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Supplementary information

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Two parallel neural ectoderm progenitors contribute to the developing brain

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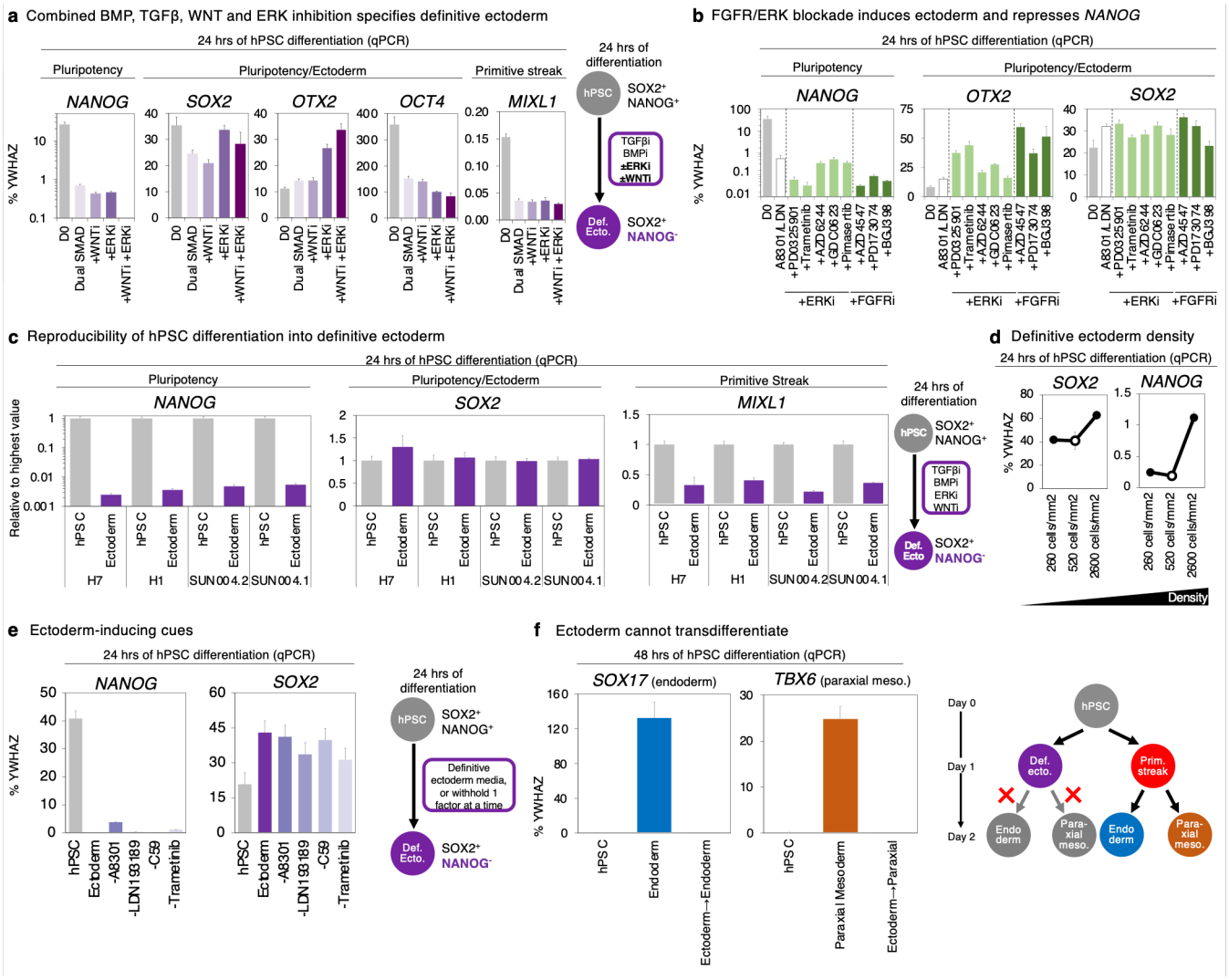


Supplementary Figure 1: Optimization of hPSC differentiation into definitive ectoderm

A) qPCR of H7 hPSCs differentiated for 24 hours with "dual SMAD inhibitors" (TGFβ inhibitor A-83-01 [1 μM] and BMP inhibitor LDN-193189 [250 nM]), in the presence or absence of WNT inhibitor (C59, 1 μM) and ERK inhibitor (Trametinib, 250 nM). Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. "D0" = day-0 undifferentiated hPSCs. i: inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

B) qPCR of H7 hPSCs differentiated for 24 hours with "dual SMAD inhibitors" (TGFβ inhibitor A-83-01 [1 μM] and BMP inhibitor LDN-193189 [250 nM]), in the presence or absence of individual ERK inhibitors (PD0325901, Trametinib, AZD6244, GDC0623 or Pimasertib) or FGFR inhibitors (AZD4547, PD173074 or BGJ398). Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. D0: day-0 undifferentiated hPSCs. i: inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

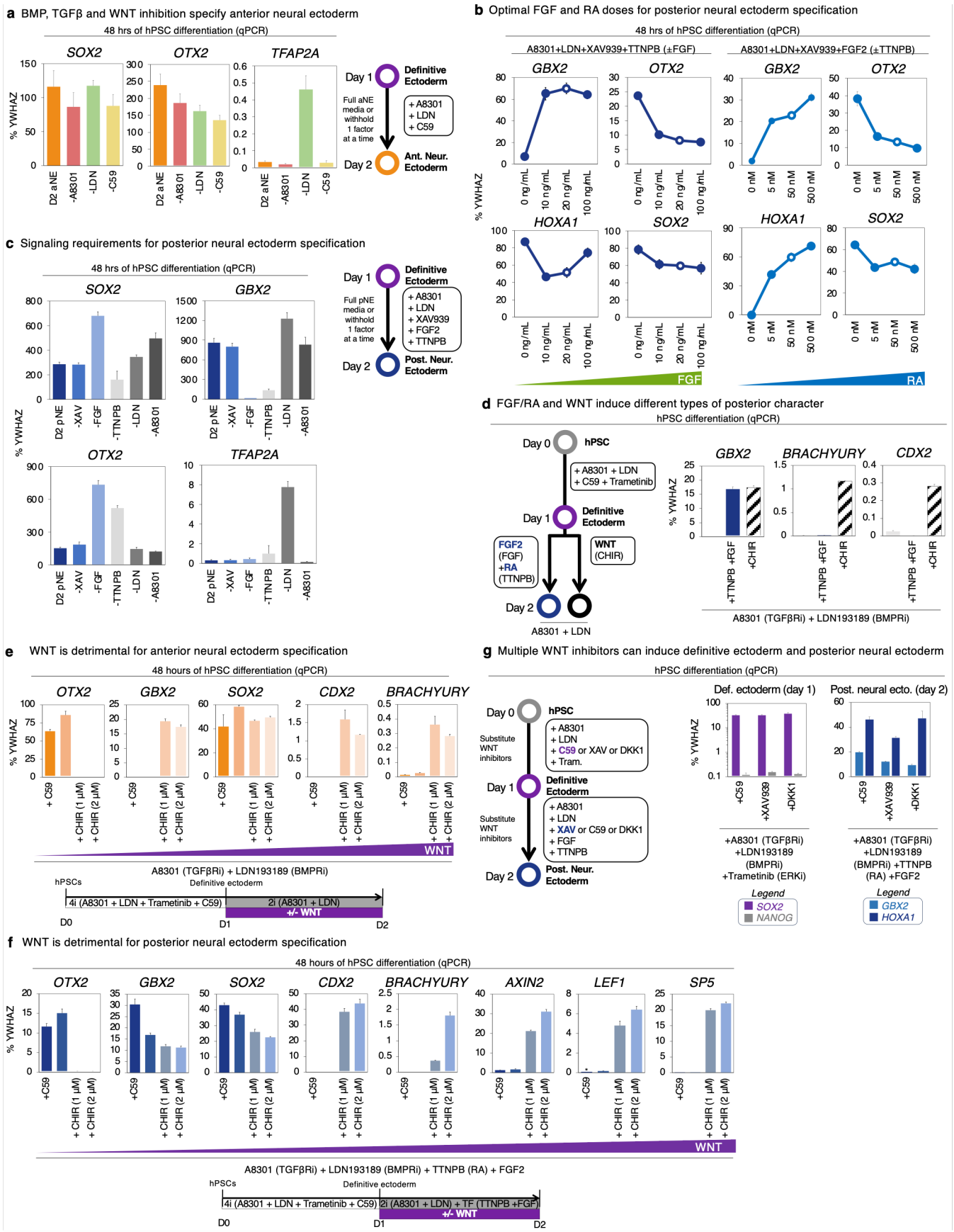
C) qPCR of H1, H7, SUN004.1 and SUN004.2 hPSCs prior to, or after, differentiation into definitive ectoderm with BMP, ERK, TGFβ and WNT inhibitors (LDN-193189, Trametinib, A-83-01 and C59, respectively) for 24 hours. Gene expression is generally shown relative to the sample with the highest expression in each cell line. Data depicts the mean of two biological

replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

D) qPCR of H1 hPSCs seeded at different densities, and then differentiated into definitive ectoderm with BMP, ERK, TGFβ and WNT inhibitors (LDN-193189, Trametinib, A-83-01 and C59, respectively) for 24 hours. qPCR data normalized to undifferentiated hPSCs. Open circle indicates a seeding density of 520 cells/mm^2, which was the baseline used throughout this study. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

E) qPCR of H1 hPSCs differentiated for 24 hours into definitive ectoderm with BMP, ERK, TGFβ and WNT inhibitors (LDN-193189, Trametinib, A-83-01 and C59, respectively), or with each ectoderm-inducing signal individually withheld. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

F) qPCR of H1 hPSCs that were either differentiated into anterior primitive streak [30] or definitive ectoderm for 24 hours, followed by treatment with either endoderm-inducing media [29] (Activin A [100 ng/mL] + LDN193189 [250 nM]) for 24 hours, or paraxial mesoderm-inducing media[30] (A-83-01 [1 μM] + LDN193189 [250 nM] + CHIR99021 [3 μM] + FGF2 [20 ng/mL]) for 24 hours. This revealed that hPSC-derived ectoderm largely failed to express either endoderm or paraxial mesoderm markers. By contrast, hPSC-derived primitive streak exposed to the same signals turned on either endoderm or paraxial mesoderm markers. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 2: Optimization of hPSC differentiation into anterior or posterior neural ectoderm

A) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by BMP, TGFβ and WNT inhibitors (LDN-193189, A-83-01 and C59, respectively) for 24 hours to generate anterior neural ectoderm. To assess the importance of each anterior neural ectoderm-inducing signal, each factor was individually withheld. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. D2 aNE: day-2 anterior neural ectoderm. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

B) qPCR of H1 hPSCs were differentiated into definitive ectoderm for 24 hours, followed by differentiation into posterior neural ectoderm for 24 hours. During posterior neural ectoderm induction, different doses of FGF agonist (FGF2) or RA agonist (TTNPB) were added to assess dose-dependent effects. Open circles indicate the optimal doses of FGF2 (20 ng/mL) and TTNPB (50 nM) for posterior neural ectoderm induction used as a baseline throughout this study. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

C) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by a combination of FGF and RA agonists (FGF2 and TTNPB, respectively) together with BMP, TGFβ and WNT inhibitors (LDN-193189, A-83-01 and C59, respectively) for 24 hours to generate posterior neural ectoderm. To assess the importance of each posterior neural ectoderm-inducing signal, each factor was individually withheld. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. "D2 pNE": day-2 posterior neural ectoderm. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

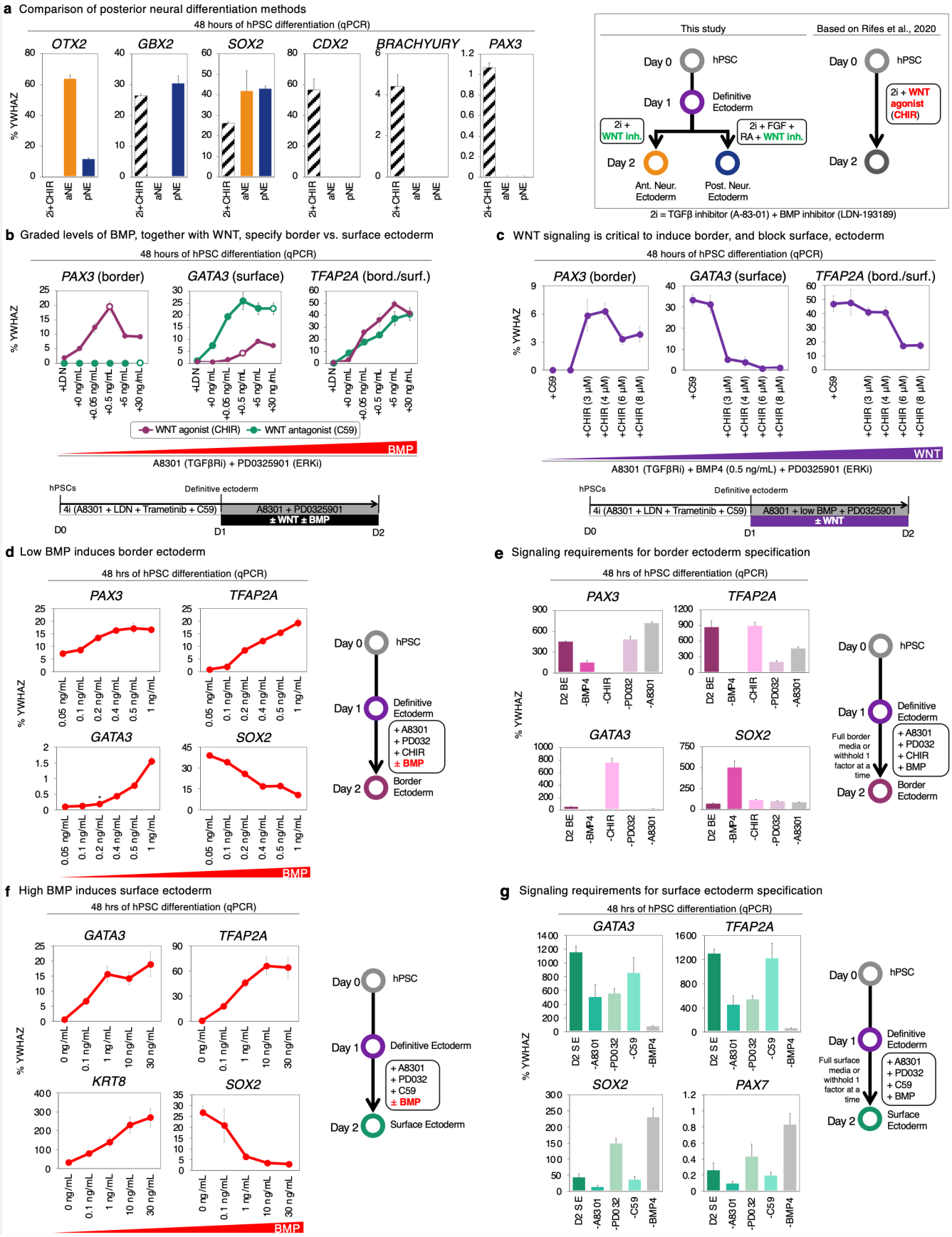
D) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by exposure to (1) neural-inducing signals (A-83-01 + LDN-193189), (2) neural-inducing signals (A-83-01 + LDN-193189) + FGF2 (20 ng/mL) + TTNPB (50 nM) or (3) neural-inducing signals (A-83-01 + LDN-193189) + CHIR99021 (2 μM) for 24 hours. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

E) qPCR of H1 hPSCs were differentiated into definitive ectoderm for 24 hours, followed by differentiation into anterior neural ectoderm for 24 hours (with A-83-01, LDN-193189, and C59). Alternatively, to test the effect of WNT signaling on anterior neural ectoderm induction, WNT inhibitor (C59) was withheld, and WNT agonist (CHIR99021) was added instead. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

F) qPCR of H1 hPSCs were differentiated into definitive ectoderm for 24 hours, followed by differentiation into posterior neural ectoderm for 24 hours (with A-83-01, LDN-193189, C59, FGF2 and TTNPB). Alternatively, to test the effect of WNT signaling on posterior neural ectoderm induction, WNT inhibitor (C59) was withheld, and WNT agonist (CHIR99021) was added instead. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological

replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

G) First, qPCR was performed of H1 hPSCs that were differentiated into definitive ectoderm for 24 hours with A-83-01, LDN193189, Trametinib, and WNT inhibitor (either C59 [1 μM], XAV939 [1 μM], or DKK1 [300 ng/mL]), revealing that all three WNT inhibitors comparably induced definitive ectoderm (*left*). Second, qPCR was performed of H1 hPSCs that were differentiated into definitive ectoderm for 24 hours with A-83-01, LDN193189, Trametinib, and XAV939, followed by differentiation into posterior neural ectoderm for 24 hours with A-83-01, LDN193189, TTNPB, FGF2, and WNT inhibitor (either C59 [1 μM], XAV939 [1 μM], or DKK1 [300 ng/mL]), revealing that all three WNT inhibitors comparably induced posterior neural ectoderm (*right*). Given these comparable results, throughout the rest of this study we applied C59 for definitive ectoderm induction and XAV939 for posterior neural ectoderm induction, as indicated in bold in the accompanying cartoon. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 3: Optimization of hPSC differentiation into neural ectoderm and non-neural ectoderm

A) qPCR of H1 hPSCs differentiated for 48 hours, using two separate strategies. First, hPSCs were differentiated into definitive ectoderm for 24 hours, followed by either anterior or posterior neural ectoderm induction for 24 hours (as described in this study). Alternatively, hPSCs were differentiated with BMP and TGFβ inhibitors (LDN-193189 and A-83-01, respectively; "2i") together with WNT agonist (CHIR99021, 2 μM) for 48 hours, similarly to a previous report[20]. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

B) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by treatment with non-neural signals (A-83-01 and PD0325901), in combination with different levels of BMP signaling (BMP agonist BMP4 [0.05-30 ng/mL] or alternatively, BMP inhibitor LDN-193189), in the presence or absence of either WNT agonist (CHIR99021, 3 μM) or WNT inhibitor (C59, 1 μM), for 24 hours. White circles indicate optimal BMP doses for border ectoderm induction (0.5 ng/mL BMP4) or surface ectoderm induction (30 ng/mL BMP4). Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

C) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by border ectoderm-inducing signals (A-83-01, BMP4 [0.5 ng/mL], and PD0325901), in combination with different levels of WNT signaling (WNT agonist CHIR99021 [3-8 μM] or WNT inhibitor C59), for 24 hours. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

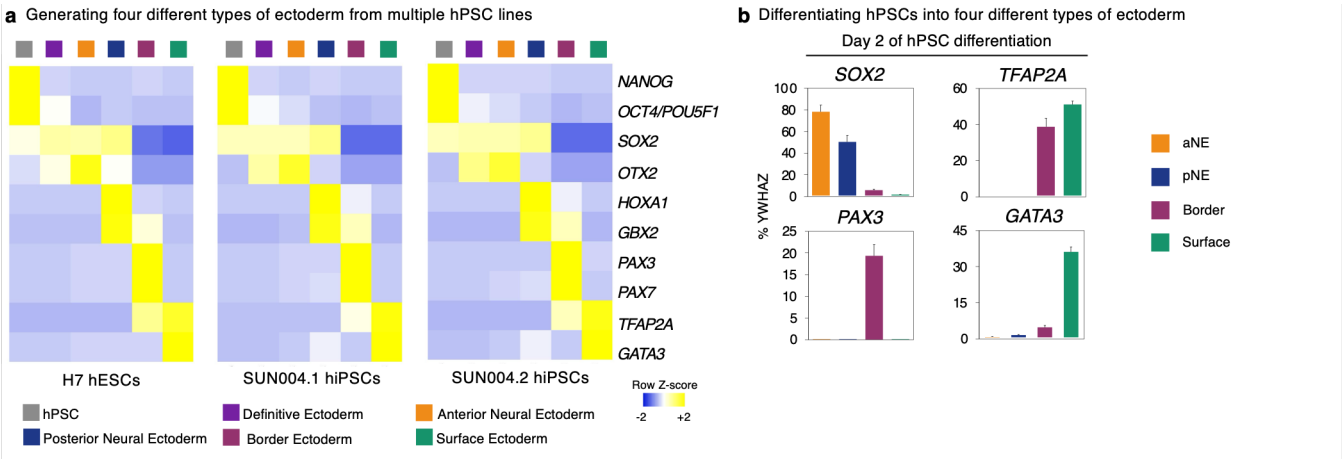
D) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by border ectoderm-inducing signals (A-83-01, CHIR99021 [3 μM], and PD0325901), in combination with different levels of BMP signaling (BMP agonist BMP4 [0.05-30 ng/mL] or alternatively, BMP inhibitor LDN-193189), for 24 hours. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

E) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by border ectoderm-inducing signals (A-83-01, BMP4, CHIR99021, and PD0325901) for 24 hours to generate border ectoderm. To assess the importance of each border ectoderm-inducing signal, each factor was individually withheld. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. D2 BE: day-2 border ectoderm. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

F) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by surface ectoderm-inducing signals (A-83-01, C59, and PD0325901), in the presence or absence of BMP4 (0-30 ng/mL), for 24 hours. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

G) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by surface ectoderm-inducing signals (A-83-01, BMP4, C59, and PD0325901) for 24 hours. To assess

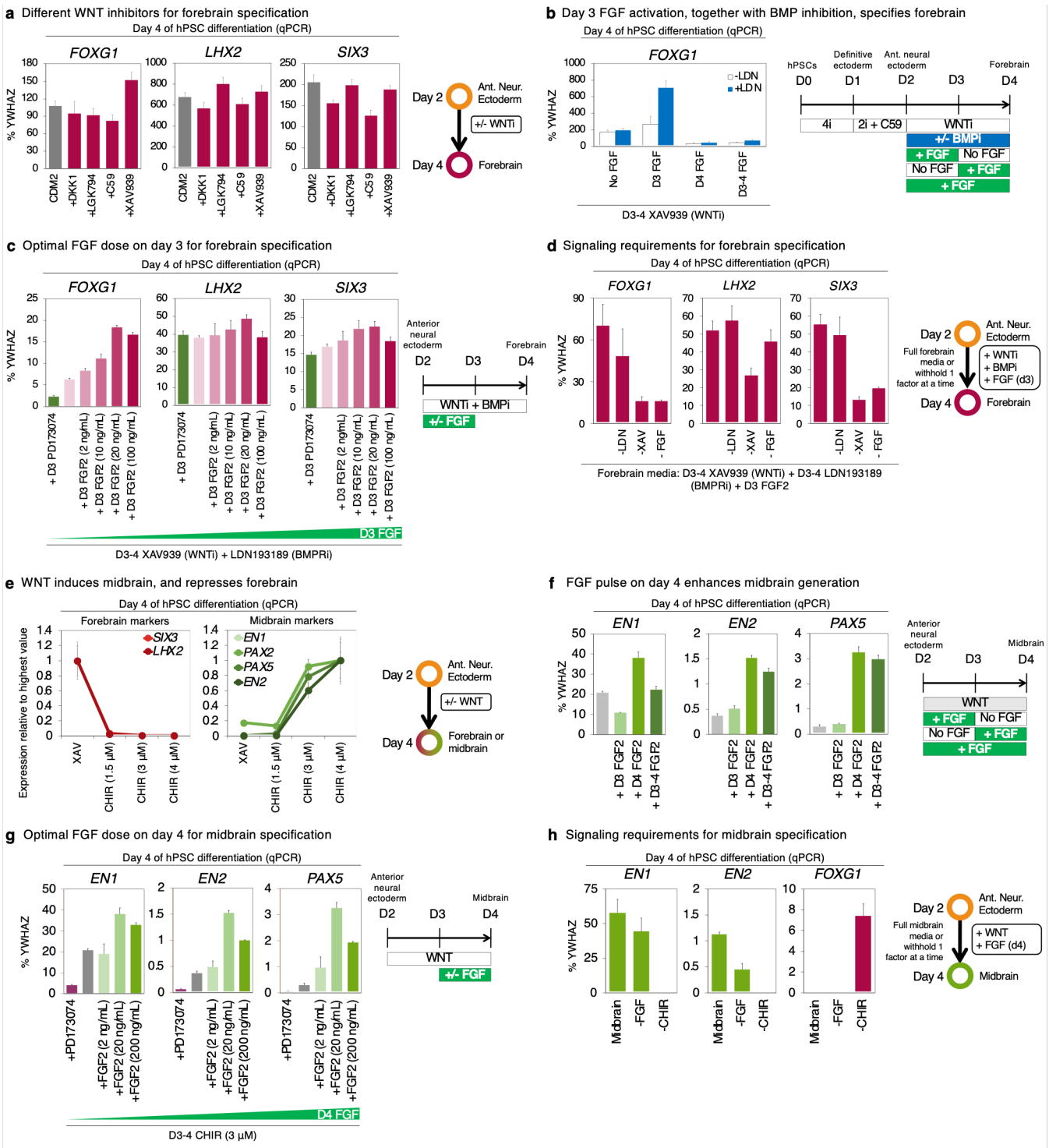
the importance of each border ectoderm-inducing signal, each factor was individually withheld. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. D2 SE: day-2 surface ectoderm. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 4: Differentiation of hPSCs into neural ectoderm and non-neural ectoderm

A) qPCR of H7 human embryonic stem cells (hESCs), SUN004.1 human induced pluripotent stem cells (hiPSCs), and SUN004.2 hiPSCs differentiated into definitive ectoderm for 24 hours, followed by either anterior neural ectoderm, posterior neural ectoderm, border ectoderm or surface ectoderm, for 24 hours. Heatmaps depict row Z-score, calculated within each hPSC line across the six cell-types from the mean normalized expression of two biological replicates from one experiment.

B) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by differentiation into either aNE, pNE, border ectoderm or surface ectoderm for 24 hours. Gene expression is shown relative to reference gene *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 5: Optimization of hPSC differentiation into forebrain or midbrain progenitors within 4 days

A) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently treated with either basal medium (CDM2) for 48 hours, or supplemented with WNT inhibitor DKK1 (LRP5/6 inhibitor, 300 ng/mL), LGK974 (Porcupine inhibitor, 100 nM), C59 (Porcupine inhibitor, 1 µM), or XAV939 (Tankyrase inhibitor, 1 µM). This revealed that XAV939 was most effective at upregulating forebrain marker *FOXG1*. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean

of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

B) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently treated with XAV939 for 48 hours to generate forebrain progenitors. During the 48 hour period of forebrain induction, FGF2 (20 ng/mL) was added for the first 24 hour interval ("day 3"), the second 24 hour interval ("day 4"), or the entirety of the 48 hour interval ("days 3-4"), in the presence or absence of BMP receptor inhibitor LDN193189 (100 nM). This revealed that day 3 FGF2 treatment, together with BMP inhibition on days 3-4, was most effective at upregulating forebrain markers *FOXG1* and *SIX3*. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

C) qPCR of H7 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently treated with XAV939 + LDN193189 + FGF2 (2-100 ng/mL) for 24 hours, and finally XAV939 + LDN193189 for 24 hours to generate forebrain progenitors. On the third day of differentiation ("D3"), different doses of FGF2 (2-100 ng/mL) were tested, or alternatively, exogenous FGF2 was withheld (no notation), in the presence or absence of FGFR inhibitor PD173074 (100 ng/mL). This revealed that 20 ng/mL of FGF2 on day 3 of differentiation was most effective at upregulating forebrain markers *SIX3* and *FOXG1*, consistent with how FGF induces *Foxg1* in the mouse embryonic forebrain[126]. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

D) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently treated with XAV939 + LDN193189 + FGF2 for 24 hours, and finally XAV939 + LDN193189 for 24 hours to generate forebrain progenitors. To assess the importance of each forebrain-inducing signal (XAV939, LDN193189 or FGF2), each individual signal was respectively withheld during the 48 hour interval of forebrain induction. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

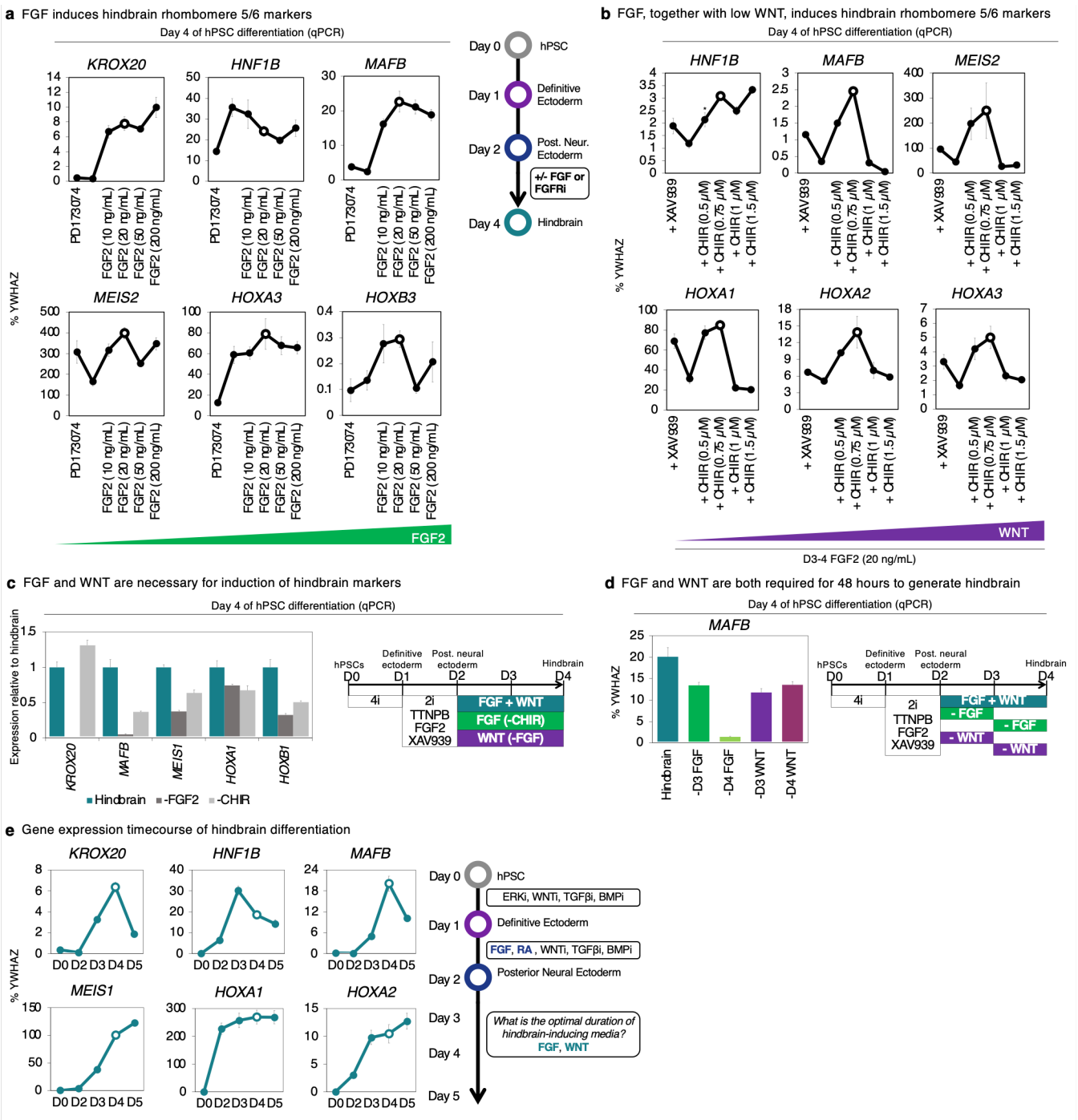
E) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for an additional 24 hours, and then subsequently treated with either WNT inhibitor (XAV939, 1 μM) or WNT agonist (CHIR99021, 1.5-4 μM) for an additional 48 hours. This showed that WNT induced midbrain progenitors, whereas WNT inhibitor specified forebrain progenitors. Gene expression is shown relative to the sample with the highest expression in this experiment. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

F) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently differentiated towards midbrain with WNT agonist (CHIR99021, 3 μM) for 48 hours, in the presence or absence of FGF2 (20 ng/mL) on the first 24 hour interval, the second 24 hour interval, or the entire 48 interval of midbrain induction. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of

two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

G) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently differentiated towards midbrain with WNT agonist (CHIR99021, 3 μM) for 48 hours, in the presence or absence of FGF2 (2-200 ng/mL) on last 24 hours of midbrain induction. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

H) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by anterior neural ectoderm for 24 hours, and then subsequently differentiated towards midbrain for 48 hours (CHIR99021 on days 3-4, and FGF2 on day 4). To assess the importance of each midbrain-inducing signal (CHIR99021 or FGF2), each individual signal was respectively withheld during the 48 hour interval of midbrain induction. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 6: Optimization of hPSC differentiation into hindbrain progenitors within 4 days

A) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by posterior neural ectoderm for 24 hours, and then further differentiated for 48 hours in the presence or absence of FGF2 (10-200 ng/mL) or FGFR inhibitor (PD173074, 100 nM). qPCR data normalized to undifferentiated hPSCs. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. FGFR: FGF receptor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

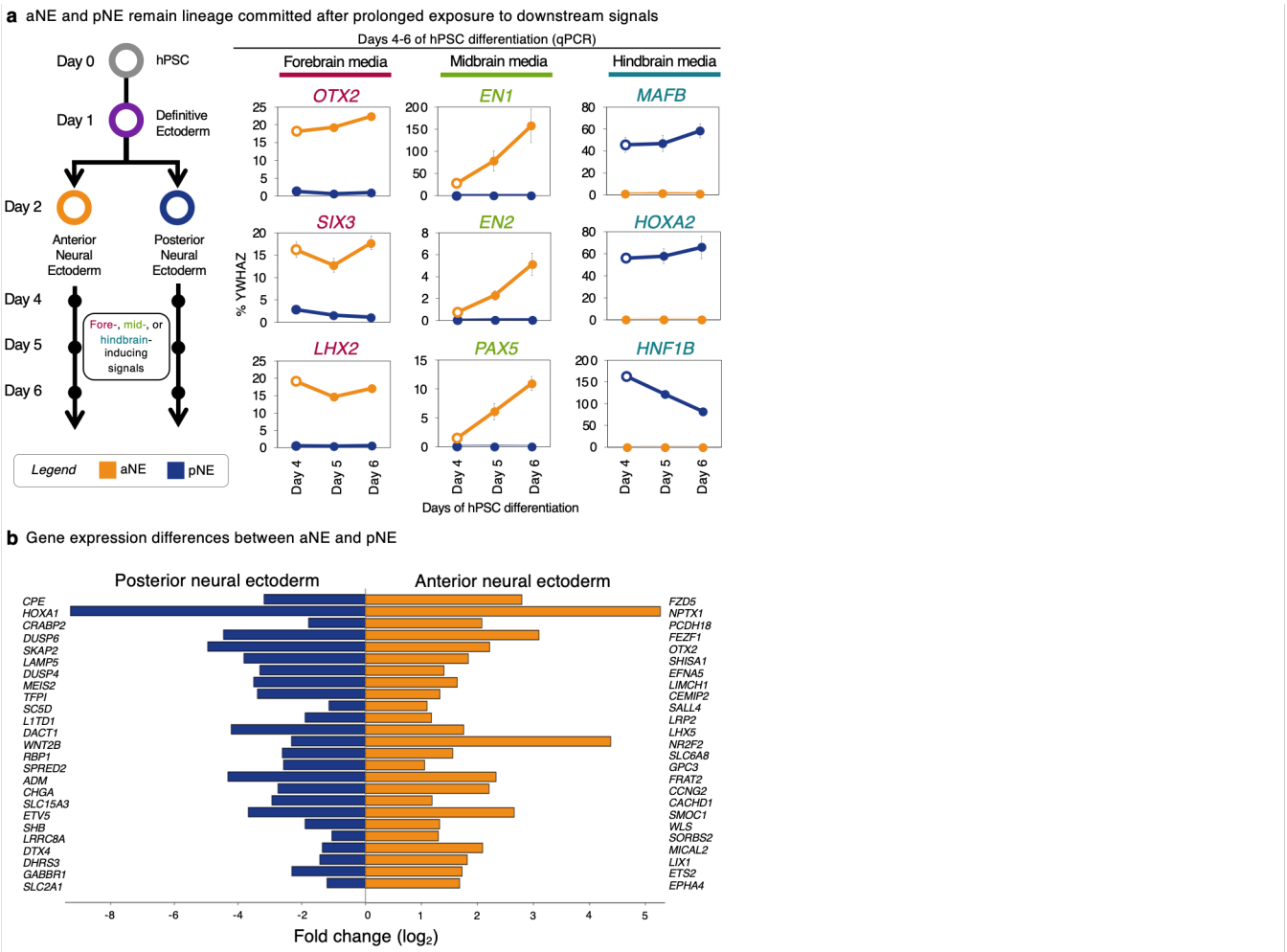
B) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by posterior neural ectoderm for 24 hours, and then further differentiated for 48 hours in FGF2 (20 ng/mL), in the presence or absence of WNT agonist (CHIR99021, 0.5-1.5 μM) or WNT inhibitor

(XAV939, 1 μM). Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

C) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by posterior neural ectoderm for 24 hours, and then further differentiated into hindbrain for 48 hours by FGF2 + CHIR99021. Alternatively, during the 48 hour interval of hindbrain induction, either FGF2 or CHIR99021 were individually withheld. This revealed that both FGF and WNT signaling are required for maximal *MAFB* expression. qPCR data normalized to levels found in hindbrain progenitors (e.g., cells treated with FGF2 + CHIR99021). Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

D) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by posterior neural ectoderm for 24 hours, and then further differentiated into hindbrain for 48 hours by FGF2 + CHIR99021. Alternatively, during the 48 hour interval of hindbrain induction, either FGF2 or CHIR99021 were individually withheld for 24 intervals ("day 3" = the first 24 hour interval, whereas "day 4" = the second 24 hour interval). This revealed that both FGF and WNT signaling are continuously required for 48 hours to achieve maximal *MAFB* expression. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

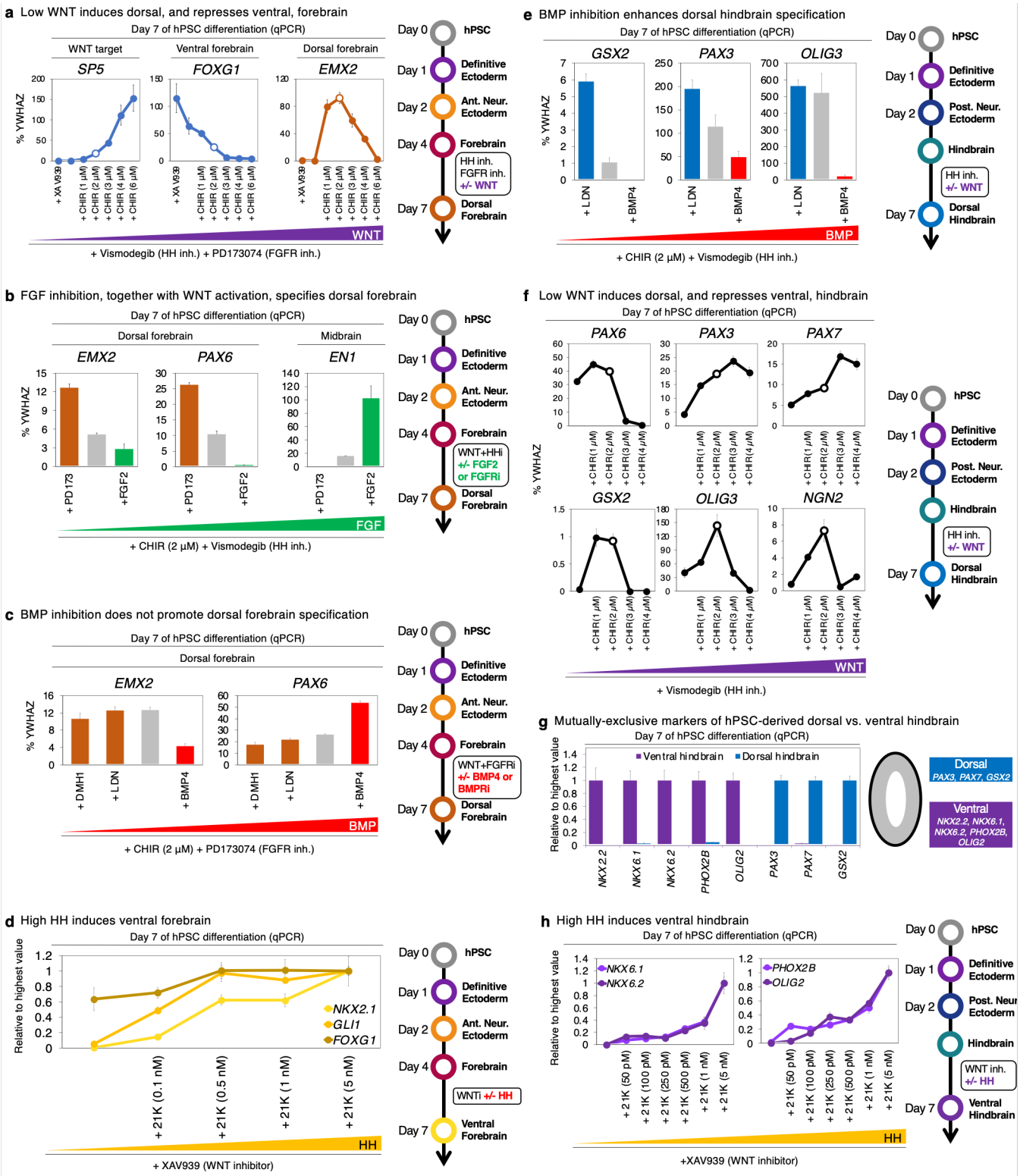
E) qPCR of H1 hPSCs differentiated into definitive ectoderm for 24 hours, followed by posterior neural ectoderm for 24 hours, and then further differentiated into hindbrain by FGF2 + CHIR99021 for 24, 48 or 72 hours (*left*). This revealed that FGF and WNT treatment for 48 hours was optimal for hindbrain specification (as denoted by a white circle), which was used as a baseline for the remainder of this study. qPCR data normalized to undifferentiated hPSCs. Cartoon of hPSC differentiation into hindbrain progenitors, with various signaling pathways activated or inhibited in a temporally dynamic way at each stage (*right*). Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 7: Accessible chromatin landscapes and lineage commitment of hPSC-derived anterior vs. posterior neural ectoderm

A) H1 hPSCs were differentiated into either anterior or posterior neural ectoderm for 2 days. They were then respectively challenged with either forebrain-, midbrain-, or hindbrain-inducing signals for 2, 3 or 4 days. qPCR was performed on days 4, 5 and 6 of hPSC differentiation. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

B) Top 25 differentially expressed genes between H7 hPSC-derived day-2 anterior neural ectoderm vs. posterior neural ectoderm, as determined by scRNAseq. The top 25 differentially expressed genes were determined by P value, and all these genes showed statistically significant expression differences ($P<0.05$). Data from one biological replicate per cell-type.



Supplementary Figure 8: Differentiation of hPSCs into dorsal forebrain and ventral forebrain progenitors within 7 days

A) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then anterior neural ectoderm within 24 hours, followed by forebrain progenitors within 48 hours. Forebrain progenitors were then treated with Vismodegib + PD173074 for 72 hours, in the presence or absence of WNT agonist (CHIR99021, 1-6 μM) or WNT inhibitor (XAV939, 1 μM). This revealed that 2 μM of CHIR99021 (indicated by white circle) was optimal for inducing dorsal forebrain marker *EMX2*. Gene expression is shown relative to reference gene *YWHAZ*; 100%

indicates that a gene is expressed at the same level as *YWHAZ*. HH inh.: HEDGEHOG inhibitor. FGFR inh.: FGF receptor inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

C) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then anterior neural ectoderm within 24 hours, followed by forebrain progenitors within 48 hours. Forebrain progenitors were then treated with CHIR99021 (2 μM) + PD173074 (100 nM) for 72 hours, in the presence or absence of FGF2 (20 ng/mL) or FGFR inhibitor (PD173074, 100 nM). This revealed that FGF inhibition enhanced dorsal forebrain markers *EMX2* and *PAX6*, while preventing erroneous WNT-induced expression of midbrain marker *EN1*. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. FGFRi: FGF receptor inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

D) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then anterior neural ectoderm within 24 hours, followed by forebrain progenitors within 48 hours. Forebrain progenitors were then treated with CHIR99021 (2 μM) + PD173074 (100 nM) for 72 hours, in the presence or absence of BMP4 (10 ng/mL) or BMPR inhibitors (DMH1 [250 nM] or LDN193189 [100 nM]). Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. BMPRi: BMP receptor inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

E) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then anterior neural ectoderm within 24 hours, followed by forebrain progenitors within 48 hours. Forebrain progenitors were then treated with XAV939 (1 μM) in the presence or absence of increasing doses of HEDGEHOG pathway agonist 21K (0.1-5 nM) for 72 hours to generate ventral forebrain. This showed that high HEDGEHOG pathway activation, together with WNT inhibition, led to maximum expression of ventral forebrain markers. Gene expression is shown relative to the sample with the highest expression in this experiment. HH: Hedgehog pathway agonist. WNTi: WNT inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

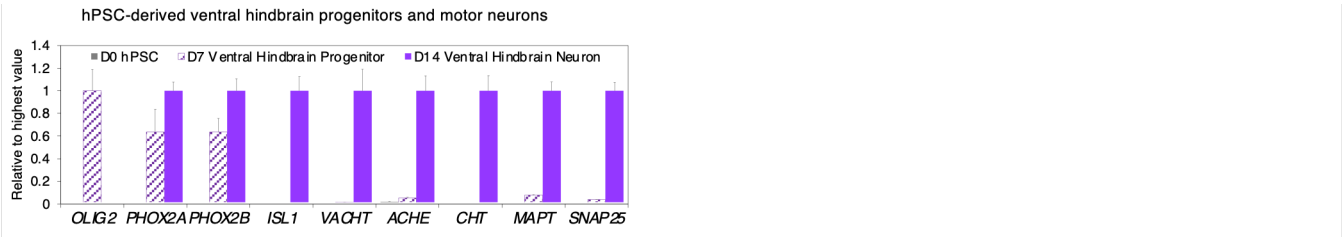
F) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then posterior neural ectoderm within 24 hours, followed by hindbrain progenitors within 48 hours. Hindbrain progenitors were then treated with HEDGEHOG inhibitor (Vismodegib, 150 nM) and WNT agonist (CHIR99021, 2 μM) for 72 hours to generate dorsal forebrain, in the presence or absence of BMP agonist (BMP4, 10 ng/mL) or BMPR inhibitor (LDN193189, 100 nM). This showed that BMP inhibition promoted expression of dorsal forebrain markers *GSX2* and *PAX3*. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. BMPRi: BMP receptor inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

G) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then posterior neural ectoderm within 24 hours, followed by hindbrain progenitors within 48 hours. Hindbrain progenitors were then treated with HEDGEHOG pathway inhibitor Vismodegib (150 nM) in the presence or absence of increasing doses of WNT pathway agonist CHIR99021 (1-4 μM) for 72 hours to generate dorsal forebrain. This showed that moderate WNT pathway activation, together with HH inhibition, led to maximum expression of dorsal hindbrain markers

GSX2 and *OLIG3*. Gene expression is shown relative to reference gene *YWHAZ*; 100% indicates that a gene is expressed at the same level as *YWHAZ*. BMPRi: BMP receptor inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

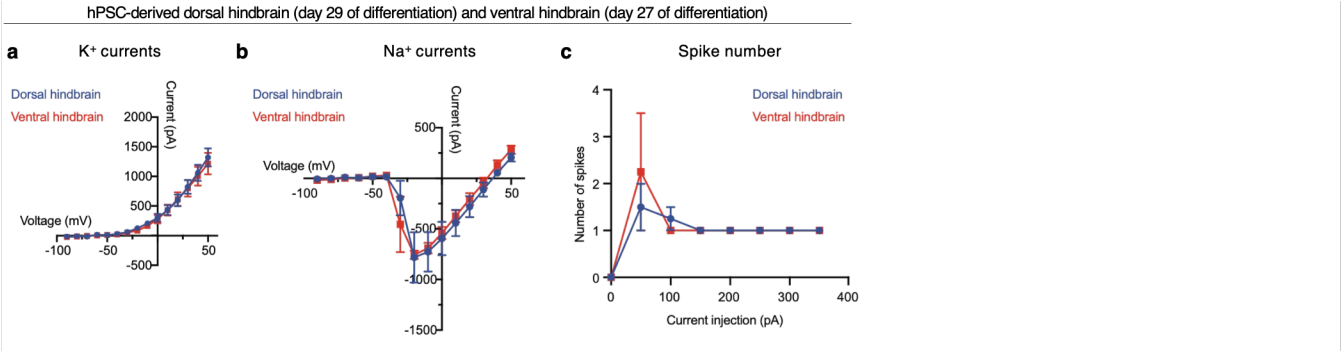
H) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then posterior neural ectoderm within 24 hours, followed by hindbrain progenitors within 48 hours. Hindbrain progenitors were then treated with either Vismodegib (150 nM) + CHIR99021 (2 μM) + DMH1 (250 nM) for 72 hours to generate dorsal forebrain, or alternatively, 21K (5 nM) + XAV939 (1 μM) for 72 hours to generate ventral forebrain. Gene expression is shown relative to the sample with the highest expression in this experiment.

I) H1 hPSCs were differentiated into definitive ectoderm within 24 hours, and then posterior neural ectoderm within 24 hours, followed by hindbrain progenitors within 48 hours. Hindbrain progenitors were then treated with WNT pathway inhibitor XAV939 (1 μM) in the presence or absence of increasing doses of HEDGEHOG pathway agonist 21K (50 pM-5 nM) for 72 hours to generate ventral hindbrain. This showed that high doses of HEDGEHOG agonist, together with WNT inhibitor, were required to maximally induce ventral hindbrain markers. Gene expression is shown relative to the sample with the highest expression in this experiment. WNT inh.: WNT inhibitor. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.



Supplementary Figure 9: Gene expression of hPSC-derived ventral hindbrain progenitors and ventral hindbrain neurons

qPCR of H1 hPSC-derived day-7 ventral hindbrain progenitors differentiated towards neurons for 7 additional days. Gene expression is shown relative to the sample with the highest expression in this experiment. Data depicts the mean of two biological replicates from one experiment, with two technical replicates per biological replicate. Error bars depict standard error of the mean.

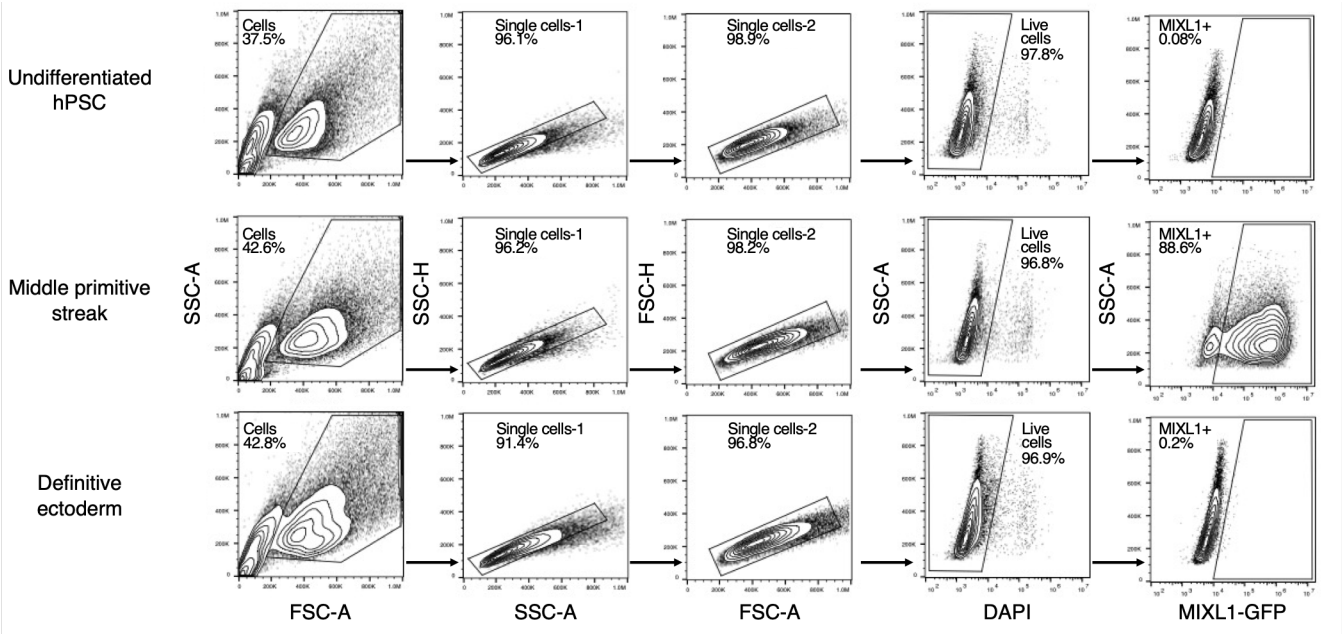


Supplementary Figure 10: **Electrophysiology of hPSC-derived dorsal and ventral hindbrain neurons**

A) Summary of the current-voltage relationship of voltage-dependent K^+ currents from dorsal and ventral hindbrain neurons. Currents were measured from -90 mV to +50 mV holding potentials. Data depicts the mean of four independent cells per cell-type, from one experiment. Error bars depict standard error of the mean.

B) Summary of the current-voltage relationship of voltage-dependent Na^+ currents from dorsal and ventral hindbrain neurons. Currents were measured from -90 mV to +50 mV holding potentials. Data depicts the mean of four single cells per cell-type, from one experiment. Error bars depict standard error of the mean.

C) Summary of the number of spikes elicited by prolonged current injection. Data depicts the mean of four single cells per cell-type, from one experiment. Error bars depict standard error of the mean.



Representative flow cytometry gating schema

Depiction of flow cytometry thresholds used to identify viable cells and MIXL1-GFP+ cells, as shown in Extended Data Figure 1.