended dataFig.7d, lower in HES7-null), but it is difficult to follow. How is A/P random patterning in HES7-null established?

Author Rebuttals to Initial Comments:

Detailed responses are listed below:

Referee #1 (Remarks to the Author):

In this work, Miao, Pourquie and colleagues present two 3D models of human somitogenesis: somitoids and segmentoids. Whereas somitoids only recapitulate the temporal component of somitogenesis, segmentoids recapitulate both space and time. The authors then use the modularity and accessibility of the models to demonstrate that an active cell sorting based on differential expression levels of Mesp2 underlies the establishment of anterior and posterior somite compartments. The authors conduct some beautiful experiments to dissect the (in)dependence of distinct patterning modules involved in somitogenesis. In general, the work elegantly demonstrates the power of in vitro systems. However, I have some significant concerns regarding:
i) The initial characterisation of the in vitro systems, which is not fully up to the standards in the field.
ii) The lack of mechanistic understanding what drives the cell sorting.
iii) The claim that the sorting mechanisms identified appear to be conserved in vivo.
I will detail these concerns, as well as some other points, below.

Major points:

1. In the meantime, the Budjan et al. "somitoids" manuscript the authors added to the submission has been published in eLife (DOI 10.7554/eLife.68925). The authors should therefore: i) include this publication in the introduction of the submitted work; ii) discuss the differences and commonalities between the published somitoids and the somitoid system presented in the submitted work; iii) demonstrate and discuss the additional value of the new somitoids system.

We now discuss both Budjan et al, Elife 2022 and Sanaki-Matsumiya et al, Nat. Commun. 2022 in the introduction of the revised paper. Budjan et al reported a very low efficiency in rosette formation without clear AP patterning, compared to the Somitoid protocol described in our paper. Sanaki-Matsumiya et al used a protocol different from ours to capture the sequential somite formation coupled with the segmentation clock. This study only contains the description of the experimental protocol allowing to generate paraxial mesoderm organoids resembling the Segmentoids described in our paper. These two papers are mostly technical and do not really use the systems to perform mechanistic studies and answer a biological question, which is in our view the strength of our manuscript.

2. The somitoid and segmentoid system should be much better characterised; proper quantifications should be performed:
i) The authors should provide a thorough morphometric analysis for both the whole structure as well as the substructures (similar to the one performed in Veenvliet et al, Science 2020 for trunk-

like-structures). E.g. area, perimeter, circularity, somite size, somite number, etc
ii) These morphometric data should be compared to the embryo
iii) The authors should report reproducibility metrics: they e.g. state that “the posterior end sometimes appears bifurcated”. Which % of the segmentoids develops a single axis? Which % forms a double axis? What is the elongation efficiency? What is the somite formation efficiency?

We have performed extensive morphometric analysis shown in Extended Data Fig.1d-f and Extended Data Fig.4c-g. We show that the somite-like rosettes in both models displayed a median projected area of ~5000 µm^2 (Extended Data Fig.1d, Extended Data Fig.4c) and relatively circular shape (Extended Data Fig.1f, Extended Data Fig.4d), which are consistent with those in mouse embryos and TLS described in Veenvliet et al, Science 2020. Interestingly, the rosettes had a fixed length scale which did not scale with the overall Somitoid size (Extended Data Fig.1e). Furthermore, both models have a nearly 100% efficiency in somite-like rosette formation. Segmentoids had a >90% efficiency in elongation and 20-30% had more than one axis in 10% Matrigel (Extended Data Fig.4f,g). All these new data have been added to the revised manuscript.

iv) From the (live) imaging data it seems that, at least in some cases, the segmentoids produce multiple somites with each cycle of the segmentation clock (similar to the TLS-CL reported by Veenvliet et al., Science 2020). Is this indeed the case? In what percentage of the structures does this happen? How many somites are formed with each cycle? How does this evolve over time?

We quantified the number of rosettes generated in each row of segments that displayed correct AP polarity and found that 1-4 adjacent rosettes were produced in each segment during each cycle of the segmentation clock (Extended Data Fig.8e). In contrast, only ~1 rosette per segment was produced in the AP dimension (Extended data Figure 8d). The caudal domain of the Segmentoids displayed a shift towards the lower end of the distribution compared to the rostral region, suggesting that fewer rosettes were formed in each segment over time. The majority of the rosettes of the segment displayed correct AP polarity patterning irrespective of the number of rosettes along medial-lateral axis. This is in contrast from the TLS-CL reported by Veenvliet et al which showed disrupted polarity patterning. These new data have been added in the revised version of the manuscript.

v) For immunostainings, the authors should report the penetrance of the shown staining (n structures with similar staining pattern / n structures analysed)

We added this information in the figure legends in the revised version.

vi) Instead of (or complementary to) quantifications of the individual structures shown for AP somite polarity (eg 4c, S7c) the authors should demonstrate reproducibility for a high(er) number of structures, showing intra-/inter-experimental variability, provide a statistical testing for e.g. CTRL vs ROCKi, etc

We added the quantification of the percentage of Segmentoids with somite AP polarity in different conditions (Fig.4k) and the corresponding statistics in the legend of Fig.4k of the revised manuscript.

3. Despite some beautiful experiments, it remains unclear what drives the self-sorting. I feel there is a missed opportunity here to provide mechanistic insights with the unique experimental systems the authors have at hand. The experiments the authors performed should already provide hints: is there differential expression of morphogenetic regulators (adhesion, actin-cytoskeleton, …) in Mesp2-high vs Mesp2-low cells? The authors might also be able to leverage the scRNA-seq data, by e.g. performing "in silico sorting" of aPSM cells and/or check for genes that covary with Mesp2 expression levels. They should then check if these differences are also observed in vivo (see point below).

We thank the reviewer for these suggestions. As the sorting capacity of cells appear transient during differentiation (72-80hr) (Fig.2l-n), our original RNAseq experiments of mature Anterior or Posterior cell fractions performed at 120h (Fig.2c) did not reflect the molecular signature during the earlier process of cell sorting. Thus, we conducted another RNAseq experiment on purified MESP2-high and MESP2-low cell fractions at the onset of cell sorting (72h) (Extended Data Fig.3, Supplementary Table2). We found that cells with high or low MESP2 expression displayed differential adhesion codes (Extended Data Fig.3a,b) as well as differential expression patterns of Ephrin family members (Extended Data Fig.3c,d). This analysis identified PCDH8, EPHA4, EPHB3 which have been previously implicated in somitogenesis in vivo (Rhee et al, Dev Biol 2003; Chal et al, Development 2017; Durbin et al, Development 2000; Nakajima et al, Development 2006; Watanabe et al, PNAS 2009). Additionally, a range of genes encoding regulators of the cytoskeleton also showed differential expression patterns (Extended Data Fig.3e). Most of the differential expression patterns had disappeared in the 120h RNAseq dataset (Extended Data Fig.3a,c,e). Thus, these differential expression patterns paralleled the transient sorting ability of the cells suggesting that they might provide a genetic basis for this behavior (Fig.2l-n). This also demonstrated the power of the Somitoid model in which cells are synchronized with respect to their developmental stage. Such experiments would not be possible using the Segmentoid-like or embryo models.

These new experiments led to the identification of the gene *TIAM1*, which encodes an important regulator of RAC1 activity, as enriched in the anterior MESP2+ compartment (Fig.2s). RAC1 is a key regulator of the cytoskeleton which has been shown to play a role in somitogenesis (Nakaya et al, Dev Cell 2004). Overexpressing Tiam1 has been widely used to modulate RAC1 activity (Inoue et al, Nat Methods 2005; Mehidi et al, Current Biol 2019). We introduced a DOX-inducible form of Tiam1 in the background of the triple HES7-Venus, MESP2-mCherry, UNCX-Venus iPS reporter line. Ubiquitous overexpression of Tiam1 during the sorting phase did not prevent acquisition of an anterior or posterior identity in PSM cells but it prevented the formation of anterior and posterior

compartments in both Somitoids (Fig.2t, Extended Data Fig.3f,g) and Segmentoids (Fig.4h,i,k). Remarkably, oscillations of the segmentation clock were maintained but rosette formation was abolished. This contrasts with ROCKi treatment which lack rosette formation but display proper AP patterning (Somitoid - Fig.2d, Extended Data Fig.3g; Segmentoid - Fig.4j,k, Extended Data Fig.9f). These experiments point to a role for cytoskeleton remodeling downstream of RAC1 in the sorting process. We have added these important new results hinting at a molecular mechanism underlying cell sorting in the revised version.

4. The authors claim in vivo relevance, but the experimental evidence for this (HCR on in fixed embryos) is extremely limited (and in fact does not show any cell sorting since the data are not time-resolved). The authors should generate a Mesp2 reporter line similar to the human one and culture embryos ex vivo to demonstrate that cell sorting takes place in vivo. This would also e.g. allow for the sorting of Mesp2-high vs Mesp2-low cells to show that differential expression of morphogenetic regulators (see point 3) is conserved in vivo.

To introduce time resolution, we performed experiments where chicken embryos were split into halves with one half fixed immediately while the other was subjected to in vitro culture before fixation as described in Palmeirim et al., Cell (1997). Both halves were then hybridized with a fluorescent MESP2 probe using the HCR protocol. An important point demonstrated by this analysis is the fact that, in vivo, MESP2 activation in the segmental domain is not uniform as commonly represented but rather salt and pepper as observed in vitro. We next compared the distribution of *MESP2* expression in time. Our analysis showed that after culture, more cells with high MESP2 signals were located in the anterior portion of the segmental domain resulting in decreased pixel heterogeneity both at the anterior and posterior portions (Fig.4m, Extended Data Fig.10c-f). This is similar to what we observed in the Somitoids where at 84h, the surrounding mCherry intensities of the tracked MESP2-high cells were higher than for MESP2-low cells (Fig.2k, Extended Data Fig.2n). These results are consistent with an involvement of cell sorting in vivo.

5. To me, it still remains unclear why the segmentation clock would be needed to achieve AP patterning within single somites. I understand how the segmentation clock will be important to allocate pre-somitic tissue (segmental stripes), but as I pointed out above it appears that in many cases more somites than expected (i.e. >2) are formed during the same cycle. Why do these somites (generated in parallel) nevertheless establish A and P domains (and not form separate "full A" and "full P" somites as in the somitoids)?

In the embryo, the width of the segment is defined by the width of the PSM, which is highly constrained in vivo (by the adjacent neural tube/notochord and lateral plate), but not in vitro. While the normal width of the PSM is approximately that of one somite, ie ~100 microns, in Segmentoids, the PSM can be much larger. In contrast, the AP size (length) of the segment appears constrained in vitro, independently of the width, and it corresponds

most of the time to one rosette (Extended data Figure 8d). Given that the rosette size is largely invariant, if the available PSM is larger than one rosette, then several aligned rosettes will form the segmental row, with each rosette showing a normal AP pattern (Extended data Figure 8e). This nicely illustrates the independence of the three patterning mechanisms (clock, AP and rosettes).

The constrain on the segment AP length can be easily explained by the clock and wavefront model which stipulates that the length is defined by the distance traveled by the wavefront during one oscillation of the clock. As differentiation is synchronized at the different AP levels of the Segmentoid, one would predict that in all Segmentoids, the posterior regression of the wavefront proceeds at the same pace, independently of their width. As the clock parameters appear conserved among the Segmentoids, then one predicts that the length of the segments specified by the clock and wavefront will be completely independent of the width of these segments. We showed that rosette formation is an intrinsic property of the cells and display a size invariant. Thus, once the segment is specified, the rosette (s) subsequently form in this geometrically constrained environment defined by one rosette in length, and a variable number of rosettes in width.

The segmentation clock determines the successive segmental domains where cell maturation is initiated in a synchronized manner. Inside each region, the activation of the cell sorting process rearranges MESP2-high cells to the anterior part and MESP2-low cells to the posterior, which completes rapidly in 1-1.5 clock cycle before rosette formation. Thus, as long as the AP length of the forming somite is one typical somite length as controlled by the segmentation clock, somite morphogenesis will be associated with correct AP patterning. The number of somites generated per segment only depends on the lateral width of the tissue and is independent of AP patterning.

6. I have multiple concerns and suggestions regarding the scRNA-seq analysis:
i) in both segmentoids and somitoids the authors report the identification of NMPs. However, are these cells truly bipotent. Their progeny surely suggests the cells are mono-fated, at least at earlier timepoints (also see comment below)? The authors should carefully analyse if these cells are true NMPs (e.g. compare to in vivo transcriptional signatures), or if they are already fate-biased (similar to what was reported for the “NMPs” in chemically modulated TLS (TLS-CL) in Veenvliet et al., Science 2020.

In vivo, the NMPs constitute a cell population with a well-defined transcriptional identity but whose fate is highly variable and depends on their developmental history. We have performed lineage tracing analysis of the NMPs in the chicken embryo and some are clearly bipotent while others only give rise to one or the other lineage (Guillot et al, elife 2021), yet all these cells belong to the same population expressing T and SOX2 as well as other markers such as NKX1.2. The cells identified as NMPs in our Somitoids and Segmentoids express these markers, strongly suggesting that they are NMPs. To further confirm the similarity of these cells to NMPs in vivo, we have trained a machine learning classifier with clusters identified in the posterior region of mouse embryos at E9.5 that we

recently published (Dias-Cuadros et al, Nature 2020). This classifier was then able to accurately recognize the corresponding clusters of human cells including the NMPs. We now include this new data in the revised version of the paper (Extended data figure 7c-g).

ii) Are the authors sure the 24h clusters are NMPs? Based on marker expression (Pou5f1, Sox2, T) these could also be epiblast(-like) cells.

NMPs are definitely epiblast cells. They occupy the anterior region of the epiblast, adjacent to the anterior primitive streak and the node in mouse as shown by Val Wilson and in chicken as shown by us and others.

There clearly seem to be three clusters of NMPs in 3e (left, 24h; middle, 48h; right, 72/96h). The authors should carefully check the transcriptional differences between these clusters, and how these compare to the in vivo signatures.

These three clusters clearly reflect the maturation of the NMP population in time. We have performed detailed analyses of the maturation of NMPs using scRNAseq both in chicken and mouse embryos (Guillot et al elife 2020) and identified some changes in time such as the onset of Hox activation. This is also observed in vitro in the Segmentoids further supporting the similarity between NMPs in vivo and in vitro. This new data is now shown in extended Figure 7k.

iii) The PAGA graphs in Fig. 3g are an extreme oversimplification of the data, and in fact do not match well with the scvelo data in Fig. S5b (also see comments below)

The PAGA data is robust and identifies the main developmental trajectories. Indeed the identified trajectory arising from the NMP population is well-characterized in vivo and in vitro now. As correctly identified by the reviewer, there was a problem with the scVelo data which we have corrected in the revised version.

iv) The proportions quantified in Fig. S5a do not match the data shown in S5b: at 24h the proportions bar graph only reports NMPs, whereas in b it is clear there are also PSM cells. Same for the neural cells at 72h, that are there in the velo map, but not in the proportion bar graphs.

We apologize for the oversight as a wrong graph ended up in S5b. We have now replaced it with the correct bar graph showing the proportion of cells of each cluster in the total population.

v) Fig S5b: at 24h the velocity errors point from the PSM to the NMP cluster? This contradicts the known in vivo trajectory.

We thank the reviewer for pointing out this issue. We have redone the scVelo analysis and obtained the correct trajectories. We have introduced these new data in the revised version.

vi) Fig S5b indeed shows that the NMPs at 24h and 48h are mono-fated. So are these cells really NMPs?

As discussed above, in the embryo, many NMPs are also monofated.

vii) FigS5b: at 98h, no single velo arrow points from the NMPs to the PSM, which contradicts known in vivo biology and the notion that the NMPs at this timepoints give rise to somites. Also, arrows from the neural cluster point to the NMPs.

As mentioned above, the new scVelo analysis in the revised version now shows the expected relationships between clusters.

viii) The authors claim that "the cell types generated in the two systems are similar". The authors should provide a more detailed analysis to provide support for this claim: A) direct comparison of cell clusters (conserved and differential markers); B) comparative analysis of proportions of cell types in both systems (including somitic cell states A vs P, D vs V, …)

We have used a machine learning classifier which was trained on the Segmentoid clusters and show that it accurately recognizes the equivalent clusters from the Somitoid dataset (Extended Data Fig.7f,g).
The second type of comparison requested is interesting but given that the samples don't have exactly the same age, it is unlikely to be very informative. The limited number of UNCX and TBX18-positive cells identified in the dataset suggests that the most mature stage of the Somitoid series is quite immature (presomite) compared to the most mature stage of the Segmentoid series which harbors a much larger number of these cells. At these stages, there is no DV polarity in the somites yet. We now discuss these issues in the revised text.

ix) The authors claim the presence of anterior (Tbx18+) and posterior (Uncx+) somitic cells in the merged data-set. It is unclear to me why the authors performed this analysis on the merged instead of the separate data-sets (segmentoids vs somitoids). Also, given the total number of somitic cells, the fraction of cells expressing (one of) the markers appears very low, especially in somitoids. The authors should quantify this, and explain why so few somitic cells express A or P markers. Also, could the low fraction of somitic cells expressing A or P markers reflect somites being in an immature state? Of course I acknowledge that the authors show in Fig 1 that a large fraction of the somites expresses Uncx; why this discrepancy between the imaging and scRNA-seq data? Finally, A and P identities being dispersed suggests the sub clustering is driven by a different distinction of somitic cell states, which the authors should check (see point below)

As explained above and correctly hinted by the reviewer, the difference in cells expressing AP markers between Somitoids and Segmentoids is most likely due to the immaturity of the cells of the last data point of the Somitoid dataset which are at the presomite stage. The cells of the Segmentoid dataset appear to be slightly more mature than the Somitoid cells at 98h. While the late time points analyzed are similar (98h), the protocols are quite different,

and it suggests that the maturation speed is faster in the Segmentoid context. We introduced this description in the revised text.

x) Do the data provide evidence for dorsal and ventral transcriptional cell identities? Dermomyotome vs sclerotome signatures? Migratory precursors, syndetome, endotome? How do the somitoids and segmentoids compare with regards to these signatures?

We do not detect any differentiation markers in our Somitoid or Segmentoid dataset suggesting that the most mature stages reached are forming/newly formed somite. This is now discussed in the revised text.

Minor points:

1. The authors should make clear that the stable expression of mCherry in the anterior part of the more mature somites is observed owing to the use of an H2B reporter. This is not explained clearly in the manuscript, and for non-specialists the discrepancy between the in vivo expression of MESP2 and the reporter expression will be extremely confusing.

We now provide a more detailed description of the reporter in the revised text (line 84-88, 157-158).

2. For the somitoids protocol the authors transfer the spheroids to laminin coated substrates according to the main text, but in the Methods they describe the coating as laminin or gelatin. Same for the somitoid dissociation-aggregation experiments. The authors should quantify and discuss if there are differences between the use of laminin and gelatin. Given that laminin is much more expensive than gelatin, one wonders why laminin would be used at all if gelatin also Weworks?

We found that culturing Somitoids on laminin, gelatin, or in suspension without any substrate generated similar rosettes as shown in Extended Data Fig.1i,j. The coated substrates were used to promote adhesion and spreading of the spheroids to perform live-cell imaging. For time lapse confocal imaging we chose laminin instead of gelatin as we had to use glass or the ibidi thin polymer bottom plates instead of classical plastic vessels. Gelatin coating on glass or polymer did not support efficient spreading of the tissue often leading to retraction during image acquisition. We have introduced these details in the Methods section of the revised paper.

3. The authors describe that the somite-like rosettes in somitoids have a laminin-rich basal lamina. Were these somitoids generated using gelatin or laminin as a substrate? In case of the latter, is the laminin detected at the basal lamina native or exogenous?

Even suspension culture gave rise to laminin-rich basal lamina (Extended Data Fig.1j), thus the laminin detected must be endogenously generated by the cells.

4. Could the authors speculate why the expression of the HES7 reporter ceases after ~72h in somitoids?
In vivo, once NMPs differentiate into posterior PSM, they experience a fixed number of oscillations until they reach the determination front and acquire an anterior PSM fate. A similar situation occurs in the Somitoid cultures. The posterior PSM cells can oscillate between the NMP and the anterior PSM stage which in Somitoids corresponds to a time window of ~4-5 oscillations. In the Segmentoids, oscillations are sustained because the NMP population is maintained, constantly adding new PSM cells posteriorly.

5. For the somitoids dissociation-reaggregation experiments, dissociations were performed at several timepoints. From the methods section it seems that the subsequent culture conditions were always the same, independent of the timepoint of dissociation. Is this correct? The authors should clarify this in the Methods.

Yes, the same subsequent culture conditions were used, since all the timepoints of dissociation are after 72h in the protocol where the same culture media is used. We further clarified this in the Methods.

6. The authors should perform dissociation-reaggregation experiments with the segmentoids at similar timepoints as the somitoids. One would expect that if the already formed somites are dissociated and reaggregated individual somites would not display AP polarity (as in the somitoids). On the other hand, when dissociating and reaggregating cells from structures prior to somite formation, one might expect that the somites still display AP polarity (as opposed to somitoids).

We thank the reviewer for this suggestion, but we would like to argue against this experiment considering the differences between the two models. The synchronized differentiation of the Somitoid model allowed a defined input population that is important for the interpretation of re-aggregation phenotypes, while the heterogenous cell composition at any timepoint of the Segmentoids would obscure the interpretation of the results of such an experiment.

7. The authors suggest that "rosette formation is an actomyosin dependent self-organising property of cells differentiated to the somite stage and does not depend on a prior prepatmtern established by the clock and wavefront system". These findings should be discussed in the light of similar findings reported in Dias et al., 2013 ("Somites without a clock") and Veenvliet et al, 2020 (TLS modulated with CHIR and LDN that produce multiple self-organised somites with each cycle).

We agree and have included such a discussion in the revised version (line 123-125).

8. Did the authors titrate the % of Matrigel to demonstrate the concentration range permissive for segmentoid formation?

In the revised version, we now show the effect of titrating the percentage of Matrigel on Segmentoids formation (Extended Data Fig.4e-g). We found that 1% is sufficient to support rosette formation, while a minimum of 5% is required for proper elongation.

9. For somitoids, laminin or gelatin substrate is permissive for morphological somite formation. Did the authors test if for segmentoids Matrigel can be substituted with laminin and/or gelatin supplementation? If not, could the authors speculate why segmentoids, but not somitoids, need Matrigel for morphological somite formation?

We found that supplementing laminin to Segmentoids did not support proper elongation or rosette formation (Extended Data Fig.4e). At least 1% Matrigel was needed to form rosettes in Segmentoids. We speculate that the CHIR and LDN present throughout the Somitoid culture promoted rosette formation without Matrigel, consistent with the findings reported in Veenvliet et al, Science 2020.

10. The authors show Hox collinearity in the segmentoids. Did the authors perform this analysis (Fig S6a) on all the cells, or a subset (NMP, PSM?). The latter would be more informative (is the Hox code indeed layed out in the axial progenitors as is the case in vivo?). Also, what is the Hox code of the somites produced in the somitoids? Are these representing the somites at a single or at multiple axial levels? This is not clear from the analysis performed in Fig S6b, but instead requires in situ / immuno labelling experiments.

The Hox gene analysis presented was performed on all the cells for both Somitoids and Segmentoids (Extended data Figure 7 i-j). We now also show the onset of HOX genes in the NMPs cluster of the Segmentoids in time indicating that most genes are first activated in these cells. Collinear activation of HOX genes is most obvious for the A and D clusters.

Referee #2 (Remarks to the Author):

In order to elucidate the mechanism of human somite formation, the authors reproduced human somite formation in vitro by constructing two different types of 3D culture systems using iPS cells. One is termed Somitoid, in which oscillatory expression of the segmental clock gene Hes7 was observed, followed by initiation of Mesp2 expression, and formation of Pax3-positive somite-like rosettes. By using this system, the authors found that the rosettes are formed independently of Hes7 and Mesp2, but depend on ROCK and myosin II. In Somitoids, Mesp2-expressing cells with an anterior identity and Uncx4-expressing cells with a posterior identity aggregated exclusively. A line of evidence strongly support that the sorting of Mesp2-high cells forms a mutually exclusive pattern of rosettes. Next, the authors examined the mechanism of

antero-posterior (A-P) polarity formation using Segmentoid, another iPS-derived organoid that recapitulates the special pattern of somites, including the A-P patterning within a somite. In Segmentoids, Hes7 was oscillated in the region corresponding to the PSM, and the expression of Mesp2 was localized to the anterior region within each somite. In contrast, in Hes7-deficient Segmentoid, Mesp2- and Uncx4-expressing cells did not form a clear nested A-P pattern. These results suggest that the segmentation clock is not essential for the specification of the anterior and posterior identities, but for the formation of their striped spatial patterns. Finally, the authors noted that Mesp2 mRNA expression is detected in salt and paper in mouse and chicken PSM, suggesting that cell sorting actually occurs even in in vivo somitogenesis. Based on these results, they concluded that cell sorting plays an important role in the formation of A-P polarity within a somite.

This paper presents two important results for elucidating the mechanism of somite segmentation. One is that the authors successfully reproduced human somite formation using two different types of 3D in vitro systems. Another one is that the authors proposed a new model of vertebrate somite formation that involves two steps: "cell fate determination, which is independent of the segmental clock," and "cell rearrangement along the anterior-posterior polarity by cell sorting”.

The experiments were carefully designed, and the results obtained with their in vitro systems were accurately described and rationally interpreted. Especially, I did not find any serious problem in the description of the generation and characterization of the organoids. As for the formation of A-P polarity of somite, the result that A-P polarity is formed without clock propagation is very surprising and will have a great impact on our understanding of somite patterning mechanisms. On the other hand, I think there is still room for improvement to make the conclusions more certain.

1. Fig. 4a: Judging from this data, it seems that the wave of oscillatory expression of Hes7 was not traveled from posterior to anterior in the PSM. I would like the authors to clarify this point in the text and discuss the possibility that the lack of wave propagation emphasizes the effect of cell sorting in A-P patterning of somites. I am also curious how the segmented clock is reflected in the A-P patterning without forward propagation of clock oscillations, and would like to ask the authors for a possible explanation.

The absence of wave detection is due to the kymograph analysis which might not have the resolution to capture the wave dynamics. Clear HES7 waves traveling along the PSM are visible in the Supplementary videos 7 and 9. We have modified the revised text to make this point clear.

2. Fig. 4g: In order to clarify the role of Hes7 in A-P patterning, I suggest that the authors analyze the abnormalities of A-P patterning in the Segmentoid system of the Hes7 mutant in more detail. For example, the time-course analysis shown in Figure 4H of wild-type segmentation may also be useful for that of the Hes7 mutant.

As suggested by the reviewer, in the revised version, we have added new time lapse analysis data of an *HES7*-null Segmentoid in Fig.4a and Extended Data Fig.8h. Movies of the MESP2 reporter showed that no clear segment could be observed in the null mutant as the MESP2 domain progressed continuously toward the posterior end (Fig.4a). This resulted in the formation of cell clusters with high and low MESP2 expression which were distributed randomly (Extended Data Fig.8h). We propose that the segmentation clock contributes to AP patterning by defining regular boundaries of maturation which organize cell sorting. In each cycle, the segmentation clock defines a stripe where the salt and pepper onset of MESP2 expression is synchronized among all cells; Since the cell sorting ability is transient, this synchronization restricts the sorting within the stripe. Within each stripe, the same cell sorting process rearranges MESP2-high and -low cells into an anterior and a posterior domain respectively in response to an AP cue that remains to be characterized. As this process repeats in time, the modular compartmentalization in each segment eventually constitutes the spatially regular, alternative stripes defining somite polarity along the body axis.

3. Fig. 4j right: In this cell tracing analysis, the authors should increase the number of Mesp2-high cells and also examine Mesp2-low cells to verify whether the sorted patterns of Mesp-high and -low cells are significantly different.

To allow efficient tracking of the MESP2-expressing cells, we generated Somitoids and Segmentoids from H2B-GFP/MESP2-mCherry reporter cells combined with MESP2-mCherry reporter cells at a 1:40 ratio. We performed single cell tracking of the H2B-labeled cells and recorded MESP2 intensity in the same cells (Methods). We showed that MESP2 reporter intensities in individual cells, high or low, remained unchanged during AP patterning both in Somitoids (Fig.2j,k, Extended Data Fig.2m,n) and Segmentoids (Fig.4f,g), as predicted by a cell sorting mechanism and arguing against an identity switch.

4. Fig. 4k, l, It would be better to trace the movement of Mesp2-high cells in vivo as well. If this is difficult, how about showing the staining patterns of Mesp2-positive cells shifted to the anterior side using embryos at different phases of the segmentation cycle?

To address the reviewer suggestion, we have split chicken embryos into their two halves, with one half fixed immediately while the other half was cultured in vitro for 45min before fixation. Then we performed fluorescent HCR staining on *MESP2* RNA on the two halves. As observed in Somitoids and Segmentoids and contrary to the classic view, we noted that *MESP2* initiation in the segmental domain is salt and pepper. We next compared the evolution of *MESP2* expression in time. Our analysis showed that after culture, more cells with high MESP2 signals were located in the anterior portion of the segmental domain resulting in decreased pixel heterogeneity both in the anterior and posterior portions (Fig.4m, Extended Data Fig.10c-f). This is similar to what we observed in the Somitoids where at 84h, the surrounding mCherry intensities of the tracked MESP2-high cells were higher than for MESP2-low cells (Fig.2k, Extended Data Fig.2n). These results are

consistent with the involvement of cell sorting in vivo.

Specific point
1. Extended data Fig.2: "No MESP expression" shown at the top of this figure is not correct. My understanding is that Mesp2 is still being expressed, but perhaps its devo production has stopped during this time.
Thank you for pointing this out. The reporter we used is t2a-H2B-mCherry inserted at the end of the endogenous MESP2 coding sequence. Given the long lifetime of mCherry protein, the constant red fluorescent signal during the indicated time window (after 72h) represent the absence of new MESP2 translation. Thus, we clarified the label as "No new MESP2 expression" shown in Extended Data Fig.2o. We have also included a better description of the MESP2 reporter in the revised text.

Referee #3 (Remarks to the Author):

In this manuscript, Miao et al describe two novel systems to study human somitogenesis. In the first, termed somitoids, iPSC spheroids were induced to PSM fates and then plated on laminin. Over the next 48-72 hours, epithelial structures resembling somites emerge. The authors show that this process involves 3-4 oscillations of the segmentation clock (as read out by a HES7 reporter), followed by upregulation of MESP2 and then PAX3. Interestingly, the culture does not form structures with AP polarity but rather separate rosettes with the characteristics of anterior and posterior segment halves. This separation is achieved through cell sorting, is still present in HES7-/- mutants, and is blocked by Rock-Inhibitor. In the second model, termed segmentoids, iPSCs are first differentiated towards PSM fates, then aggregated and placed in 3D culture in Matrigel. These segmentoids then elongated and create clusters of somite like objects at one end, which is identified as the anterior side. In contrast to the somitoid model, the segments in these structures have AP polarity. It is argued that the cell sorting mechanism which creates separate structures in somitoids, also creates this AP pattern in segmentoids. Overall, these are interesting models which will be very useful for studying human somitogenesis, and the experiments are rigorously performed and clearly presented. However, there are some technical issues with the manuscript, and I am not convinced that the data support that the cell sorting mechanism is operating in segmentoids, and by extension, in vivo. The authors should also address a recently published paper which creates similar structures also called 'somitoids'. These concerns are listed below:

1. With regard to the cell sorting mechanism, some of the authors' data argue against it being the primary mechanism in segmentoids. Rock inhibitor, which should disrupt this sorting, does disrupt the separation of anterior and posterior structures in somitoids but does not disrupt AP patterning in segmentoids. Further, disruption of the segmentation clock through HES7 knockout does not affect the separation of anterior and posterior structures in somitoids, but does abrogate AP patterning of segments in segmentoids. Together, these data seem to argue that while cell sorting separates the two types of structures in somitoids and the segmentation clock is dispensable for this, the progression of the segmentation clock is essential for delineating the A-

P pattern in segmentoids, and in this case cell sorting is dispensable (although looking at the images in Figure S7, it may sharpen boundaries).

We appreciate the reasoning, but we believe the reviewer misunderstood the data obtained with ROCK inhibitor (ROCKi) treatment. We showed that ROCKi blocks epithelial rosette formation but does NOT block the separation of anterior and posterior cells in Somitoids. This is demonstrated by the fact that the spatial auto-correlation profile of the Somitoids treated with ROCKi (as shown in Extended Data Fig.3g) is similar to WT (Extended Data Fig.2k). Similarly in Segmentoids, rosette formation is abrogated by ROCKi but not the separation of anterior and posterior cells (Fig.4j,k, Extended Data Fig.9f). Thus, in both Somitoids and Segmentoids, ROCKi prevents epithelial rosette morphogenesis but not AP patterning.

In the revision we added new data shedding light on the mechanism of cell sorting/separation. We performed RNAseq experiments on MESP2-high and MESP2-low cell fractions separated at the onset of cell sorting (Extended Data Fig.3, Supplementary Table2). This led us to identify cell surface proteins and a range of cytoskeleton regulators differentially expressed in the MESP2-high and low cells (Extended Data Fig.3a-e). These differential expression signatures were not present when the same experiment was performed a 120h, after cell epithelization (Extended Data Fig.3a-e), suggesting that some of these genes might be involved in the sorting process. This strategy identified TIAM1, an important regulator of RAC1, as a gene differentially regulated during the sorting phase. We introduced a Dox-inducible Tiam1 construct in the triple reporter iPS line HES7/MESP2/UNCX. Overexpression of Tiam1 did not interfere with AP fate acquisition but it abolished their segregation into distinct compartments in both Somitoids (Fig.2t, Extended Data Fig.3f,g) and Segmentoids (Fig.4h,i,k). These experiments suggest a role for cytoskeleton remodeling downstream of RAC1 in the remodeling process.

Our new experiments with Tiam1 overexpression presented in Figure 4h and in Extended Figure 9f show that despite normal HES7 oscillations, staggered MESP2 expression, and AP fate acquisition, AP patterning does not take place. We now summarized the phenotypes of HES7 deletion in both Somitoids and Segmentoids in Extended Data Fig.8j. HES7 deletion does not affect the acquisition of AP fates in individual cells or rosette assembly in both models. It abrogates AP patterning in Segmentoids but not in Somitoids. The discrepancy in AP patterning between the two models is correlated with distinct MESP2 dynamics in each context. In Somitoids, MESP2 expression occurs simultaneously in all cells and importantly, the same mode of MESP2 induction occurs with or without HES7 (Fig.1b,c,j,k). As a result, HES7-null Somitoids undergo the same downstream cell sorting process as in WT and give rise to the same AP patterning (Fig.2e, Supplementary Video3). This suggests that the segmentation clock does not directly control the cell sorting process. On the other hand, in Segmentoids, HES7 deletion leads to a change in MESP2 induction. In WT, each HES7 oscillation leads to a segmental domain of MESP2 expression (Fig.3l,m), followed by the rearrangement of MESP2-high and -low cells within this modular domain (Extended Data Fig.9b). In contrast, in HES7-null, MESP2 expression

moves continuously without clear segmental domains (Fig.4a,b), leading to randomly distributed cell clusters of cells with different MESP2 expression level (Extended Data Fig.8g,h). These data suggest that the output of the clock, the regular stepwise domains of MESP2 expression, is required for AP patterning. Altogether, we propose that the segmentation clock contributes to AP patterning by defining segmental domains where differentiation is initiated simultaneously in all cells. Differentiating cells acquire either A or P identity depending on the MESP2 level and begin to sort out within this domain.

2. If the cell sorting mechanism were the primary mechanism in segmentoids and in vivo, as cell sorting is a random process, it hard to see what would guarantee that the "anterior" fated cells actually end up in the anterior. This information would seem to need to come from an external source such as the clock. Can the authors' model explain not only the separation of anterior and posterior but the correct polarity in each segment?

We agree with the reviewer and we did not mean that random cell sorting is sufficient in our model. To avoid confusion, we describe the mechanism as "Direction-biased cell sorting" in our general working model in Fig.4n. We indicated in the text that the direction enforcing mechanism is unknown. Directional cues could be provided by the traveling waves of the segmentation clock or from the signaling gradients along the AP axis.

3. Even in the somitoids, it is not completely convincing that cell sorting is the only mechanism involved in separating anterior and posterior. There are other ways that a salt-and-pepper pattern could be resolved, such as cells switching fate. Could these also play a role here? From the data in S2g, it is unclear whether the fraction of MESP2+ cells is constant over time as the sorting model would predict. Could the authors quantify this?

To systematically track single cells and their MESP2 expression level simultaneously, we mixed H2B-GFP/MESP2-mCherry reporter cells with cells expressing MESP2-mCherry at a 1:40 ratio to facilitate tracking. We used H2B-GFP for single cell tracking and normalization of MESP2 intensity in the same cell (Methods). We showed that MESP2 reporter intensities in individual cells, high or low, remained unchanged in the time window of the emergence of the AP pattern both in Somitoids (Fig.2j,k, Extended Data Fig.2m,n) and Segmentoids (Fig.4f,g), thus ruling out fate switching of the cells' identity and supporting the cell sorting hypothesis.

4. Looking at the data in movies 2, 4, it appears that MESP2 expression is biased towards the center of the somitoid while UNCX is biased towards the edge. This should be quantified. It also seems to undercut the notion that the anterior and posterior rosettes are randomly distributed resulting from cell sorting.

We performed the quantification and indeed observed a biased distribution of MESP2-high cells towards the center (Extended Data Fig.2b). Within the central domain A and P rosettes appear randomly distributed. Clearly the peripheral domain shows some

differences from the central domain which may have to do with cell density or with a different distribution of the receptors to growth factors, as observed for BMP4 in human ES colonies in Etoc et al, Dev Cell 2016 for instance. While it will be interesting to study this difference more in depth, in this paper, we have concentrated our analyses on the central domain.

5. For the in vivo data in 4k,l, it is unclear where the boundary of the forming segment that is expressing MESP2 is. Is MESP2 expressed in the entirely segment in a salt and peppter pattern or is it biased towards the anterior portion? If the sorting model is correct, one would not expect a bias in MESP2 at the outset. Could the authors indicate the segment boundary in the image?

The early phase that captures the initial salt and pepper expression of MESP2 occurs in a somite-wide domain but happens before any clear segmental boundary formation. Thus, we cannot indicate the boundary on the figure.

6. In Fig 4c, it seems from the images, that each stripe of MESP2/UNCX contains several epithelial structures that can be seen in the DAPI image. Could the authors provide a merged image or some other way to see the relationship between the stripes of MESP2/UNCX and these epithelial structures? Even better would be to show images of MESP2 or UNCX merged with N-Cad or other markers which would delineate these structures.

In many instances, several rosettes form on the same segmental row depending on the width of the Segmentoid. Therefore, the segment dimension does not appear to be constrained in width. We have now quantified this in Extended data Figure 8e. In contrast however, the vast majority of the rosettes only span one segment in length and exhibit a correct AP pattern (Extended data Figure 8d), meaning that segmental length is highly constrained to the length of rosettes (which is approximately constant) in the AP dimension. We have added these new quantifications to the revised version of the manuscript.

7. In the scRNA seq data in 3f,I why does such a small percentage of the cells labeled “somite” express MESP2, UNCX, or TBX18? Is this an issue of dropouts in the data or is it something biological?

MESP2 is strongly expressed only by a fraction of the cell population during a short time window (note that this is the RNA and not the mCherry signal which is very stable and maintained at later stages in absence of RNA expression) and thus the weaker signal on the dot-blot is not unexpected. UNCX and TBX18 are weakly expressed in these datasets indicating that the Somitoids and Segmentoids of our latest time point are slightly immature, corresponding probably to a forming/newly formed somite stage. This is supported by the fact that no differentiation markers (MYF5, PAX1…) are expressed in these datasets. This means that we would need to incubate the Somitoids and Segmentoids

longer than 98h in order to reach more mature somitic stages with stronger UNCX and TBX18 expression. We are now discussing these issues in the revised version of the paper.

8. The data in S6 are not so clear in supporting the colinear activation of HOX genes. Could the authors plot this in a more informative way, maybe with time on the x axis and the expression of different HOX genes on the y axis so that the sequence of expression can be better visualized?

We now plot the activation of HOX genes in the NMP cluster of the Segmentoids in time. This shows that the onset of Hox genes takes place in the NMPs and it also illustrates a clear collinear activation for the A and D cluster. We have added this new data in Extended Figure 7k and discuss this in the revised text.

9. In line 192, the SOX2 expressing cells are referred to as "neural tube-like structures". I have a hard time seeing this from the images. This should be better explained and higher resolutions images provided.

We have removed this description from the revised text.

10. In Fig 2k,n, I found the color of the diagrams confusing. Throughout the figure, MESP2 cells are marked in magenta, but here the magenta seems to indicate the whole population collected at 72h whether MESP2 positive or not. I would suggest using a different color.

We have changed colors as suggested in the revised figure.

11. The authors should cite and discuss Budjan et al eLife 2022 which creates different structures termed somitoids. The senior author here is also a coauthor of that manuscript and it has been included as a related manuscript here but is not discussed.

We now briefly discuss this paper, which is mostly technical, in the introduction. The quality of the Somitoids obtained with this protocol is far inferior to the ones obtained with the new protocol described in our paper. Specifically, they only observe a limited number of rosettes in each Somitoid and did not examine AP patterning.

Referee #4 (Remarks to the Author):

Using human iPS, the authors established two culture systems to produce "Somitoids" and "Segmentoids", which reproduced parts of somitogenesis in human. The major target of this article was to reveal mechanism of how anterior and posterior sub-compartment of single somite is established. Using reporter genes to mark specific events in somitogenesis, the authors clearly showed that Somitoids reproduced early temporal events for initiating somitogenesis without

special organization. However, they found Somitoids are composed of two distinct cell populations (MESP2-high and MESP2-low) and those are segregated by sorting mechanism. To reveal whether this sorting mechanism is also involved in the A/P compartmentalization in vivo, the authors examined MESP2-reporter expression in Segmentoid and proposed that this would be the case.

This article proved that most of events observed in somitogenesis are reproduced in Segmentoids and the reproduction system is useful for the further study. However, most of parts were confirmation of events observed in mice. One new finding is the possible involvement of sorting in the A/P compartmentalization in vivo. However, the evidence is rather weak and no molecular cue underling the sorting mechanism was addressed at all even though they performed single cell transcriptome analyses. In addition, there are several questions should be addressed.

Specific comments

1. Title: I could not get why the authors use the words “Reconstruction and deconstruction” in the title. I could not find any explanation in the text. What does deconstruction mean?

The rationale behind the title is that Segmentoids correspond to an attempt to reconstruct (reproduce) human somitogenesis in vitro trying to recapitulate development as it takes place in the embryo. In contrast, the Somitoids offer an opportunity to deconstruct somitogenesis by dissociating the different patterning processes involved (clock and wavefront, AP patterning and rosette formation).

2. Abstract: the following sentence is vague.
Line 35-37; we demonstrate a large degree of independence of the various patterning modules involved in somitogenesis including the segmentation clock, somite epithelialization and AP polarity patterning.

We have changed the sentence to: “Our work demonstrates that the major patterning modules involved in somitogenesis including the clock and wavefront, AP polarity patterning and somite epithelialization which are normally integrated during development, can be dissociated and operate independently in our in vitro systems”.

3. Line 51-52; The periodicity of somite formation involves a molecular oscillator called the segmentation clock. This oscillator controls the rhythmic activation of Notch, Wnt and FGF pathways which manifest as traveling waves of target gene expression in the posterior PSM. What is the oscillator?

The oscillator is a cyclic gene regulatory network. We have changed the sentence to “This gene regulatory network controls the rhythmic activation of Notch, Wnt and FGF pathways which manifest as traveling waves of target gene expression in the posterior PSM.”

4. Line 86-87; HES7 signals initiated from the peripheral region of the spreading organoid and propagated as concentric waves toward the center.
Cells proliferated actively since the size of organoid enlarged. The initiation of HES7 signal from peripheral region is interesting. What kind of cells located in the periphery?

There is cell proliferation, but it is mostly the spreading of the organoid on the flat surface which is illustrated in the movies. Cells at the periphery exhibit a somewhat different behavior compared to the central cells. This may have to do with a different distribution of the receptors to growth factors, as observed for BMP4 in human ES colonies in Etoc et al, Dev Cell 2016. This may also reflect a difference in cell density between the periphery and the center. It will be interesting to study this more in depth in the future.

Is there any counting mechanism (of cell cycle) will be responsible for the Hes7 initiation? What is the upstream of Hes7?

While we do not understand what triggers HES7 initiation, we observed that it can be initiated by the change of medium. We do not believe that there is a counting mechanism because the number of oscillations can be significantly increased by inhibiting Retinoic Acid signaling and activating FGF (Diaz-Cuadros et al, Nature 2020).

5. Line 90-91; onset of MESP2 immediately follows the arrest of HES7 oscillations.
What is the other condition for the onset of MESP2. Is there any indication from single cell transcriptome analyses? Is there any meaning for about 4 cycles of HES7? It was shown that MESP2 can be activated in the absence of HES7 protein, indicating that arrest of Hes7 oscillation is not required for MESP2 activation.
DAPT treatment prevented expression of UNCX and MESP2 expression. How about HES7 oscillation? When do the authors treat with DASP? They should indicate the time of drug treatment in each Figure legends.

We showed in 2D cultures of iPS-derived human PSM that the downregulation of FGF signaling is key for the activation of MESP2. Treating iPS-derived PSM cultures with FGF/MAPK inhibitors results in premature activation of the MESP2-mCherry reporter (Diaz-Cuadros et al, Nature 2020). We do not think there is a meaning for the 4 cycles of HES7 as this number can be substantially modified by activating FGF and inhibiting retinoic acid. This reflects the time window between the onset of HES7 oscillations in the NMPs and the time when cells reach the anterior PSM fate.

We added DAPT at 48h when the cells are in PSM stage and we have added this information in the figure. We now show in Extended Data Fig.2c that treatment of the Somitoids with DAPT leads to an arrest of the HES7 oscillations after 2 cycles. We mention this result in the revised text.

6. Line139-141; need more explanation with reference. What kind of mechanism is predicted and what can be addressed by the reporter system here.

The reviewer referred to our original lines: "How *MESP2* expression resolves from its initial wide segmental domain which marks the future somite to an anterior half-somite stripe defining the future anterior somite compartment is not understood."

In current models of antero-posterior somite specification, MESP2 is first expressed at an equivalent level by all cells of the segmental domain defined in response to the segmentation clock. Then, it becomes downregulated in cells of the posterior somitic domain to generate the somite AP compartments. Our results suggest a different model where MESP2 is initially activated in a salt and pepper fashion in a segment-wide domain in response to the clock, followed by a sorting phase (independent of the clock) positioning the high MESP2 expressors anteriorly and the low MESP2 expressors posteriorly. We have now tried to explain this mechanism more clearly in the revised text.

The reporter construction is not clearly shown here and in the reference. Is this MESP2 knockin reporter that allow MESP2 protein translation with t2a-H2B-mcherry? Or is this a transgene driven by MESP2 promoter-enhancer ?
I would like to know what is reported by this reporter. Does it report transcription activity with extended stable mcherry protein expression (this is Trajectory of MESP2 expression?)

In the revised version, illustration of the reporter is shown in Extended Data Fig.2a. We have also added the following text to better describe the reporter:

"This line was engineered by introducing a t2A-mCherry construct by homologous recombination into the *MESP2* locus and thus reports for MESP2 protein production. mCherry is however much more stable than MESP2 and thus it is retained for some time by cells that have previously expressed *MESP2* after they stop transcribing the gene."

7. 72 h Somitoid cells showed sorting ability but 96h cells lost the ability even mcherry signal intensity is different. This is very interesting but the molecular mechanism is not addressed at al. This result indicates that molecule responsible for cell sorting is transiently expressed at 72 h and disappear after 96 h. What kind of molecule would be candidate?

In the revision we added new data identifying TIAM1 as candidate effector molecule for the cell sorting. We performed RNAseq experiments on MESP2-high and MESP2-low cell fractions separated at the onset of cell sorting (Extended Data Fig.3, Supplementary Table2). This led us to identify cell surface proteins and a range of cytoskeleton regulators differentially expressed in the MESP2-high and low cells (Extended Data Fig.3a-e). These differential expression signatures were not present when the same experiment was performed a 120h, after cell epithelialization (Extended Data Fig.3a-e). This strategy

identified TIAM1, which encodes a prominent regulator of RAC1, as a gene differentially regulated during the sorting phase. We introduced a Dox-inducible Tiam1 construct in the triple reporter iPS line HES7/MESP2/UNCX. Overexpression of Tiam1 did not interfere with AP fate acquisition but it abolished their segregation into distinct compartments in both Somitoids (Fig.2t, Extended Data Fig.3f,g) and Segmentoids (Fig.4h,i,k). These experiments suggest a role for cytoskeleton remodeling downstream of RAC1 in the sorting process.

8. Segmentoid formation is also very interesting especially for the elongation with AP polarity. What is the mechanism to determine the budding point and the direction. Is there any cellular bias accounting for the initial symmetry breaking?

We have no clue about what breaks the initial symmetry of the spheroids. We observed that SOX2/T cells are initially scattered in the spheroids and then they gather at one point which will define the future posterior end of the Segmentoid. What controls this behavior is not understood at this point and while we agree that this is a very interesting question, we feel this is beyond the scope of this manuscript.

9. There are several segmental structures in Segmentoid. One has single row of segments, but others have grape-like segments. Is there any common features between rosettes formed in somitoid and somite-like structure in Segmentoid.

In the anterior-most region of Segmentoids, grape-like rosettes without clear AP identities were observed (Extended Data Fig.8c). While in the middle to posterior region, rows of rosettes mostly span one segment in length and exhibit a correct AP patterning.

The rosettes formed in Somitoids and Segmentoids displayed a similar size and shape (Extended Data Fig.1d,f; Extended Data Fig.4c,d). They also shared typical somite-like apical-basal polarity such as concentrated F-actin in the center (Fig.1f; Fig.3c,o).

10. The authors suggested a sorting mechanism between MESP2-high and low cells is responsible for the establishment of A/P polarity of a somite. It is likely but the direct evidence is weak. Because in vitro culture is possible, some single cell tracking data should be presented.

We agree that this data is important and now present it in the revised version of the manuscript. To systematically track single cells and their MESP2 expression level simultaneously, we mixed H2B-GFP/MESP2-mCherry reporter cells with cells expressing MESP2-mCherry at a 1:40 ratio to facilitate tracking. We used H2B-GFP for single cell tracking and normalization of MESP2 intensity in the same cell (Methods). We showed that MESP2 reporter intensities in individual cells, high or low, remained unchanged in the time window of the emergence of the AP pattern both in Somitoids (Fig.2j,k, Extended

Data Fig.2m,n) and Segmentoids (Fig.4f,g), thus ruling out fate switching of the cells and supporting the cell sorting hypothesis.

11. Line 262-265; the segmentation clock is not required for the expression of AP identity genes in individual cells, but its output conferring rhythmicity to MESP2 induction and segment determination appears to play an important role in the spatial organization of stripes of anterior and posterior identity in the forming somites.
This part is difficult to understand if sorting mechanism is only based on the MESP2 expression. What is the function of rhythmicity? The authors speculate A/P patterning is generated by sorting mechanism depending on the level of MESP2 expression. In the absence of segmentation clock, MESP2 expression is uniformly induced in any cells that prevent cell sorting (?). This may be explained by nematic order parameter (in extended dataFig.7d, lower in HES7-null), but it is difficult to follow. How is A/P random patterning in HES7-null established?

In the absence of the segmentation clock (ie in HES7 null mutants), in the Segmentoids and in the Somitoids, MESP2 is not uniformly induced in every cell but it still exhibits differences in expression as in wild type. This suggests that the mechanism controlling acquisition of AP identity is independent of the clock. This is very clear in the Somitoid cultures where the sorting mechanism appears fully functional in HES7-/- cultures where segregated MESP2+ and UNCX+ rosettes form as in control cultures. The main difference between WT and HES7-null Segmentoids is the fact that in WT, MESP2 expression is activated in a segment-wide domain at each clock cycle, and thus its posterior progression is staggered, while in the mutant the posterior progression of MESP2 is continuous since there are no segments. The segmental progression triggered by the clock thus allows to initiate the differentiation program simultaneously in all cells of the forming segment. This ensures that the sorting process remains confined within the segmental domain. Indeed the mechanism also implies the existence of a directional cue which instructs the MESP2+ cells to locate anteriorly rather than posteriorly. Such a cue might be provided by the clock wave or by the gradient systems of the PSM. We have revised the discussion of the paper to try to better explain these concepts.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

Overall, the authors did an excellent job in their revision of the manuscript to address my concerns. I still have a few remaining comments (except for point 1 all minor):

1. My most significant remaining concern is the evidence the authors present for in vivo relevance. Whereas I agree that the new experiments provide additional evidence that similar cell sorting mechanisms act in vivo, I would still welcome live imaging experiments, preferably in a mammalian system (mouse) & using similar reporters as used in the segmentoids/somitoids. While challenging, this is technically possible and would unequivocally demonstrate the relevance of cell sorting in vivo.

Notwithstanding this criticism, I stress that I acknowledge the great value of the (beautiful) work using the segmentoids and somitoids, and the authors could also (further) stress the limitations of their in vivo approach.

2. Not all morphometry requested was performed. In particular, information on the # somites per structure is lacking, as well as a comparison with the embryo.

3. In their rebuttal the authors state that they have added info about the penetrance of reported observations (staining patterns) to the figure legends in the revised manuscript, however I do not find this info in the legends (eg Fig 3c,d,n,o).

4. I found the authors' answer to my point 5 highly insightful ("Thus, once the segment is specified, the rosette (s) subsequently form in this geometrically constrained environment defined by one rosette in length, and a variable number of rosettes in width." - "Thus, as long as the AP length of the forming somite is one typical somite length as controlled by the segmentation clock, somite morphogenesis will be associated with correct AP patterning). I thank the authors for their explanation and suggest that they succinctly include this clarification in the manuscript.

5. I fully agree with the explanation of the authors regarding NMP bipotency. I would however suggest that they clarify this in the context of the distinct terminologies used in this excellent recent Perspective with their former lab member Charlene Guillot as one of the co-authors: https://pubmed.ncbi.nlm.nih.gov/34717142/

6. Related to point 5: yes, I agree the authors show that the in vitro NMPs have an in-vivo-like transcriptional signature, BUT what evidence do the authors have that these are the cells that produce the in vitro somites?

7. Of course the authors are right that (early) NMPs reside in the CLE, but could the authors elaborate on the evidence they have that the Oct4/T/Sox2 triple-positive cells are NMPs and not Epiblast Stem Cells (where are they in the (pluri)potency spectrum?)?

8. The machine learning classifier for cell states is a great addition, but a more detailed comparison of the similarities (conserved markers) and differences (differential genes) between in vivo and in vitro & segmentoid and somitoid would still be very welcome.

Finally, just as a note: I had to double check myself, but in Veenvliet et al. 2020 we do not show that CL promotes rosette formation in the absence of Matrigel (as the authors state in their rebuttal).

Kind regards,
Jesse Veenvliet

Referee #2 (Remarks to the Author):

The revised version from Miao et al. generally answers the points raised by this reviewer. Prior to publication, I would recommend the following point be reconsidered, if possible.

Regarding cell tracking experiments shown Fig. 4f and g:
This cell tracking experiment revealed that Mesp2 expression was not significantly altered in individual cells during cell sorting (Fig. 4g middle). Thus, the authors concluded that the change in the pattern of Mesp2-positive cells shown on the left side of Fig. 4g was caused by sorting of both Mesp2-positive and -negative cells. However, this does not seem to be direct indication whether such cell sorting actually occurred. Since the authors have already done cell tracking at the one-cell level, it is likely that they have data on the movement of individual cells. If so, why not add a quantitative analysis of the direction of cell movement as well as Mesp2 expression?

Referee #3 (Remarks to the Author):

In this revision, the authors have added new data on single cell tracking of reporter cells and on the role of TIAM1, a cytoskeletal regulator in the cell sorting. I continue to the believe that this is in an interesting and important paper and that the data presented are overall of high quality with some very nice experiments with reporters and knockouts. Of the current papers and preprints demonstrating in vitro somite forming systems, this paper represents the most thorough characterization and some of the knockout results are novel and interesting in terms of the degree to which different processes can be decoupled in different systems. However, I also remain unconvinced that the data conclusively support the biological take home message: that a cell sorting process in pre-fated anterior and posterior cells underlies the separation of anterior and posterior fates. The new data do not directly provide evidence for a cell sorting mechanism and there are also some technical issues. I detail these concerns here.

1. The authors state that when tracking single cells, the MESP2 intensity is unchanged in both somitoids (line 177) and segmentoids (lines 329-330). This statement is an oversimplification of the data which does not entirely support it. While it appears to be true in Fig 2j, there is substantially

more mixing between the intensities of high and low MESP2 cells in S2m, which as far as I can tell is data from an identical experiment. I do not think it is justified to put the data from the same type of experiment which is less supportive of the conclusion in the supplement without comment in the main text. The correlation between initial and final levels is also weaker in the supplemental figure compared to the main one. In fact, in S2m, many of the MESP2 high cells, end up with levels of MESP2 comparable with the MESP2 low cells, and so this does not support a pure sorting mechanism. The same can be said of Figure 4g, where there is considerable mixing between the high and low trajectories and in that case there even seems to be some increase of MESP2 expression in the MESP2 low cells, past the point where the levels of MESP2 should have been fixed according to the authors' model.

2. In any event, unchanged MESP2 levels are indirect evidence for cell sorting. Analysis of the data like that in Figure S2n should be able to reveal whether cells that start in an area that will become a rosette of the opposite fate tend to leave that rosette (the slight changes in the biases of surrounding cells in the right panel of this figure do not substitute for this). Alternatively, are cells which begin with the "wrong" MESP2 levels for their eventual surroundings more likely to change this level, because it is clear that some cells do change their levels in S2m?

3. Related to the interpretation of the MESP2 reporter, the statement that "Given the long lifetime of mCherry protein, this suggests that no new MESP2 is expressed in the plateau phase of the reporter profile" requires more support. Are these cells dividing? If so, expression would be expected to decay by dilution in accordance with the division rate and the plateau would mean that it is being balanced by some new protein synthesis. mCherry is also known to be prone to photobleaching so it is also possible that under imaging some photobleaching of mCherry is balanced by newly produced protein. While this statement simplifies the interpretation of the movies, it isn't clear to me that the authors' conclusions would require that there be no new transcription of MESP2 so perhaps this statement could be qualified and the consequences for the interpretation of the data better explained.

4. I do not understand what the authors are suggesting with the new data on TIAM1. First, it appears that overexpression of TIAM1 affects the fraction of cells which adopt anterior or posterior fates (comparing 2a and 2t, there appears to be more MESP2 and less UNCX expression when TIAM1 is overexpressed). The authors should quantify whether this is the case. If TIAM1 induces a switch in cell fate proportions, it is hard to see how an argument could be made about cell sorting based on this data. Further, the authors are not specific about what they are proposing for a mechanism - does differential TIAM1 expression make some cells more likely to migrate than others or affect the mechanical properties of the cells? Can this explain the time evolution of the pattern? As the major biological message of the paper is this cell sorting mechanism which is independent of AP patterning, and these experiments are intended to shed light on this, the authors should be clearer about what they are suggesting.

5. I do not understand the authors' response regarding the differences in the HES7KO between somitoids and segmentoids. The data in 4a seem to show a failure of the sorting process in this case (although it is hard to judge from these images the degree of intermixing of single cells), but according to the authors' model, why do these not simply sort into separate rosettes of AP polarity randomly distributed throughout the segmentoid? The same appears to be true in the data in S8g, example 1. In example 2, there does appear to be some segregation of the UNCX cells, although MESP2 appears rather uniform. Altogether, these data seem to me to argue against a decoupling of the segmentation clock from the AP pattern in segmentoids. The authors also argue that having a

sliding, rather than discrete progression of MESP2 contributes to the phenotype, and this makes sense for why discrete segments aren't defined, but I would still expect a general sorting into A and P territories as much as without HES7 if the sorting was truly decoupled from the clock.
6. I still find it difficult to assess the degree of colinearity in the Hox gene expression from the data presented. Although the dot plots with fractions of cells and expression levels contain a lot information, it would be easier to see if the authors plot the average expression of each gene vs time so that the different genes can be compared. I think the authors should also mention in the main text that the HoxB and C clusters do not show this pattern.

Referee #4 (Remarks to the Author):

The authors responded to most of my concerns. But I just have some more comments on the title and interpretation of experimental results on the cell sorting mechanism.

1. The authors explained why they used the word “deconstruction” but I do not see this word except for the title.
2. The model “the initial MESP2 expression is heterogenous and cells are sorted out based on the properties defined by MESP2” was convincingly shown by separate culture experiments of MESP2-high and low cells in the Somitoids system. Since mCherry reports endogenous MESP2 expression (although it lasts much longer than the real expression), this is also demonstrated in Segmentoids. But, that means the MESP2 expression levels are predetermined or established before the expression starts. Since Hes7 oscillation was not required for the generation of this different population, what would be the cue? Since MESP2 expression appears in the salt and pepper pattern and the Notch signaling pathway is heavily involved in the somitogenesis, I speculate that some lateral inhibition mechanism may operate for the generation of the initial MESP2 expression pattern.
3. The cell sorting mechanism is this manuscript's most important finding and argument. The authors tried cell tracking in live imaging samples. I think that they analyzed many cells but the data shown are only 5 cells (Extended Data Fig.9). The movie was fantastic and I observed several times and I noticed that the MESP2-positive cells sometimes moved posteriorly and incorporated in the next anterior domain, indicating some signal gradient exists in the segmental domain. I think that it should be described in the text.
4. The authors use the word “rosette” for both Somitoids and Segmentoids, but the properties are different. It might be better not to use the word for Segmentoids.

Author Rebuttals to First Revision:

Response to the reviewers' critiques

Referee #1 (Remarks to the Author):

Overall, the authors did an excellent job in their revision of the manuscript to address my concerns. I still have a few remaining comments (except for point 1 all minor):

1. My most significant remaining concern is the evidence the authors present for in vivo relevance. Whereas I agree that the new experiments provide additional evidence that similar cell sorting mechanisms act in vivo, I would still welcome live imaging experiments, preferably in a mammalian system (mouse) & using similar reporters as used in the segmentoids/somitoids. While challenging, this is technically possible and would unequivocally demonstrate the relevance of cell sorting in vivo.

Notwithstanding this criticism, I stress that I acknowledge the great value of the (beautiful) work using the segmentoids and somitoids, and the authors could also (further) stress the limitations of their in vivo approach.

We sincerely thank Jessie for the positive assessment of our revised paper. While we appreciate this suggestion, we believe that live imaging transgenic mouse embryos in vitro is beyond the scope of this manuscript. The generation of new mouse lines and their validation would be needed, as well as the development of in vitro culture conditions allowing to perform the advanced microscopy on living mouse embryos. While these experiments are in principle possible, they would require very significant experimental work which we estimate at least to 6 months if everything works fine. In the revised text, we are careful with our conclusions and only state that "Together, these suggest that the sorting mechanism that we uncovered in vitro is likely operating in vivo". (Lines 367-368)

2. Not all morphometry requested was performed. In particular, information on the # somites per structure is lacking, as well as a comparison with the embryo.

The number of rosettes per structure is now discussed in the text and shown in Extended Data Fig. 1e and Extended Data Fig. 4c. Comparing with the embryo is difficult since in vitro there can be 1-4 rosettes (Extended Data Fig.8e) along the medial-lateral axis in segmentoids, while in vivo there is only one.

3. In their rebuttal the authors state that they have added info about the penetrance of reported observations (staining patterns) to the figure legends in the revised manuscript, however I do not find this info in the legends (eg Fig 3c,d,n,o).

We apologize for the omission and have added this information in the corresponding legends for Fig.1f,g; Fig.3c,d,n,o; Extended Data Fig.1g-j; Extended Data Fig.4e; Extended Data Fig.5; Extended Data Fig.8c,g; Extended Data Fig.9i.

4. I found the authors' answer to my point 5 highly insightful ("Thus, once the segment is specified, the rosette (s) subsequently form in this geometrically constrained environment defined by one

rosette in length, and a variable number of rosettes in width." - "Thus, as long as the AP length of the forming somite is one typical somite length as controlled by the segmentation clock, somite morphogenesis will be associated with correct AP patterning). I thank the authors for their explanation and suggest that they succinctly include this clarification in the manuscript.

We have included this clarification in the text "This suggests that antero-posterior organization of the rosettes is geometrically constrained by the forming segment along the AP axis". (Lines 310-311)

5. I fully agree with the explanation of the authors regarding NMP bipotency. I would however suggest that they clarify this in the context of the distinct terminologies used in this excellent recent Perspective with their former lab member Charlene Guillot as one of the co-authors: https://pubmed.ncbi.nlm.nih.gov/34717142/

The perspective mentioned proposed to call Neuro Mesodermal Competent cells (NMC), — individual cell with the developmental potential to give rise to both neural and mesodermal derivatives during axis elongation and to reserve the term Neuromesodermal progenitor(s) (NMP[s]) to individual NMC cell that gives rise to both neural and mesodermal derivatives during axis elongation. While this is an interesting suggestion, this nomenclature is far from being accepted in the field and we worry that changing the name NMP to NMC in our analysis would create unnecessary confusion at this stage.

6. Related to point 5: yes, I agree the authors show that the in vitro NMPs have an in-vivo-like transcriptional signature, BUT what evidence do the authors have that these are the cells that produce the in vitro somites?

The only cell population detected by scRNAseq in both day one Somitoids and Segmentoids are NMPs thus implying that somite cells are descendants of the NMPs. Moreover, our scRNAseq datasets for Somitoids and Segmentoids both show that there is one major developmental trajectory linking the somite fate to the NMP-like cells. This is indicated in the revised text as: "Velocity combined with PAGA analysis confirmed that both Neural and Mesodermal cells arise from the NMP progenitors (Fig.3g, Extended Data Fig.6b,c)." (Lines 260-261)

7. Of course the authors are right that (early) NMPs reside in the CLE, but could the authors elaborate on the evidence they have that the Oct4/T/Sox2 triple-positive cells are NMPs and not Epiblast Stem Cells (where are they in the (pluri)potency spectrum?)?

Epiblast stem cells do not express T while NMPs do. Moreover, our OCT4/T/SOX2 positive cells also express more NMP-specific markers such as NKX1.2 indicating that they are not epiblast stem cells. The similarity of the human NMPs differentiated in vitro with mouse NMPs is further supported by machine learning analyses presented in Extended data Figure 7d and e which compare the cells differentiated in vitro in the Somitoids and Segmentoids to a E9.5 single cell RNAseq mouse dataset of the posterior embryonic region that we have previously generated (Diaz-Cuadros et al, Nature 2020).

8. The machine learning classifier for cell states is a great addition, but a more detailed comparison of the similarities (conserved markers) and differences (differential genes) between in vivo and in vitro & segmentoid and somitoid would still be very welcome.

We have performed such an additional analysis and presented the similarities in the expression of somitogenesis-associated genes in Extended Data Fig. 7h in the revised manuscript.

Finally, just as a note: I had to double check myself, but in Veenvliet et al. 2020 we do not show that CL promotes rosette formation in the absence of Matrigel (as the authors state in their rebuttal).

Thanks for the correction and sorry for the oversight. This was however not included in the paper and therefore does not require to amend the text.

Kind regards,
Jesse Veenvliet

Referee #2 (Remarks to the Author):

The revised version from Miao et al. generally answers the points raised by this reviewer. Prior to publication, I would recommend the following point be reconsidered, if possible.

Regarding cell tracking experiments shown Fig. 4f and g:
This cell tracking experiment revealed that Mesp2 expression was not significantly altered in individual cells during cell sorting (Fig. 4g middle). Thus, the authors concluded that the change in the pattern of Mesp2-positive cells shown on the left side of Fig. 4g was caused by sorting of both Mesp2-positive and -negative cells. However, this does not seem to be direct indication whether such cell sorting actually occurred. Since the authors have already done cell tracking at the one-cell level, it is likely that they have data on the movement of individual cells. If so, why not add a quantitative analysis of the direction of cell movement as well as Mesp2 expression?

We have generated new movies which clearly show the sorting behavior. We provide novel examples of tracking in Somitoids (Supplementary Video6, Extended Data Fig. 2j) and Segmentoids (Supplementary Video11, Fig. 4e, Extended Data Fig.9e,f). As suggested by the reviewer, we have also performed a quantitative analysis of the cellular movements during the morphogenetic phase. To this end, we have used Particle Image Velocimetry (PIV) to reconstruct the entire velocity field of MESP2+ cells. To characterize the global pattern of cell movements, we have used simple concepts from vector calculus. Specifically, the divergence of the velocity field is the mathematical quantity that describes whether regions in a continuum are expanding or contracting. We expected MESP2+ cells to flow out of regions that become MESP2 negative (positive divergence) and flow in regions that become MESP2 positive. This prediction was confirmed with high statistical confidence by our new analysis. This new data is now shown in Fig.2j,k, Fig.4g, Extended Data Fig.2r, and Extended Data Fig.9g and discussed in the revised manuscript. Lines 184-190; 345-348.

Referee #3 (Remarks to the Author):

In this revision, the authors have added new data on single cell tracking of reporter cells and on the role of TIAM1, a cytoskeletal regulator in the cell sorting. I continue to the believe that this is in an interesting and important paper and that the data presented are overall of high quality with some very nice experiments with reporters and knockouts. Of the current papers and preprints demonstrating in vitro somite forming systems, this paper represents the most thorough characterization and some of the knockout results are novel and interesting in terms of the degree to which different processes can be decoupled in different systems. However, I also remain unconvinced that the data conclusively support the biological take home message: that a cell sorting process in pre-fated anterior and posterior cells underlies the separation of anterior and posterior fates. The new data do not directly provide evidence for a cell sorting mechanism and there are also some technical issues. I detail these concerns here.

We sincerely thank the referee's positive note on our manuscript. We are addressing the concerns raised below.

The authors state that when tracking single cells, the MESP2 intensity is unchanged in both somitoids (line 177) and segmentoids (lines 329-330). This statement is an oversimplification of the data which does not entirely support it. While it appears to be true in Fig 2j, there is substantially more mixing between the intensities of high and low MESP2 cells in S2m, which as far as I can tell is data from an identical experiment. I do not think it is justified to put the data from the same type of experiment which is less supportive of the conclusion in the supplement without comment in the main text. The correlation between initial and final levels is also weaker in the supplemental figure compared to the main one. In fact, in S2m, many of the MESP2 high cells, end up with levels of MESP2 comparable with the MESP2 low cells, and so this does not support a pure sorting mechanism. The same can be said of Figure 4g, where there is considerable mixing between the high and low trajectories and in that case there even seems to be some increase of MESP2 expression in the MESP2 low cells, past the point where the levels of MESP2 should have been fixed according to the authors' model.

The reviewer is correct that a limited amount of mixing is visible in the traces, but that is expected as there is noise in the system, and we arbitrarily separated cells in top 50% and bottom 50%. Thus, cells close to the 50% boundary can easily change category due to noise. However, there are mathematically rigorous arguments to confirm that cells retain their expression levels, and we now present several of them. First, tracking the average expression of top and bottom 50% shows that the expression levels are maintained throughout the dynamics. This maintenance is even more evident when the slight downward trend of fluorescence levels (due to photobleaching) is corrected (Figure 1a below). If instead of separating cells in top and bottom 50%, we separated them in top and bottom quartile we can show that mixing is almost completely abolished (Figure 1b below). This shows that the cells that are changing category start off with more similar levels of fluorescence.

Moreover, correlation analysis of MESP2-mCherry expression at the beginning of the movie and at the end of the movie show a high degree of correlation (R^2~0.6-0.7). The only case where correlation is lower (R^2=0.39) could be explained as a consequence of the behavior of

only 3 outliers (out of 52 cells tracked), identified by calculating Mahalanobis distance. After excluding these 3 outliers, the R^2 is 0.71 as shown in the current plot (Figure 1c below and in the manuscript Extended Data Fig.2n). Finally, we want to point out that the R^2 corresponds to the correlation coefficient squared so all our examples have correlation coefficients higher than 0.6 (in fact most data have a correlation coefficient close to 0.8) which is quite high for a complex, noisy biological process. The few outliers might either represent noise or cells that change fate. This is an open question which might be interesting to address in the future. However, these cells are rare, and the behavior of most cells is well-approximated by assuming that cells keep their expression, as supported by our rigorous statistical arguments.

Thus, our data demonstrate that the <u>vast majority</u> of cells tracked do not change their MESP2-mCherry intensity level. Our findings indicate that cell sorting is an integral part of the AP patterning process (see also below), which has never been shown before. We nonetheless agree with the reviewer that our data does not allow us to exclude that a minority of cells can significantly change their expression level during the sorting phase, possibly reflecting a change of fate or noise in the system. We have revised the text as indicated below to better reflect this possibility:
"Indeed, we found that the mCherry intensity in individual cells remained largely unchanged from 72h to 84h (Fig.2i, Extended Data Fig.2n) with very few outliers (9 out of 98). These results suggest that although fate switching may occur, the vast majority of cells kept their MESP2 levels". (Lines 179-180)

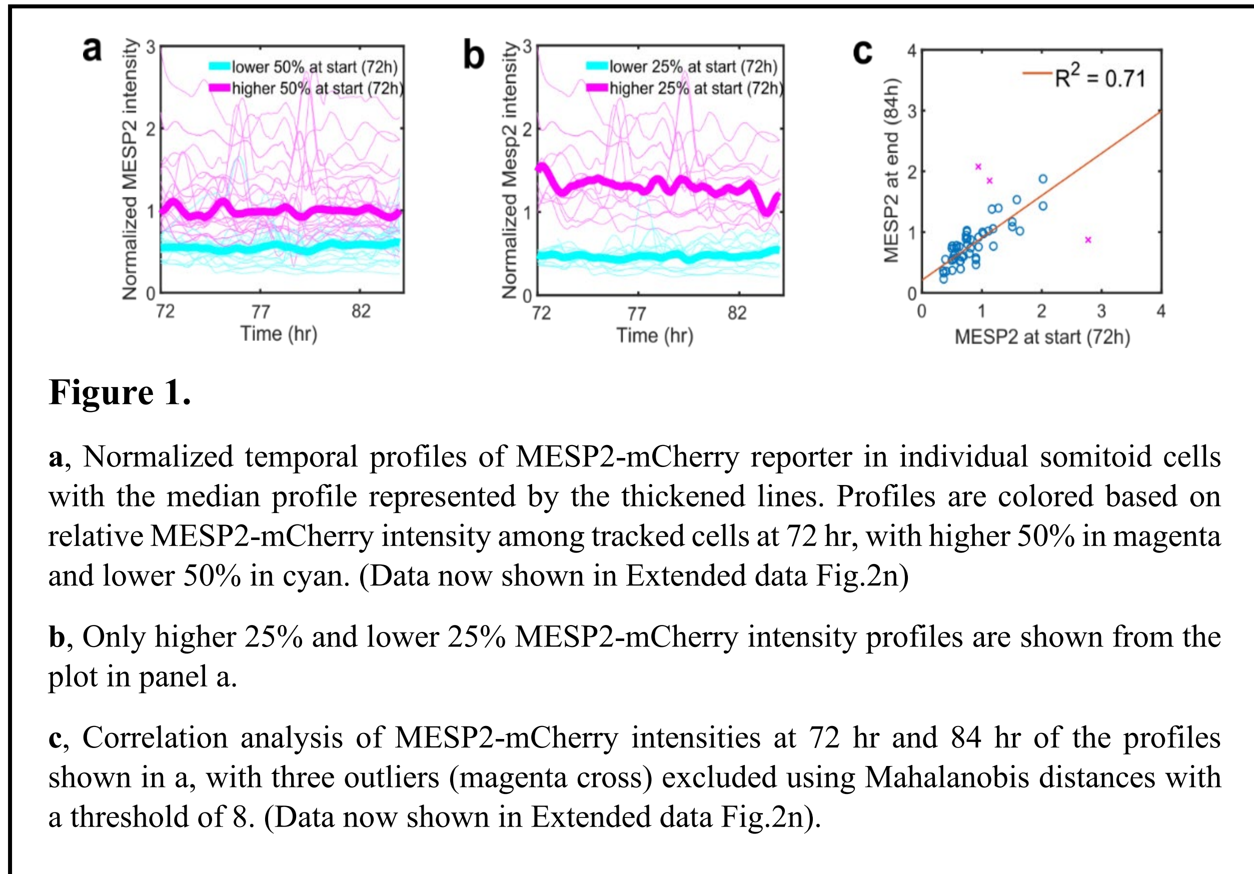

Figure 1.

a, Normalized temporal profiles of MESP2-mCherry reporter in individual somitoid cells with the median profile represented by the thickened lines. Profiles are colored based on relative MESP2-mCherry intensity among tracked cells at 72 hr, with higher 50% in magenta and lower 50% in cyan. (Data now shown in Extended data Fig.2n)

b, Only higher 25% and lower 25% MESP2-mCherry intensity profiles are shown from the plot in panel a.

c, Correlation analysis of MESP2-mCherry intensities at 72 hr and 84 hr of the profiles shown in a, with three outliers (magenta cross) excluded using Mahalanobis distances with a threshold of 8. (Data now shown in Extended data Fig.2n).

2. In any event, unchanged MESP2-mCherry levels are indirect evidence for cell sorting. Analysis of the data like that in Figure S2n should be able to reveal whether cells that start in an area that will become a rosette of the opposite fate tend to leave that rosette (the slight changes in the biases of surrounding cells in the right panel of this figure do not substitute for this). Alternatively, are cells which begin with the "wrong" MESP2-mCherry levels for their eventual surroundings more likely to change this level, because it is clear that some cells do change their levels in S2m?

We agree that unchanged MESP2-mCherry levels are indirect evidence for cell sorting. Direct evidence for cell sorting can be provided by tracking cells in the Somitoids and Segmentoids and showing that initially scattered cells with high and low MESP2-mCherry intensity levels segregate to establish domains of similar identity. We have generated new movies of the sorting behavior in both Segmentoids and Somitoids and our cell tracking data clearly argue in favor of sorting. Two examples of such movies have been added (Supplementary Video6 and Supplementary Video11). We now provide more detailed tracking analyses of these new movies in the revised manuscript to better illustrate the sorting behavior of the MESP2-expressing cells (Figure 2a below and in the revised manuscript Extended Data Fig. 2j, Fig. 4e, Extended Data Fig.9e,f).

To quantitatively support our conclusion on sorting, we have performed a rigorous mathematical analysis of the traces of MESP2+ cells. Specifically, we have used Particle Image Velocimetry (PIV) to reconstruct the entire velocity field of these cells. To characterize the global pattern of cell movements, we have then quantified the divergence of the velocity field which describes whether regions in a continuum are expanding or contracting. We expected MESP2+ cells to flow out of regions that become Mesp2- (positive divergence) and flow in regions that become MESP2+. This prediction was confirmed with high statistical confidence by our new analysis, and it is now described in the Figure 2b below, as well as Fig.2j-k, Fig.4g, Extended Data Fig.2r, and Extended Data Fig.9g in the revised manuscript (Lines 184-190; 345-348).

Finally, we have followed the reviewer suggestion and compared the behaviors of cells that start and end at the right place vs. cells that start at the “wrong” place in Somitoids. We found that while the level of expression of these two classes of cells does not change on average, their average displacement is different, with cells starting in the wrong place moving significantly more than cells starting at the right place (Figure 2c below). This analysis supports that on average cell sorting plays a more important role than changes in expression in somite rostro-caudal patterning. These new analyses are shown in Extended Data Fig.2p,q and discussed in the revised manuscript (lines 181-184).

To conclude, the number of cells whose MESP2-mCherry intensity levels are changing is very limited and thus analyzing sufficient numbers to understand their contribution to patterning would be very difficult. The novelty of our paper resides in the identification of the sorting mechanism which appears to be the main mechanism responsible involved in somite rostro-caudal patterning. Whether there is a small minority of cells which change MESP2 expression level to match the identity of their compartment or whether the identified outliers represent noise in the system is an interesting question, but we believe it is beyond the scope of our study. We nonetheless discussed explicitly this alternative possibility in the revised text (Lines 179-180).

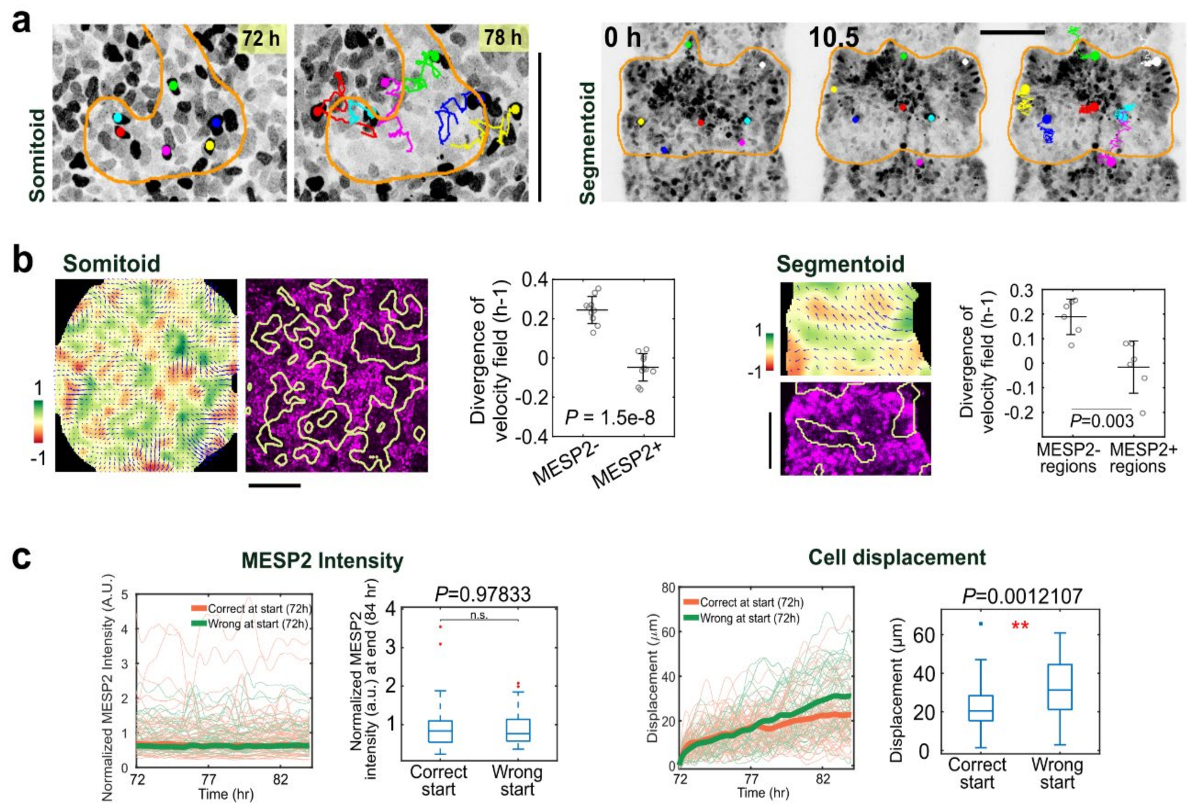

Figure 2.

a, Cell tracks overlayed on images of MESP2 in Somitoid and Segmentoid. The orange outline in the Somitoid (left, now shown in Fig.2g and Extended Data Fig.2j)) represents the forming MESP2-low cluster, while the orange outline in the Segmentoid (right, data now shown in Fig.4e) represents the forming segment.

b, For both Somitoid and Segmentoid: Left, Velocity field (arrows) and the corresponding divergence (heatmap) of Particle Image Velocimetry analysis, as well as regions of positive divergence (yellow outlines) overlayed on the MESP2 reporter image. Right, divergence of velocity field of MESP2-high and low regions (data now shown in Fig.2j,k and Fig.4g). All scale bars represent 100 μm.

c, Left, temporal profile of MESP2 intensity in cells starting in a correct (orange) or wrong (green) region, as well as plot of end-time-point MESP2 intensity of cells with correct or wrong start. Right, temporal profile of displacement in cells starting in a correct (orange) or wrong (green) region, as well as plot of end-time-point displacement of cells with correct or wrong start (data now shown in Extended data Fig.2p, q).

3. Related to the interpretation of the MESP2 reporter, the statement that "Given the long lifetime of mCherry protein, this suggests that no new MESP2 is expressed in the plateau phase of the reporter profile" requires more support. Are these cells dividing? If so, expression would be expected to decay by dilution in accordance with the division rate and the plateau would mean that it is being balanced by some new protein synthesis. mCherry is also known to be prone to photobleaching so it is also possible that under imaging some photobleaching of mCherry is balanced by newly produced protein. While this statement simplifies the interpretation of the movies, it isn't clear to me that the authors' conclusions would require that there be no new transcription of MESP2 so perhaps this statement could be qualified and the consequences for the interpretation of the data better explained.

In mouse embryos, Mesp2 expression is very transient in the PSM, with its mRNA being only expressed in the stripe at the level of the forming segment and then maintained in the anterior part of the newly formed segment during the next somitic cycle. This together represents a time window of about 4 hours. A similar very transient expression of the MESP2 mRNA is observed in our scRNA sequencing analysis for both Somitoids and Segmentoids. In the Somitoids, MESP2-mCherry expression reaches a plateau at 72h, followed by the cell sorting phase which lasts approximately 6 hours (Fig.2g). In the Segmentoids, we observe mCherry expression first in the forming segmental domain and then in the rostral domain of the forming segment consistent with the data from mouse embryos. Expression is also maintained in the anterior part of the newly formed somites due to the stability of mCherry compared to MESP2 mRNA. We have measured the cell cycle duration in 2D cultures of iPS-derived human PSM which is around 20 hours (Diaz-Cuadros et al, BioRxiv 2022). This important time difference between the sorting phase and the cell cycle suggests that dilution of mCherry due to cell division is unlikely to strongly affect the mCherry intensity profile during the sorting phase. Nevertheless, cell division together with photobleaching, likely accounts for the general slow downward trend of MESP2 plateau phase. We have introduced this discussion in the methods of the revised paper (lines 796-803).

4. I do not understand what the authors are suggesting with the new data on TIAM1. First, it appears that overexpression of TIAM1 affects the fraction of cells which adopt anterior or posterior fates (comparing 2a and 2t, there appears to be more MESP2 and less UNCX expression when TIAM1 is overexpressed). The authors should quantify whether this is the case. If TIAM1 induces a switch in cell fate proportions, it is hard to see how an argument could be made about cell sorting based on this data.

Our experiments show that an altered ratio of MESP2 high and low cells does not affect cell sorting ability in principle. This is shown in Fig. 2q, where a very small fraction of MESP2-high cells mixed in a large excess of MESP2-low cells can still sort together. Thus, even if TIAM1 would affect the fractions of cells acquiring A and P identities, this should not affect their sorting. In contrast, in TIAM1 overexpressing cells, we do not observe any sorting into MESP2-mCherry high or low clusters (compare Fig. 2t and 2a). This is quantified in Extended Data Fig.3h, which shows that TIAM1 overexpressing Somitoids exhibit altered spatial auto-correlation function, losing the damped oscillator-like shape observed in controls (Extended Data Fig.2k) which is typical for periodic patterns. This data clearly shows that the cells failed to sort out when overexpressing TIAM1. We nevertheless

performed flow cytometry analyses to quantify the ratios of UNCX-expressing cells in Somitoids after TIAM1 overexpression and observed a slight increase of the ratio (Extended Data Fig.3g). But as discussed above, this slight increase should not alter the sorting ability of the cells. We now present this new experiment in the revised manuscript.

Further, the authors are not specific about what they are proposing for a mechanism - does differential TIAM1 expression make some cells more likely to migrate than others or affect the mechanical properties of the cells? Can this explain the time evolution of the pattern? As the major biological message of the paper is this cell sorting mechanism which is independent of AP patterning, and these experiments are intended to shed light on this, the authors should be clearer about what they are suggesting.

We propose that the differential regulation of TIAM1 downstream of MESP2 leads to different cytoskeletal properties. As TIAM1 regulates the small GTPase RAC1 that plays a central role in regulating the cytoskeleton, this is likely to differentially alter the mechanical properties of the cells allowing the sorting of MESP2-high and low cells. The detailed molecular mechanism is beyond the scope of our study and will be the subject of future investigations. We nevertheless discuss the proposed mechanism more specifically in the revised text (Lines 222-225).

5. I do not understand the authors' response regarding the differences in the HES7KO between somitoids and segmentoids. The data in 4a seem to show a failure of the sorting process in this case (although it is hard to judge from these images the degree of intermixing of single cells), but according to the authors' model, why do these not simply sort into separate rosettes of AP polarity randomly distributed throughout the segmentoid? The same appears to be true in the data in S8g, example 1. In example 2, there does appear to be some segregation of the UNCX cells, although MESP2 appears rather uniform. Altogether, these data seem to me to argue against a decoupling of the segmentation clock from the AP pattern in segmentoids. The authors also argue that having a sliding, rather than discrete progression of MESP2 contributes to the phenotype, and this makes sense for why discrete segments aren't defined, but I would still expect a general sorting into A and P territories as much as without HES7 if the sorting was truly decoupled from the clock.

We agree that one could have expected to see separate rosettes of defined AP identity randomly distributed along the HES7 KO Segmentoids. However, our data indicate a somehow hybrid phenotype where some rosettes exhibit a single defined identity (see for instance Supplementary Video9) while others show a mixed identity (Fig 4a). We have clarified this in the revised text (lines 320-321). What is very clear is that, in the HES7 KO Segmentoids, cells acquire an AP identity (as shown by MESP2 and UNCX expression) but do not become organized into alternating MESP2 positive and negative stripes. This argues that acquisition of anterior and posterior somitic identities can be uncoupled from the segmentation process. We believe that this is the most important result from this experiment.

6. I still find it difficult to assess the degree of colinearity in the Hox gene expression from the data presented. Although the dot plots with fractions of cells and expression levels contain a lot information, it would be easier to see if the authors plot the average expression of each gene

vs time so that the different genes can be compared. I think the authors should also mention in the main text that the HoxB and C clusters do not show this pattern.

The difficulty with assessing the collinearity of HOX expression patterns here is due to the fact that we are looking at mRNA expression levels at steady state and not at transcription activation. We have plotted the average expression of each gene vs time as suggested (Extended data Fig7j-l) and toned down the message on collinearity which is not the focus of this study. We also mentioned that HOXB and C do not show this pattern (Lines 289-291).

Referee #4 (Remarks to the Author):

The authors responded to most of my concerns. But I just have some more comments on the title and interpretation of experimental results on the cell sorting mechanism.

1. The authors explained why they used the word "deconstruction" but I do not see this word except for the title.

The meaning of deconstructing is "to reduce (something) to its constituent parts in order to reinterpret it". We believe this accurately describes our strategy. We have now added the following sentences to the revised text: "It will allow deconstructing these patterning processes to understand how they integrate in the complex morphogenetic program of somitogenesis." Lines 374-376.

2. The model "the initial MESP2 expression is heterogenous and cells are sorted out based on the properties defined by MESP2" was convincingly shown by separate culture experiments of MESP2-high and low cells in the Somitoids system. Since mCherry reports endogenous MESP2 expression (although it lasts much longer than the real expression), this is also demonstrated in Segmentoids. But, that means the MESP2 expression levels are predetermined or established before the expression starts. Since Hes7 oscillation was not required for the generation of this different population, what would be the cue? Since MESP2 expression appears in the salt and pepper pattern and the Notch signaling pathway is heavily involved in the somitogenesis, I speculate that some lateral inhibition mechanism may operate for the generation of the initial MESP2 expression pattern.

We agree with the referee's hypothesis regarding possible lateral inhibition involvement in the initial salt and pepper expression pattern of MESP2. We have added the following sentence in the conclusion of the revised text: "In each cycle, the segmentation clock defines a stripe of Notch activation where the salt and pepper onset of MESP2 expression is synchronized among all cells, possibly involving a mechanism such as lateral inhibition". Lines 385-387.

3. The cell sorting mechanism is this manuscript's most important finding and argument. The authors tried cell tracking in live imaging samples. I think that they analyzed many cells but the data shown are only 5 cells (Extended Data Fig.9). The movie was fantastic and I observed several times and I noticed that the MESP2-positive cells sometimes moved posteriorly and incorporated in the next anterior domain, indicating some signal gradient exists in the segmental domain. I think that it should be described in the text.

We are delighted that the reviewer enjoyed the movie and we have generated several similar ones (Supplementary Video6 and Supplementary Video11) on which we have performed additional cell tracking analysis (Extended Data Fig. 2j, Fig. 4e, Extended Data Fig.9e,f). These allowed us to characterize the sorting in more detail. Indeed, we observed that 26 out of 111 tracked MESP2-high cells moved posteriorly toward the next anterior MESP2-high domain. This is summarized in Extended Data Fig.9f and described in the revised text (Lines 338-342).

4. The authors use the word “rosette” for both Somitoids and Segmentoids, but the properties are different. It might be better not to use the word for Segmentoids.

We have used the word “rosette” to refer to somite-like epithelial blocks, regardless of their AP identities or patterning.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The author responded sincerely and accurately to my claims. The convincing power of the newly added cell tracking movies is excellent！

Referee #3 (Remarks to the Author):

I appreciate the authors' responses and in particular softening the text where appropriate to allow that some data may be explained by alternate mechanisms. I agree that the data presented now support that most (but possibly not all) of the resolution of cells into AP domains within somites is governed by cell sorting. I support publication of the paper in its current form.

Referee #4 (Remarks to the Author):

I was satisfied with the responses by the authors. I do not have further concerns.