
Supplementary information

**Reconstituting human somitogenesis
in vitro**

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Reconstituting human somitogenesis *in vitro*

Yoshihiro Yamanaka^{1,7}, Sofiane Hamidi^{1,7}, Kumiko Yoshioka-Kobayashi¹, Sirajam Munira¹, Kazunori Sunadome^{1,2}, Yi Zhang¹, Yuzuru Kurokawa¹, Rolf Ericsson¹, Ai Mieda¹, Jamie L. Thompson³, Janet Kerwin⁴, Steven Lisgo⁴, Takuya Yamamoto^{1,2,5}, Naomi Moris³, Alfonso Martinez-Arias⁶, Taro Tsujimura¹, Cantas Alev^{1*}

¹Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto 606-8501, Japan

²Department of Life Science Frontiers, Center for iPS Cell Research and Application (CiRA), Kyoto University, 606-8507, Kyoto 108-8639, Japan.

³The Francis Crick Institute, 1 Midland Road, Somers Town, London, NW1 1AT, UK.

⁴HDBR, Newcastle University, Newcastle Upon Tyne, NE1 4EP, UK

⁵Medical-risk Avoidance based on iPS Cells Team, RIKEN Center for Advanced Intelligence Project (AIP), Kyoto 606-8507, Japan

⁶Department of Medicine and Life Sciences Universitat Pompeu Fabra and ICREA, Pag Lluís Companys 23, Barcelona, Spain

⁷These authors contributed equally: Yoshihiro Yamanaka, Sofiane Hamidi

*Correspondence to: alev.cantas.8m@kyoto-u.ac.jp

SUPPLEMENTARY DISCUSSION

Midline and bilateral somite formation in axioids

We typically observed in MG and RA treated axioids a single axis of sequentially forming epithelial somites with single central somitocoels. Intriguingly, axioids also frequently displayed a superficial groove or midline-like structure, starting in the PSM and going through most of the forming segments (Supplementary Fig. 3a, b), though it typically remained superficial, without separating the segments into two fully segregated somites. (Supplementary Fig. 3a, b, Supplementary Video 8). True bilateral, fully segregated somites, each with their respective central somitocoel, were nevertheless occasionally present in retinoid treated axioids, sometimes as an isolated fully segregated bilateral single pair of somites and in some rare cases also as a sequence of two or more somite pairs along the AP-axis of an axioid (Supplementary Fig. 3c-e, Supplementary Video 8). These bilateral somites showed usually normal protein and gene expression patterns, including proper rostrocaudal polarity (Supplementary Fig. 3f-k). The exact molecular nature and possible mechanism which may underlie the formation of single vs. bilateral somites in axioids remains to be elucidated.

SUPPLEMENTARY TABLES

Supplementary Table 1: Genes expressed in identified clusters of scRNA-seq data.

Supplementary Table 2: NMP and NE module score genes.

List of neuromesodermal progenitor (NMP) and neuroectoderm (NE) associated genes used to calculate the module scores for Extended Data Fig. 3g.

Supplementary Table 3: Recombinant proteins, small molecules, reagents & consumables/equipment used in this study.

List of utilized recombinant proteins & small molecules (3.1), modulators of signaling pathways (3.2) reagents (3.3) and consumables/equipment (3.4).

Supplementary Table 4: Antibodies used in this study.

List of utilized primary antibodies for immunohistochemistry (4.1), secondary antibodies for immunohistochemistry (4.2), primary antibodies for CUT&Tag library preparation (4.3), secondary antibodies for CUT&Tag library preparation (4.4).

Supplementary Table 5: List of probes used for HCR.

List of utilized probes for HCR-based whole-mount in situ hybridization analysis of human axioid samples.

Supplementary Table 6: List of probe sequences used for HybISS analysis.

List of utilized probe sequences used for HybISS-based spatial transcriptomic analysis of human axioid samples.

Supplementary Table 7: List of primers used for CUT&Tag experiments.

List of utilized primers used for CUT&Tag-based analysis of human axioids.

SUPPLEMENTARY VIDEOS

Supplementary Video 1: Symmetry breaking and initial elongation of axioids.

Live imaging of axioids derived from 409B2 (upper) and 201B7 Luc (lower) between 24 h and 72 h of culture. Data shown is representative of at least three independent experiments. Scale bar is 200 μm .

Supplementary Video 2: Effect of Matrigel on axioid morphology.

Live imaging of axioids derived from 409B2 (upper) and 201B7 Luc (lower) with +MG (right) or without -MG (left) embedding into Matrigel (MG) between 72 h and 120 h of culture. Data shown is representative of at least three independent experiments. Scale bar is 200 μm .

Supplementary Video 3: *HES7* gene expression dynamics in MG embedded axioids.

Live imaging of the spatiotemporal morphogenetic expression of the *HES7* gene in a 201B7 Luc-derived axioid embedded in MG from 72 h to 120 h of culture. BF video (left) and *HES7*:Luciferase signal (right). Data shown is representative of at least three independent experiments. Scale bar is 200 μm .

Supplementary Video 4: Effect of retinoid signaling on axioid growth and morphology.

Live imaging of axioids derived from 409B2 (top panels) and 201B7 Luc (bottom panels) after embedding in MG only (left) or in MG supplemented with retinol (ROL), retinal (RAL) or retinoic acid (RA) (right) from 72 h to 120 h of culture. Data shown is representative of at least three independent experiments. Scale bar is 200 μm .

Supplementary Video 5: 3D reconstruction of retinoid treated axioids.

3D reconstruction of 409B2-derived axioids embedded in MG only (upper left) or in MG supplemented with retinol (ROL) (lower left), retinal (RAL) (lower right) or retinoic acid (RA) (upper right) at 120 h of culture stained for F-actin (Phalloidin) in gray, TBXT (BRA) in blue, Fibronectin (FN1) in green and MEOX1 in red.

Supplementary Video 6: Effect of RA pathway inhibition on axioid growth and morphology

Live imaging of axioids derived from 201B7 Luc after embedding in +MG +RAL supplemented with (from left to right) DMSO, BMS493, AGN193109 or ER50891 from 72 h to 120 h of culture. Data shown is representative of at least three independent experiments. Scale bar is 200 μm .

Supplementary Video 7: 3D visualization of axioids treated with RA pathway inhibitors.

3D reconstruction of axioids derived from 201B7 Luc embedded in +MG +RAL (upper left) or in +MG +RAL supplemented with three different RA inhibitors, including BMS493 (upper right), AGN193109 (lower left) or ER50891 (lower right) at 120 h of culture stained for F-actin (Phalloidin) in gray, TBXT (BRA) in blue, Fibronectin (FN1) in green and MEOX1 in red.

Supplementary Video 8: Midline and bilateral somite formation in axioids.

Visualization of midline formation in a 409B2-derived axioid embedded in +MG +RAL (top) and of formation of a single bilateral somite in 409B2-derived axioid embedded in +MG +ROL (bottom). Live imaging was performed between 72 and 120 h of culture. Scale bar is 200 μm .

Supplementary Video 9: 3D reconstruction of somites in axioids and human CS9-11 embryos.

3D reconstruction of somites formed in 409B2-derived MG embedded axioids (top) treated retinol (ROL) (left), retinal (RAL) (middle) and retinoic acid (RA); and 3D reconstructions of somites in human embryos (bottom) found in CS9 (left), CS10 (middle) and CS11 (right) human embryos. Each somite-like structure is highlighted by a different color depending on its position along the antero-posterior axis.

Supplementary Video 10: Effect of ROL, RAL or RA on *HES7* gene expression dynamics.

Live imaging of the spatiotemporal morphogenetic expression of the *HES7* gene in 201B7 Luc-derived axioids embedded in MG only (upper left pair) or in MG supplemented with retinol (ROL) (lower left pair), retinal (RAL) (lower right pair) or retinoic acid (RA) (upper right pair) from 72 h to 120 h of culture. BF video (left) and *HES7*:Luciferase signal (right) for each condition. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m.

Supplementary Video 11: Effect of BMS493 supplementation on *HES7* gene expression dynamics.

Live imaging of the spatiotemporal morphogenetic expression of the *HES7* gene in 201B7 Luc-derived axioids embedded in MG supplemented with +RAL+DMSO (left pair) or +RAL+BMS493 (right pair), from 72 h to 120 h of culture. BF video (left) and *HES7*:Luciferase signal (right) for each condition. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m.

Supplementary Video 12: Effect of NOTCH, FGF and WNT pathway inhibition on axioids.

Live imaging of axioids derived from 201B7 Luc after embedding in +MG +RAL supplemented with DMSO (top left), DAPT (top right), PD173074 (middle left), PD0325901 (middle right), XAV939 (bottom left) or IWP2 (bottom right) from 72 h to 120 h of culture. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m.

Supplementary Video 13: Effect of *HES7* gene KO on axioid growth and morphology.

Top panel, live imaging of axioids embedded in +MG +RAL derived from 201B7 Luc (top left), *HES7* KO1 (top middle) and *HES7* KO2 (top right) cell lines. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m. Bottom panel, 3D reconstruction of axioids embedded in +MG +RAL derived from *HES7* KO1 (middle) and *HES7* KO2 (bottom) stained with F-actin (Phalloidin) in gray, TBXT (BRA) in blue, Fibronectin (FN1) in green and MEOX1 in red.

Supplementary Video 14: Effect of *HES7* gene KO on *HES7* gene expression dynamics.

Live imaging of the spatiotemporal expression of the *HES7* gene in 201B7 Luc, (top pair), *HES7* KO1 (middle pair) and *HES7* KO2 (bottom pair)-derived axioids embedded in +MG +RAL. BF video (left) and *HES7*:Luciferase signal (right) for each condition. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m.

Supplementary Video 15: Effect of *HES7* point mutation (rs113994160: c.73C>T; *HES7*^{R25W}) on axioid growth and morphology.

Top panel, live imaging of axioids embedded in +MG +RAL derived from 201B7 Luc (top left), *HES7*^{R25W} MT1 (top middle) and *HES7*^{R25W} MT2 (top right) cell lines. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m. Bottom panel, 3D reconstruction of axioids embedded in +MG +RAL derived from *HES7*^{R25W} MT1 (middle) and *HES7*^{R25W} MT2 (bottom) stained with F-actin (Phalloidin) in gray, TBXT (BRA) in blue, Fibronectin (FN1) in green and MEOX1 in red.

Supplementary Video 16: Effect of *HES7* point mutation (rs113994160: c.73C>T; *HES7*^{R25W}) on *HES7* gene expression dynamics.

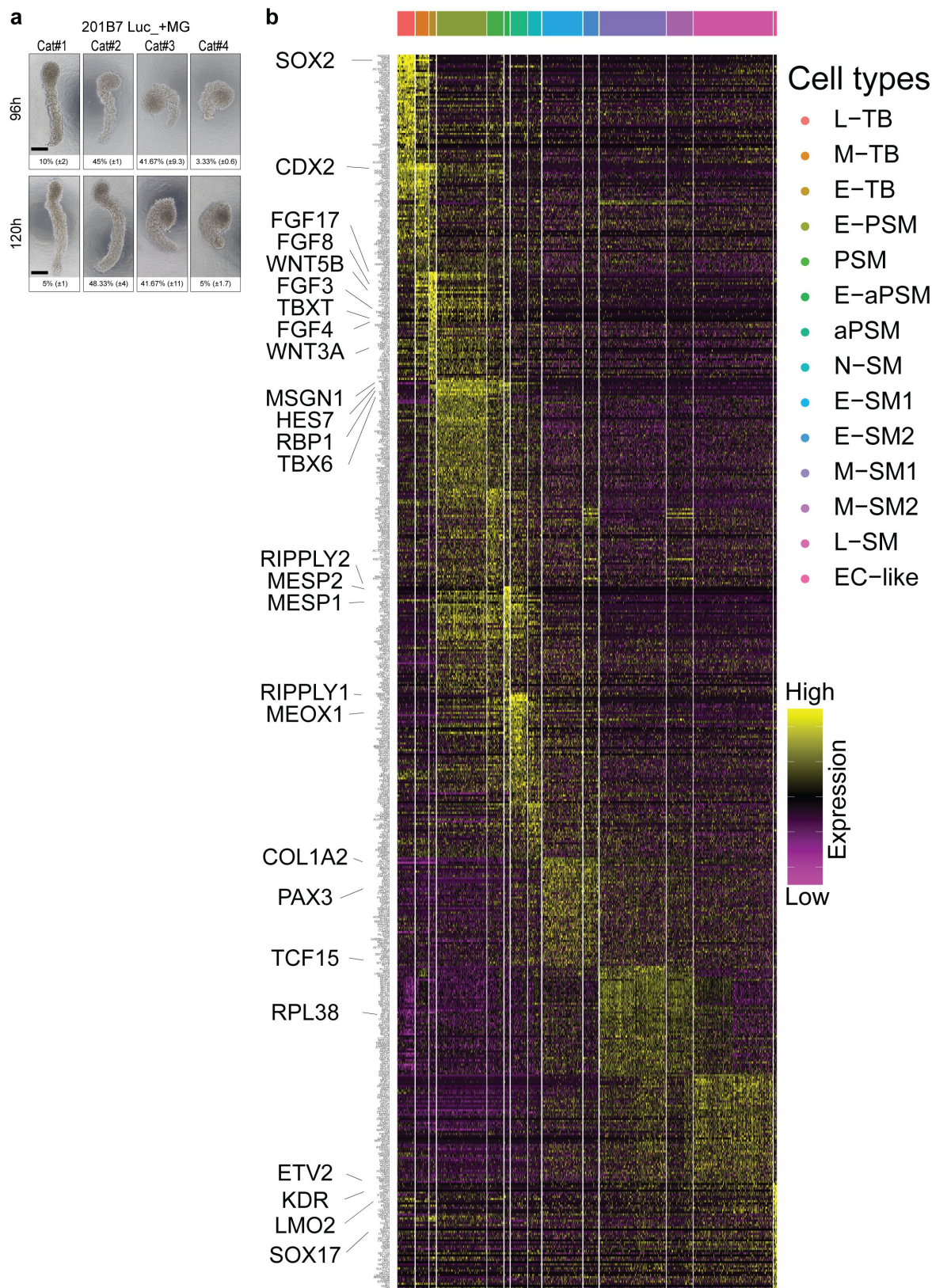
Live imaging of the spatiotemporal expression of the *HES7* gene in 201B7 Luc, (top pair), *HES7*^{R25W} MT1 (middle pair) and *HES7*^{R25W} MT2 (bottom pair)-derived axioids. BF video (left) and *HES7*:Luciferase signal (right) for each condition. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m.

Supplementary Video 17: Effect of *MESP2* gene KO on axioid growth and morphology.

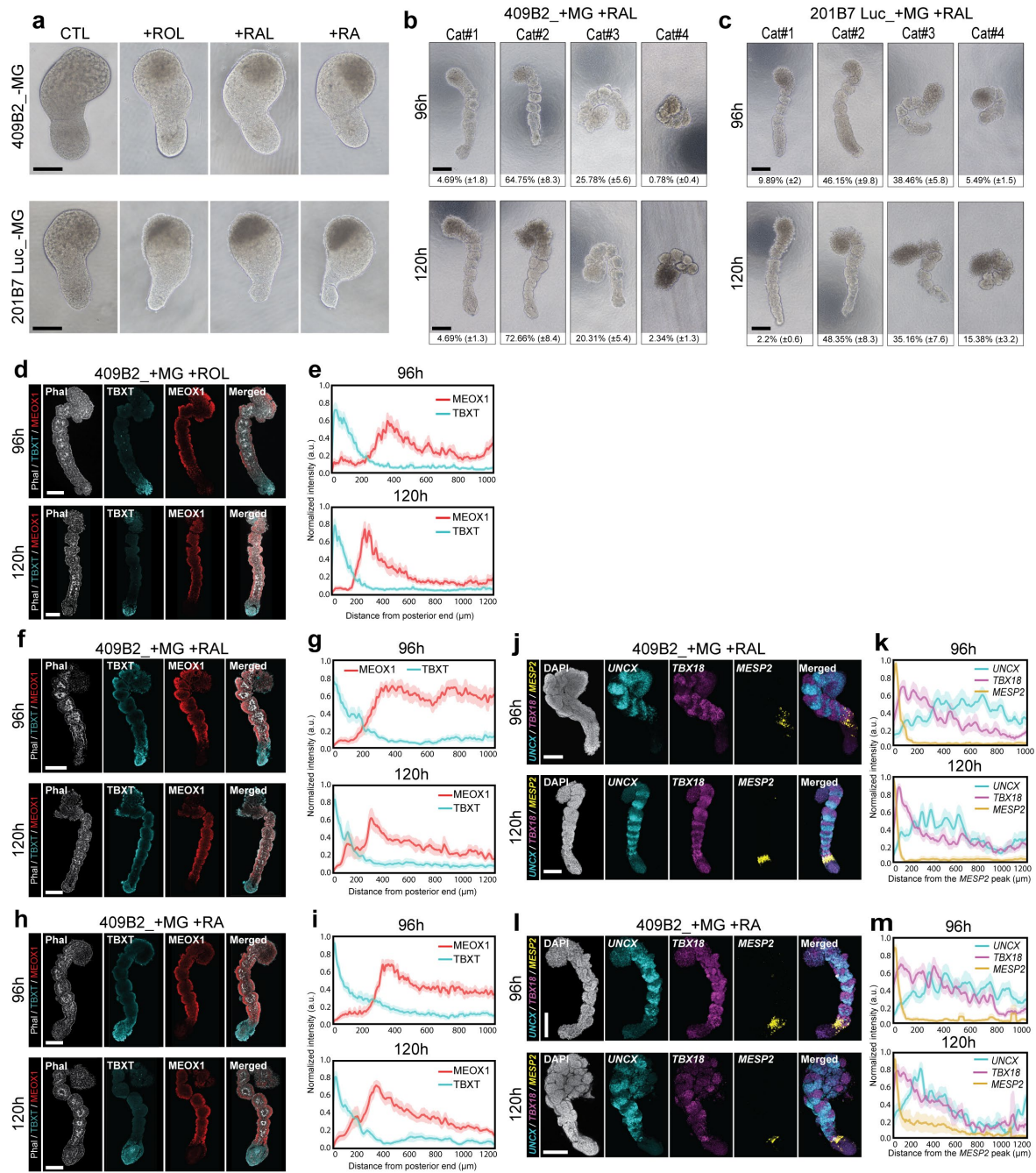
Top panel, live imaging of axioids embedded in +MG +RAL derived from 201B7 Luc (top left), *MESP2* KO1 (top middle) and *MESP2* KO2 (top right) cell lines. Data shown is representative of at least three independent experiments. Scale bar is 200 μ m. Bottom panel, 3D reconstruction of axioids embedded in +MG +RAL derived from *MESP2* KO1 (middle) and *MESP2* KO2 (bottom) stained with F-actin (Phalloidin) in gray, TBXT (BRA) in blue, Fibronectin (FN1) in green and MEOX1 in red.

Supplementary Video 18: Effect of *MESP2* gene KO on *HES7* gene expression dynamics.

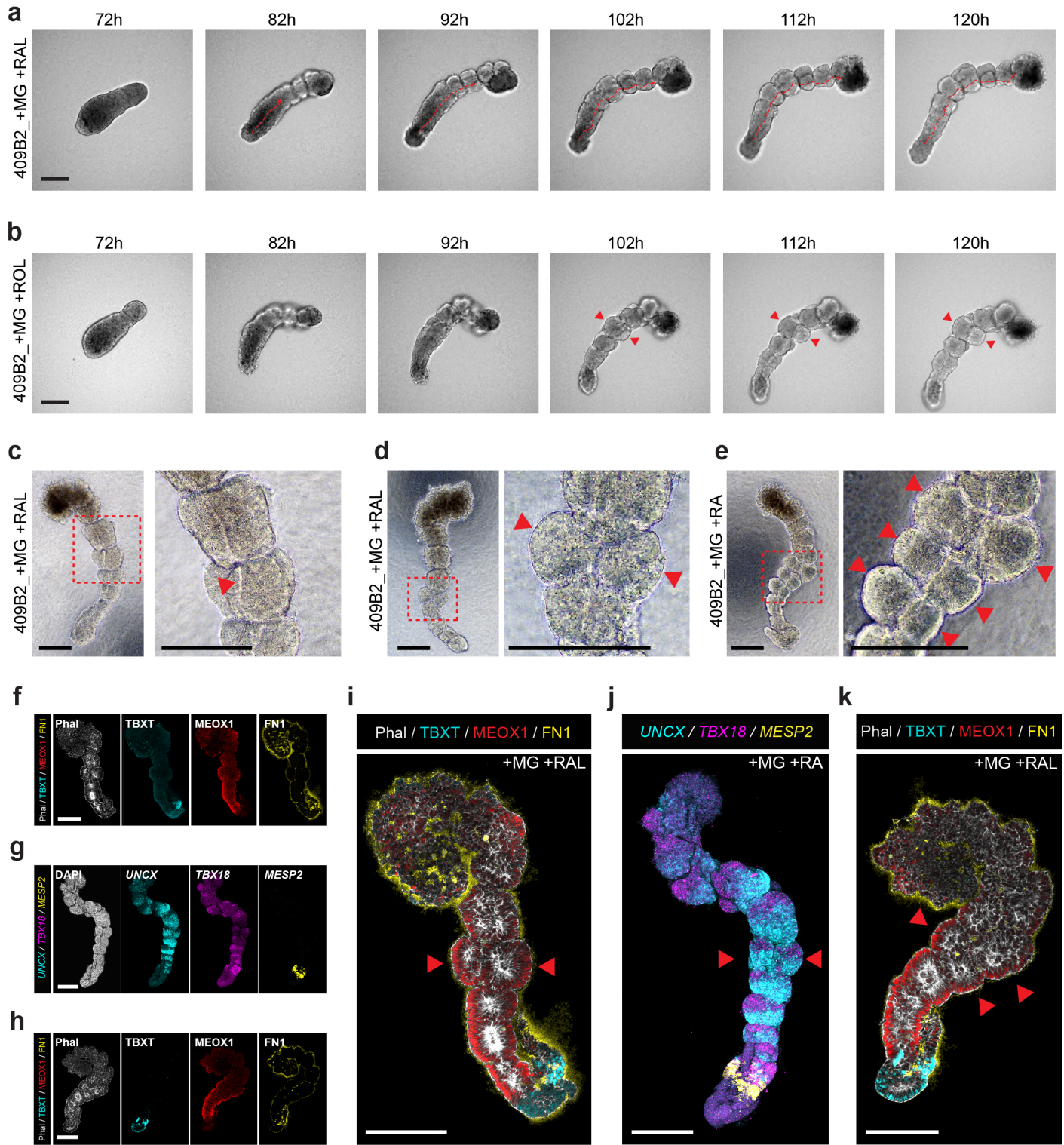
Live imaging of the spatiotemporal expression of the *HES7* gene in 201B7 Luc (top pair), *MESP2* KO1 (middle pair) and *MESP2* KO2 (bottom pair)-derived axioids embedded in +MG +RAL. BF video (left) and *HES7*:Luciferase signal (right) for each condition. Data shown is representative of at least three independent experiments. Scale bar is 200 μm .



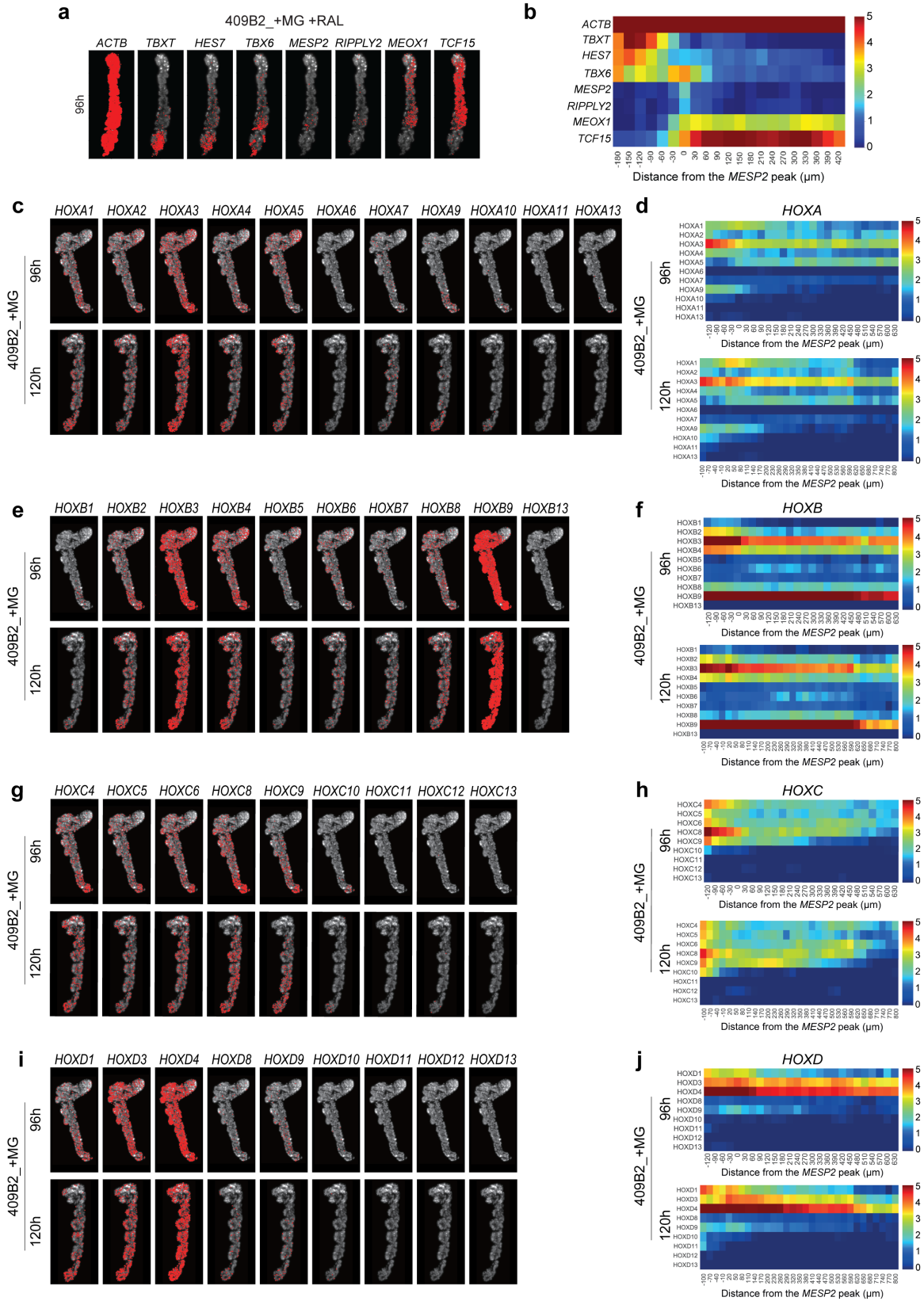
Supplementary Figure 1 | Morphogenetic characterization of axioids embedded in +MG only and scRNA-seq expression profiles of human axioids. a, Representative bright field images reflecting the different morphology-based categories (Cat#1: straight, minimal curvature, clear segments; Cat#2: curved but clear segments; Cat#3: very curved, segment borders are still distinguishable; Cat#4: not properly elongated or completely collapsed; segment borders not clear) of MG embedded axioids at 96 h and 120 h derived using the 201B7 Luc cell line (3 independent experiments, $n = 60$ axioids). Cat#1 corresponds to Fig.7b, 96 h, CTL. **b,** scRNA-seq expression profiles of identified marker genes for each cell cluster described in Fig. 2b, except for E-SM2, M-SM2, and presumably apoptotic cells. The top 50 (or less) genes of higher fold changes are shown. Scale bar is 200 μm .



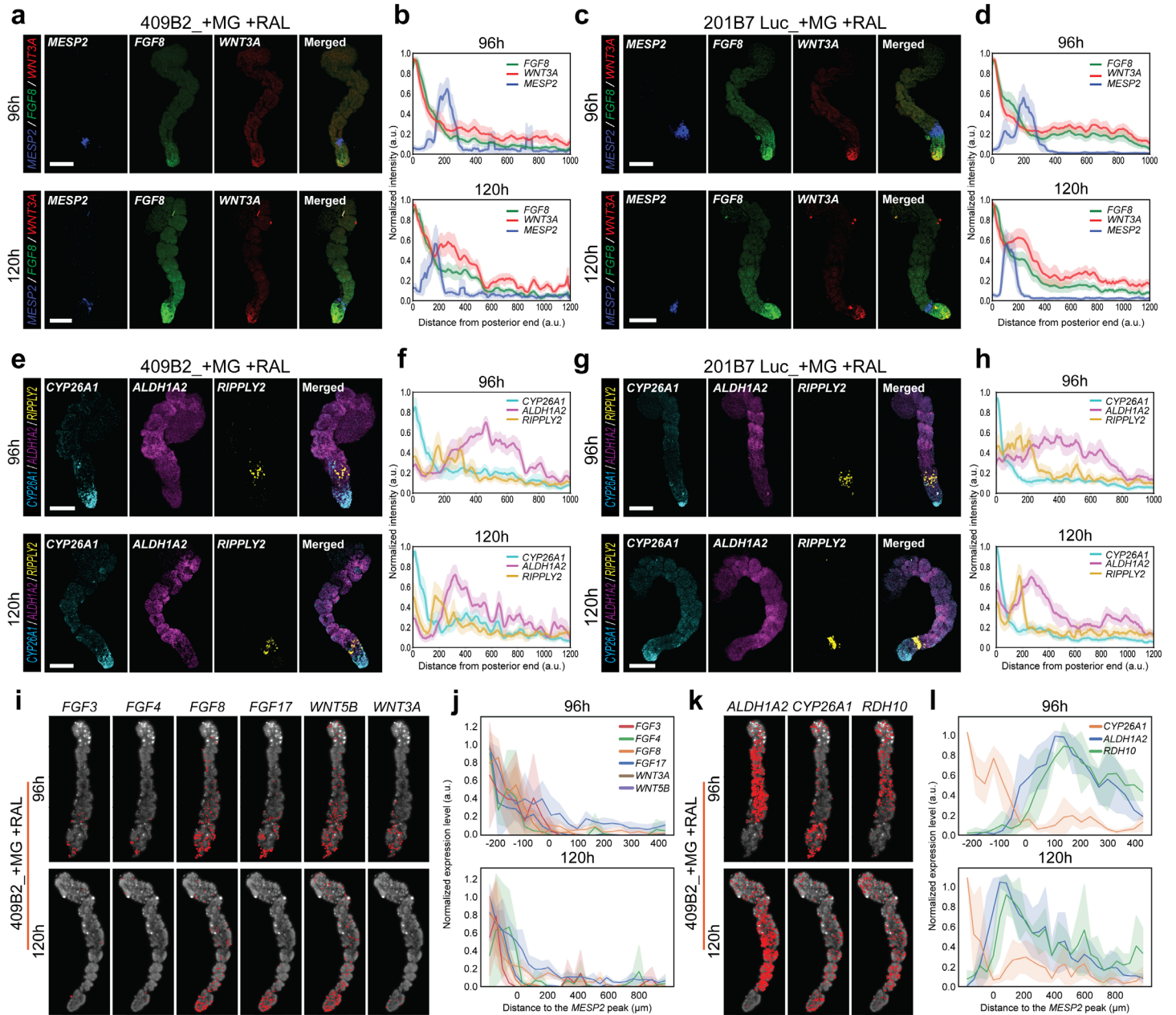
Supplementary Figure 2 | Characterization of retinoid treated human axioids. **a**, Bright field images of axioids at 120 h without MG embedding, after addition of RAL only, ROL only and RA only (no MG in all cases) in 409B2, top panel and 201B7 Luc, bottom panel. **b**, and **c**, Representative bright field images reflecting the different morphology-based categories (Cat#1: straight, minimal curvature, clear somites; Cat#2: curved but clear segments & somites; Cat#3: very curved, somites & segment borders are still distinguishable; Cat#4: not properly elongated or completely collapsed; somites & segment borders not clear) of +MG+RAL embedded axioids at 96 h and 120 h in 2 different cell lines **b**, the 409B2 (5 independent experiments, $n = 128$ axioids, mean $\pm$ SD) and **c**, the 201B7 Luc hiPSC line (96 h, 3 independent experiments, $n = 91$ axioids and 120 h, 3 independent experiments, $n = 92$ axioids, mean $\pm$ SD). **d-i**, Representative images of immunofluorescence staining of F-actin (Phalloidin) in gray, TBXT in cyan, and MEOX1 in red and corresponding quantification of signal intensity of 409B2 derived axioids embedded in **d-e**, +MG+ROL at 96 h (3 independent experiments, $n = 9$ axioids) and 120 h (3 independent experiments, $n = 9$ axioids), **f-g**, in +MG+RAL at 96 h (4 independent experiments, $n = 10$ axioids) and 120 h (4 independent experiments, $n = 9$ axioids), **h-i**, in +MG+RA 409B2 at 96 h (4 independent experiments, $n = 15$ axioids) and 120 h (4 independent experiments, $n = 12$ axioids). **j-m**, Representative images of HCR staining of UNCX in cyan, TBX18 in magenta and MESP2 in yellow, and corresponding signal intensity measurements along the posterior to anterior axis normalized to the position of the MESP2 signal peak of axioids embedded in +MG+RAL 409B2 at 96 h (3 independent experiments, $n = 9$ axioids) and 120 h (3 independent experiments, $n = 9$ axioids) shown in **j-k**, and of axioids embedded in +MG+RA at 96 h (3 independent experiments, $n = 9$ axioids) and 120 h (4 independent experiments, $n = 8$ axioids) shown in **l-m**. Single channel images shown in **j** correspond to the merged channel image shown in Fig. 4f. Lines in **e**, **g**, **i**, **k** and **m** correspond to mean values, error bands represent the 95% confidence interval, of which the top and bottom show the 2.5 and 97.5 percentiles for each data point. Scale bar is 200 μ m. a.u., arbitrary units.



Supplementary Figure 3 | Midline and bilateral somite formation in human axioids. **a**, and **b**, Serial images of elongating human axioids (409B2) at 8-10 h intervals, from 72 h to 120 h (extracted from **Supplementary Video 5**). **a**, Red dotted arrow shows formation and extension of superficial midline along the posterior to anterior axis of an axioid embedded in +MG +RAL. **b**, Red arrowheads show the initiation of bilateral somite formation in an axioid embedded in +MG +ROL. **c-e**, Left, brightfield images of axioids (409B2) at 120 h, red dotted square encompasses the area enlarged on the right. **c**, +MG +RAL embedded axioid showing a noticeable midline highlighted by an arrowhead in the right image. **c**, and **d**, +MG +RAL and +MG +RA embedded axioids respectively showing in **c**, a single bilateral somite and in **d**, multiple bilateral somites highlighted by red arrowheads. **f-k**, Immunofluorescence and in situ-based assessment of +MG +RAL and +MG +RA treated axioids with bilateral somites. **f**, **h**, **i**, **k**, Immunofluorescence staining of F-actin (Phalloidin) in gray, TBXT (BRA) in cyan, MEOX1 in red and FN1 in yellow. **g**, and **j**, HCR staining of *UNCX* in cyan, *TBX18* and *MESP2* in yellow in magenta of human axioid (409B2) at 120 h. Axioids in **f** and **g** present a single bilateral somite and in **h** and **k** multiple bilateral somites. **i**, **j**, and **k**, Correspond to an enlarged view of the merged channel images of **f**, **g** and **h** respectively. Images shown in **c-h** are representative of 3 independent experiments. Scale bar is 200 μm .



Supplementary Figure 4 | *HOX* gene expression in axioids in +MG only supplemented medium. **a**, Visualization of the spatial distribution of the *ACTB*, *TBXT*, *HES7*, *TBX6*, *MESP2*, *RIPPLY2*, *MEOX1*, *TCF15* transcripts in a section of a 409B2-derived axioid embedded in +MG +RAL at 96 h using HybISS and **b**, corresponding heatmap plot showing the average gene expression along the posterior to anterior axis normalized to the position of the *MESP2* signal peak ($n = 3$ axioids). **c-j**, HybISS-based visualization and quantification of *HOX* loci. **c**, **e**, **g**, and **i**, Visualization of the spatial distribution of the *HOXA*, *HOXB*, *HOXC* and *HOXD* transcripts using HybISS analysis of all the members of the respective *HOX* clusters using sections of 96 h and 120 h axioids cultured in MG only. **d**, **f**, **h**, and **j**, Heatmap plots of the HybISS data shown in **c**, **e**, **g** and **i** showing the average *HOXA*, *HOXB*, *HOXC* and *HOXD* cluster gene expression along the posterior to anterior axis of 96 h and 120 h axioids cultured in MG only ($n = 3$ axioids).



Supplementary Fig. 5 | Expression gradients of FGF, WNT and RA signaling pathway members in human axioids visualized and quantified via HCR and HybISS. **a-h**, HCR whole mount in situ hybridization images and signal quantification of +MG +RAL embedded axioids at 96 h and 120 h derived from **a, b, e, f**, 409B2 and **c, d, g, h**, 201B7 Luc iPSC lines. Shown images are representative of 3 independent experiments. **a**, and **c**, HCR staining of *FGF8* in green, *WNT3A* in red and *MESP2* in blue. **b**, and **d**, Corresponding quantification along the posterior to anterior axis of the signal intensity normalized to the position of the *WNT3a* signal peak (409B2 96 h: 4 independent experiments, $n = 7$ axioids and 120 h: 3 independent experiments, $n = 6$ axioids, 201B7 Luc 96 h: 3 independent experiments, $n = 9$ axioids and 120 h: 3 independent experiments, $n = 8$ axioids). **e**, and **g**, HCR staining of *CYP26A1* in cyan, *ALDH1A2* in magenta and *RIPPLY2* in yellow. **f**, and **h**, Corresponding quantification along the posterior to anterior axis of the signal intensity (409B2 96 h: 4 independent experiments, $n = 10$ axioids and 120 h: 3 independent experiments, $n = 7$ axioids, 201B7 Luc 96 h: 3 independent experiments, $n = 8$ axioids and 120 h: 3 independent experiments, $n = 10$ axioids). Lines in **b**, **d**, **f** and **h** correspond to mean values, error bands represent the 95% confidence interval, of which the top and bottom show the 2.5 and 97.5 percentiles for each data point. Scale bar is 200 μm . **i-l**, HybISS based visualization and quantification of the spatial distribution of FGF/WNT and RA signaling pathway transcripts in human 409B2-derived axioids at 96 h (top) and 120 h (bottom) of culture in +MG +RAL. **i**, and **j**, HybISS images and quantification of spatial expression of *FGF3*, *FGF4*, *FGF8*, *FGF17*, *WNT3A* and *WNT5B*. **k**, and **l**, HybISS images and quantification of spatial expression of *ALDH1A2*, *CYP26A1* and *RDH10*. Lines in **j** and **l** correspond to mean values and error bands represent the SD for each data point ($n = 3$ axioids). a.u., arbitrary units.