Specification of Functional Cranial Placode Derivatives from Human Pluripotent Stem Cells.

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SUPPLEMENTAL FIGURES: Figures S1-S7:

FIGURE S1

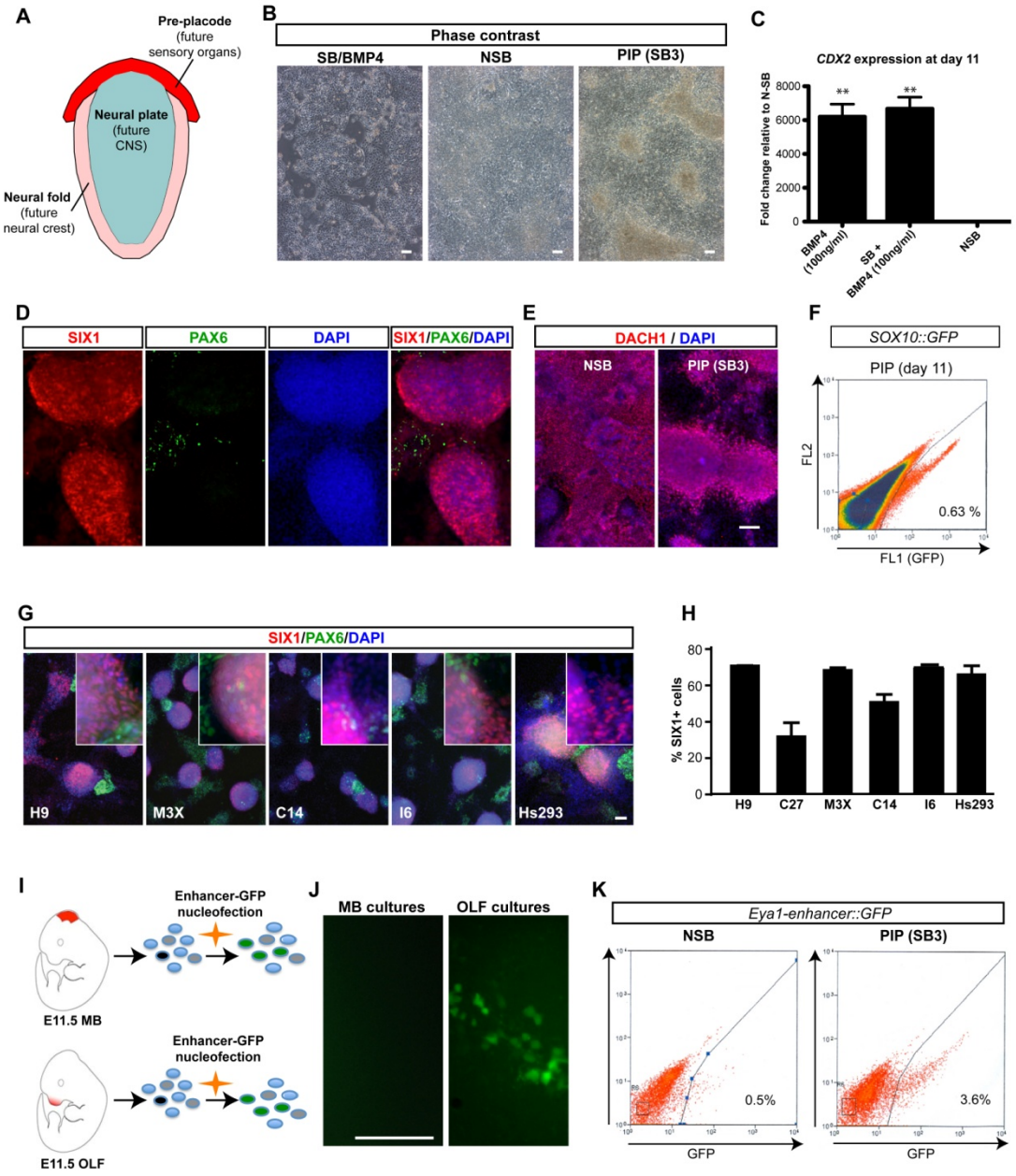

Figure S1 - Placode induction, characterization and validation of protocol across various human ESC and iPSC lines (related to **Figure 1**): **A**) Schematic illustration of the dorsal view of a human neural plate stage embryo (based on (O'Rahilly, 1987)). Fate studies in model organisms have identified a unique horseshoe-shaped territory in the head ectoderm that contains all placode precursors, called the pre-placodal region (PPR; marked here in red). The neuroectoderm forms the neural plate (marked light blue). The most lateral aspects of the neural plate form the neural folds (marked in pink) that give rise to the future neural crest cells. **B**) BMP4 treatment induces trophectoderm–like lineage by morphology **C**) *CDX2* expression at day 11 following BMP4 treatment or SB + BMP4 treatment. Data represent fold changes of mRNA

expression by qRT-PCR as compared to N-SB condition. **D)** SIX1 (red) and PAX6 (green) expression at day 11 of differentiation. **E**) Immunocytochemistry for DACH1 (red) expression in N-SB and PIP conditions. **F**) Flow analysis for SOX10 expression at day 11 of PIP using a *SOX10::GFP* hESC reporter line. **G, H**) The placode induction protocol (PIP) was robust across multiple hESC (H9, I6 and Hs293) and hiPSC lines (C14, M3X, and C27). Representative images of SIX1/PAX6 immunocytochemistry are shown in (**G**) for each line with higher magnification insets and with quantification of the percentage of $SIX1^+$ cells under PIP condition in (**H**). **I**) Scheme of experimental design to test specificity of *EYA1::GFP enhancer* in the mammalian system. The primary cultures olfactory (OLF) area and midbrain (MB) area from E11.5 mouse embryo were isolated and nucleofected with placode specific *EYA1::GFP enhancer* **J**) Only the primary olfactory culture (OLF) showed activation of *EYA1* enhancer, while midbrain primary cultures showed no GFP expression. **K**) The GFP expression is quantified by FACS analysis under PIP and N-SB condition. Error bar represents SEM. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ compared with control N-SB condition (n = 3 independent experiments). Scale bars correspond to 50 μm.

FIGURE S2

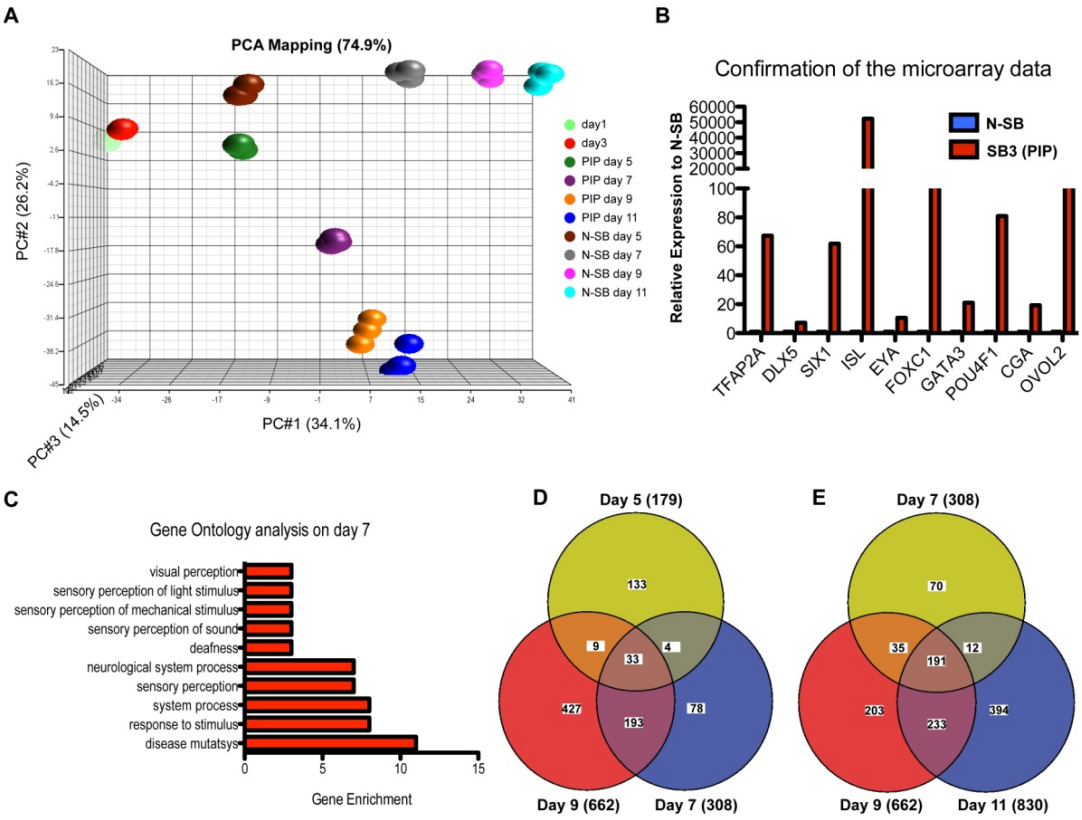

Figure S2 - Temporal gene expression analysis of placodal (PIP condition) versus CNS (NSB condition) fates (related to **Figure 2**)**: A**) Principal component analysis of data confirms close temporal correlation of samples during hESC differentiation with increasing separation of PIP versus N-SB treated cells at later differentiation stages. **B)** Confirmation of the microarray data by qRT-PCR. **C)** Gene ontology analysis of genes that are upregulated at day 7 of PIP protocol. **D)** Venn Diagram of comparison of the genes that are upregulated at day 5, 7, and 9 in PIP. **E)** Venn Diagram of comparison of the genes that are upregulated at day 7, 9, and 11 in PIP.

FIGURE S3

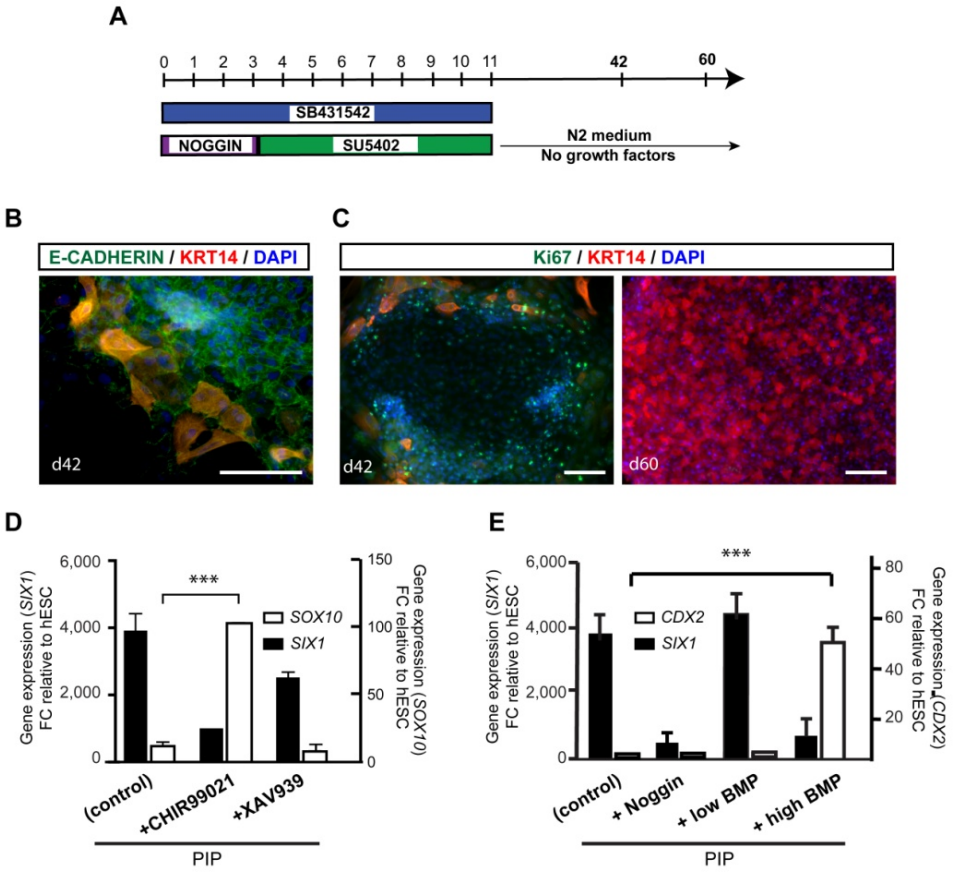

Figure S3 - Induction of epidermal versus placodal fates upon modulating FGF signaling and the role of BMP and WNT signaling during human placode induction (related to **Figure 3**)**: A**) Schematic summary of differentiation condition used for early keratinocyte induction. **B)** Patches of keratinocyte co-express E-CADHERIN (green) and KRT14 (red) at day 42. **C)** The center of the patches expresses KI67 at day 42, while KI67 positive cells diminish by day 60. KRT14 positive cells are negative for KI67. Scale bars correspond to 50 µm. **D)** Analysis of *SIX1* and *SOX10* expression at day 11 following treatment with pharmacological inhibitor (XAV939) and activator (CHIR99021) of WNT signaling starting at day 3 of PIP. **E)** Analysis of *CDX2* and *SIX1* expression at day 11 following treatment with inhibitor (Noggin) and activator (BMP4) of BMP signaling starting at day 3 of PIP. Error bar represents SEM. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ (n = 3 independent experiments).

FIGURE S4

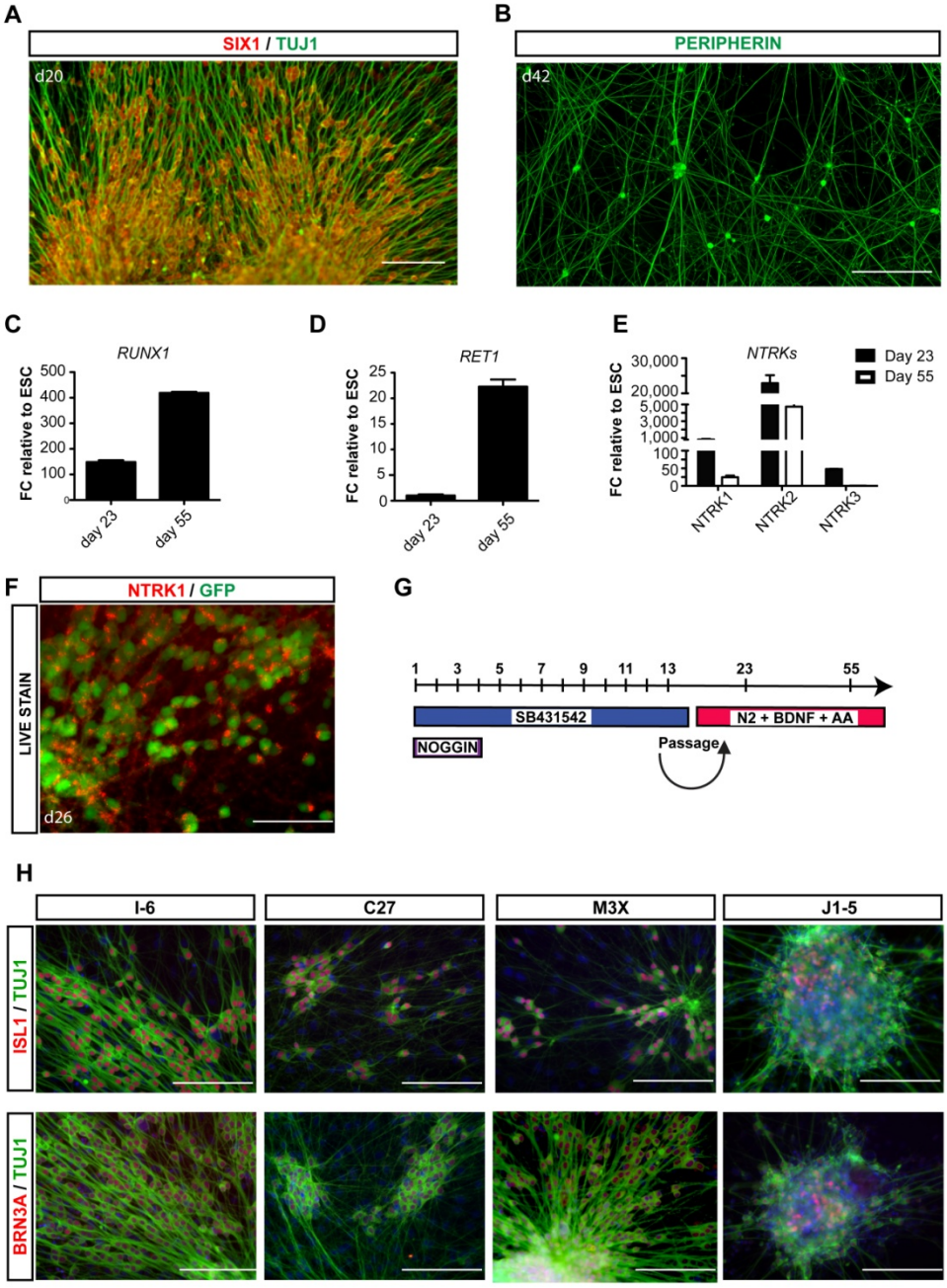

Figure S4 - Characterization of placode-derived trigeminal sensory neuron lineage across multiple human ESC and iPSC lineages (related to **Figure 4**): **A**) Immunocytochemical analysis at day 20 of differentiation demonstrates that SIX1^{+} placodal clusters efficiently yield large numbers of TUJ1 positive neurons that initially retain SIX1 expression. **B**) At day 42 differentiated neurons express Peripherin (green). **C-E**) Short (day 23) and long term (day 55) cultures of trigeminal neuron mRNA expression of **C)** *RUNX1* **D)** *RET1* **E)** *TRK* receptors, Data represent fold changes (FC) of mRNA expression, normalized to hESC. **F)** Trigeminal neurons stain for TRKA (live stains) **G**) Schematic representation of differentiation protocol for trigeminal

sensory neurons. The clusters are manually passaged at day 13-17 **H**) Trigeminal-type sensory neurons can be obtained under the same PIP conditions at high efficiencies from various hESC (I6) and hiPSC (C27, M3X, J1-5) lines. Error bar represents SEM (n = 3 independent experiments).

FIGURE S5

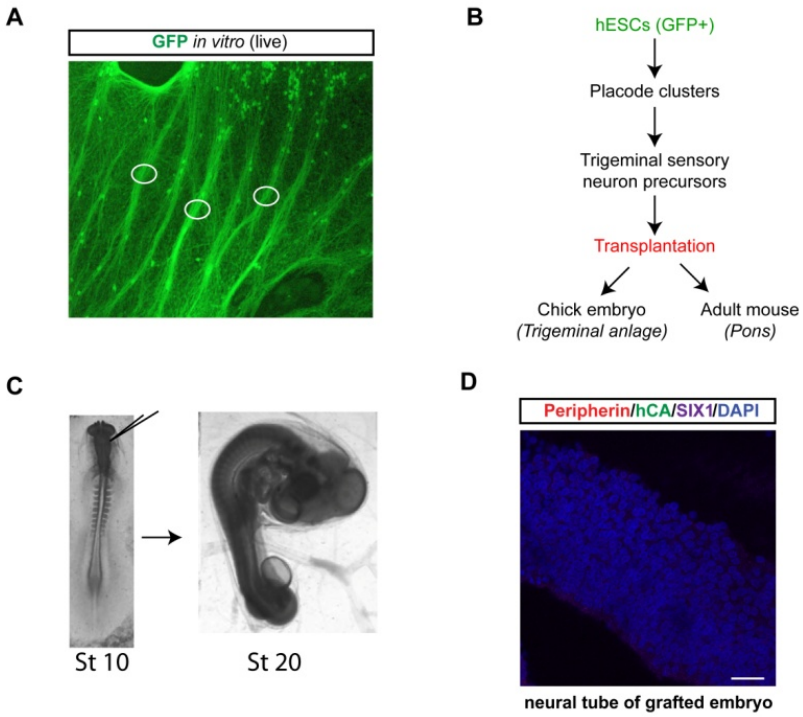

Figure S5 - Paradigm for assessing the *in vivo* properties of hESC-derived trigeminal neuron precursors: (related to **Figure 4**)**: A**) hESC-derived trigeminal neurons form bundles *in vitro* during differentiation (day 30) as shown by white circles. **B**) Schematic of chick and mouse transplantation experiments. **C**) Transplantation time and collection time of chick embryo experiments **D)** The negative staining control for transplantation in the chick sections at neural plate region. **E-H)** Transplantation of trigeminal neuron precursors into the adult mouse pons to test the ability of grafted hESC-derived trigeminal neuron axonal arbors to reach targets in trigeminal nuclei. **E**) Location and morphology of GFP (green) labeled human trigeminal neurons grafted within the pontine nuclei (Pn) after 1 month transplantation **F**) human GFP neuronal processes traveling towards the trigeminal nuclei Aqueduct (Aq). **G)** Immunohistochemistry for co-expression of BRN3A (red), GFP (green) and DAPI (blue) in grafted cells. **H**) Human neuronal fiber bundles co-express hNCAM and GFP.

FIGURE S6

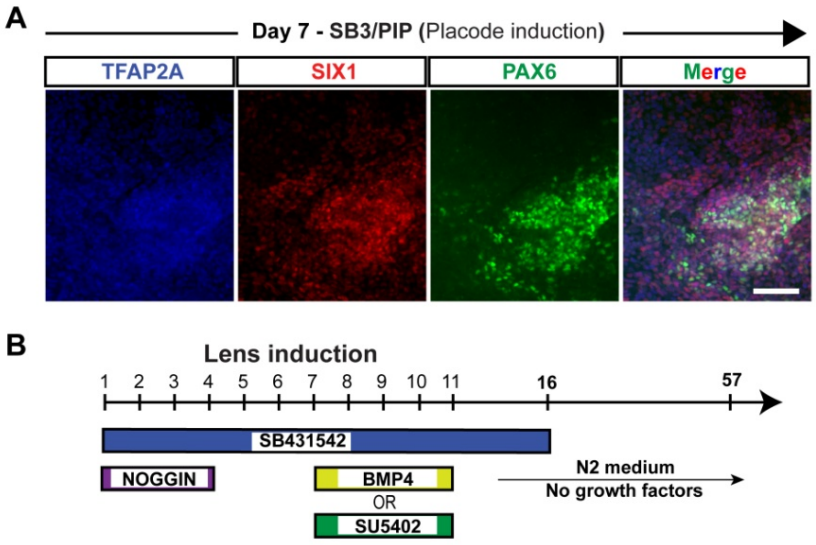

Figure S6 - Pre-placodal cells can be further differentiated into lens placode upon treatment with BMPs (related to **Figure 5**)**: A)** TFAP2A, PAX6 and SIX1 co-expression mark a transient putative pre-placode stage under PIP conditions. Immunocytochemical analysis for SIX1 (red), TFAP2A (blue) and PAX6 (green) at day 7 of differentiation under PIP condition. Scale bars correspond to 50 µm. **B**) Schematic representation of the differentiation condition used for early lens induction.

FIGURE S7

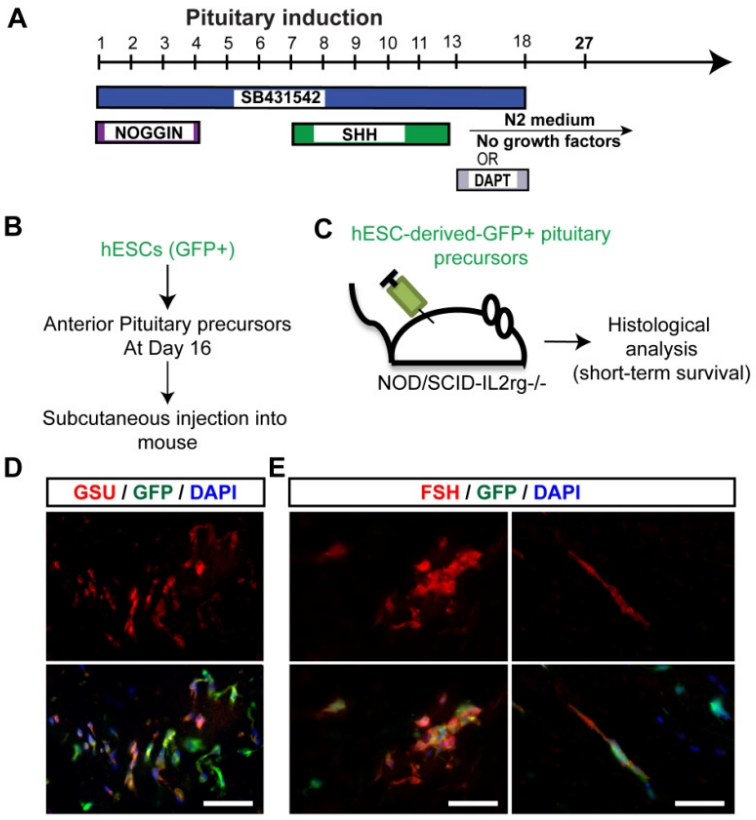

Figure S7 - Protocol to generate anterior pituitary placode and hormone producing cells for *in vivo* transplantation studies (related to **Figure 6**)**: A**) Schematic representation of the differentiation condition used for early pituitary induction. **B, C)** Short-term *in vivo* analysis upon subcutaneous injections of hESC-derived GFP^{+} early pituitary cells in NOD-SCID mice. **D**) Co-expression of GSU (CGA) and GFP labeled cells in mouse grafts. **E**) Co-expression of FSU and GFP labeled cells in mouse grafts. Scale bars correspond to 50 μm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Cells and culture conditions: hESCs (WA-09; XX, passages 35-45), hiPSC lines (iPS-14, iPS-27; passages 20–30) (Chambers et al., 2009), and I6 (Amit and Itskovitz-Eldor, 2002) were cultured on mouse embryonic fibroblasts plated at 12- 15,000 cells/cm^2 (MEFs, Global Stem) and maintained in medium consisting of DMEM/F12, 20% knockout serum replacement (GIBCO), 0.1 mM b-mercaptoethanol, 6 ng/mL FGF-2 changed daily or in mTeSR™1 (StemCell Technologies, Inc., Vancouver, Canada) on hESC-qualified Matrigel™ (BD Biosciences, San Jose, CA) coated plates. Cells were passaged using 6 U/mL of dispase in hESCs media, washed and re-plated at a dilution of 1:10 to 1:15.

Neural Induction and placode induction: Feeder-free neural induction was carried out as previously described (Chambers et al., 2009). Briefly, hESCs cultures were disaggregated using accutase for 20 minutes, washed using hESCs media and pre-plated on gelatin for 1 hour at 37°C in the presence of ROCK inhibitor to remove MEFs. The non-adherent hESCs were washed and plated on matrigel at a density of 60,000 cells/cm^2 on matrigel (BD) coated dishes in MEF conditioned hESCs media (CM) spiked with 10 ng/mL of FGF-2 and ROCK-inhibitor. The ROCK inhibitor was withdrawn after 24 hours, and hESCs were allowed to expand in CM for 2 days or until they were 95% confluent. The initial differentiation media conditions included knock out serum replacement (KSR) media with 10 μM TGF-β inhibitor (SB431542, Tocris) and 250 ng/mL of Noggin (R&D). Upon day 5 of differentiation, increasing amounts of N2 media (25%, 50%, and 75%) were added to the KSR media every two days while maintaining 500 ng/mL of Noggin and TGF-β inhibitor. Similar results could be obtained when using KSR-free conditions (E6 medium (Chen et al., 2011)) following adjustments in BMP signaling; data not shown). For placode induction, Noggin was removed at day3 of differentiation. In some experiments, BMP-4 (R&D 50 ng/ml), Noggin (R&D 250 ng/ml), DKK-1 (R&D 100 ng/ml), FGF8 (R&D 50 ng/ml), SU5402 (Tocris 10 μM), Wnt-3a (R&D 50 ng/ml), DAPT (Tocris, 10 μM), CHIR99021 (Stemgent, 3 μM), Cyclopamine (Tocris, 10 μM), Sonic Hedgehog (C25II-R&D 100 ng/ml), and Purmorphamine (Stemgent, 1μM), were added.

Terminal differentiation of trigeminal sensory neurons: The placode clusters were isolated manually at day 13-17. Clusters were replated onto culture dishes pre-coated with 15 μg/mL polyornithine, 1 μg/mL laminin (Po/Lam) and maintained in N2 medium supplemented with ascorbic acid (AA, 0.2 mM), and BDNF (20 ng/mL). For electrophysiology experiments, NGF and ROCK inhibitor was used in the ACSF, which increases the survival of cells within the chamber.

Differentiation of hormone producing cells: During placode induction protocol, Sonic Hedgehog (C25II-R&D 100 ng/ml) and Purmorphamine (Stemgent, 1μM) were added between days 7-11 of PIP to promote differentiation towards pituitary placode anlage. The cells were maintained without passaging in N2 medium and treated with additional factors such as DAPT (Tocris, 10 μM), between day 13 and day 17 of differentiation to induce PIT1$^+$ and GATA2$^+$ precursor cells (see **Figure S7A)**.

Quantitative Real-time PCR: Total RNA was extracted using an RNeasy kit (Qiagen) or Trizol. For each sample, 1 μg of total RNA was DNAase treated and reverse transcribed using the Quantitect RT kit (Qiagen). Amplified material was detected using Taqman probes and PCR mix (ABI) on a Mastercycler RealPlex2 (Eppendorf). All results were normalized to a HPRT housekeeping gene control and are based on 4–6 technical replicates from each of at least 3 independent experiments.

Microarray Analysis: Total RNA was isolated at day 1, 3, 5, 7, 9 and 11 of differentiation from both control (NSB) and PIP using Trizol (Invitrogen). Three biological replicates per time point were used. All samples were processed by the MSKCC Genomics Core Facility and hybridized on Illumina Human-6 oligonucleotide arrays. Quantile Normalization and model-based expression measurements were performed by using the Partek Genomic Suite (Partek GS) (Downey, 2006). A pair-wise comparison between NSB and PIP was performed. Genes found to have an adjusted p-value < .001 and a fold change greater than 2 were considered significant. Expression differences are reported as the log2 of the fold change. Gene Ontology enrichment was determined by entering gene lists into the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://www.david.niaid.nih.gov) (Dennis et al., 2003; Huang et al., 2009).

Microscopy, antibodies, and flow cytometry: Cells were fixed using 4% paraformaldehyde for 15 minutes, washed with PBS, permeabilized using 0.3% Triton X in PBS, and blocked using 1% BSA. Primary antibodies used for microscopy included PAX6 (Covance, DSHB), TUJ1 (Covance), BRN3A (Chemicon), AP2a (DSHB), HNK1 (Sigma), PAX3 (DSHB), SIX1 (ABR, Atlas), ISL1 (DSHB), PERIPHERIN (Santa Cruz), GSU (gift A. McNeilly), CRYAB (Chemicon), DACH1 (Proteintech), EYA1 (gift Kawakami), E-CADHERIN (Abcam), FSH (gift A. McNeilly), GATA2 (Abcam), chick GFP (Abcam), hNCAM (Santa Cruz), GLUTAMATE (Sigma), KRT14 (Labvision), SIX6 (Atlas), LHX3 (Abcam), OVOL2 (Aviva), TFAP2A (DSHB), FOXG1 (gift E. Lai), hCA (Stem Cells), and Ki67 (Sigma). Appropriate Alexa 488, Alexa 568, Alexa 647 secondary antibodies (Molecular Probes) and/or DAPI counterstaining was used for visualization. For Flow Cytometry, cells were mechanically dissociated after exposure to accutase for 20 min at 25 °C. To eliminate dead cell populations in FACS analysis, we used DAPI or 7-AAD according to manufacturer's recommendation. Cells were analyzed using FACScan (Becton Dickinson) and FlowJo software (Tree Star, Inc.).

Electrophysiology: The clusters were seeded on glass slides. Slides are recovered in artificial CSF (ACSF) containing the following: 119 mM NaCl, 2.5 mM KCl, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1 mM NaH_2PO_4, and 20 mM glucose (pH 7.4, osmolarity 300 mOsm), bubbled with 5% CO_2/95% O_2 at room temperature for a minimum of 1 h before recording. Slices were constantly perfused with ACSF during recordings. Whole-cell recordings were made from the neurons migrating from the clusters, which tended to be more mature than cells in the center of the clusters. Patch electrodes (5–8 M) were filled with intracellular solution containing

the following: 130 mM K-gluconate, 16 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 4 mM Na_2-ATP, and 0.4 mM Na_3-GTP (pH 7.25, osmolarity 290 mOsm). The membrane potential of each cell was identified shortly after rupturing the patch and periodically during the course of the experiment to ensure there was no significant deterioration of the health of the cell. Spontaneous miniature synaptic currents were recorded in voltage-clamp mode held at –60 mV. Depolarizing and hyperpolarizing current steps (0.2 Hz; duration 500 ms) were applied to the cells to help characterize their electrophysiological profile. All parameters were measured for a minimum of three trials for each cell, and the average value was calculated.

***In vivo* transplantation**:
All animal experiments were done in accordance with protocols approved by our institutional Animal Care and Use Committee and following NIH guidelines for animal welfare.

Murine subcutaneous injections of hormone producing cells: For subcutaneous transplantations, $1x10^6$ hESC-derived pituitary cells (at day 16 and day 32 of differentiation) in 0.5 ml of matrigel or matrigel alone (as controls) were injected into adult 8-weeks-old male *NOD-SCID IL2Rgc* null strain mice and adult 8-weeks-old male nude rats (Taconic). The whole blood collections or retrorbital blood collection per RARC guidelines were performed a week, 4 weeks and 6 weeks after the transplantations for hormone measurements from the plasma. Animals were sacrificed at 2 days, 1 week, and 6 weeks after transplantation and processed for histology. Matrigel plugs from adult mice were fixed in 4% paraformaldehyde and cryosectioned for immunohistochemical analysis.

Transplantation into chick trigeminal ganglion anlage: For *in ovo* transplantation, fertile eggs (Charles River) were incubated at 37°C in a humidified incubator. The GFP hESC-placode derived neurons were transplanted into the prospective trigeminal ganglion of HH Stage 9-10 chick embryos. Eggs are incubated until HH Stage 20. The chick embryo was sectioned transverse at the level of the midbrain neural tube to visualize the trigeminal ganglia.

Transplantation into mouse pons region:
The GFP expressing hESC derived trigeminal neuron/progenitor cells at day 22 of differentiation were transplanted into the right pons of nine *NOD-SCID IL2Rgc* null strain mouse by using stereotactic surgery; the coordinates are Lambda: -0.77, Bregma: -4.16, D/V: 4.65 and M/L: +0.5(Right). For each animal 200,000 cells were transplanted in a 100K/µl density. Tissues from chick embryos and adult mice were fixed in 4% paraformaldehyde and cryosectioned for immunohistochemical analysis.

In vitro and *in vivo* analysis of ACTH and GH release:
Hormone producing hESC-derived cells were analyzed at day 36 of differentiation. The cells were rinsed with HBSS and subsequently exposed to fresh HBSS (500 µl at 37°C for 10 min). The supernatant was subsequently collected and subjected to ELISA using the ACTH LumELISA kit (Calbiotech). ACTH was also readily detected from conditioned medium subjected to ELISA. For *in vivo* studies, blood samples from mice and rats engrafted with hESC-

derived anterior pituitary cells and matrigel-only controls were collected into K2 EDTA-treated BD Microtainer MAP (BD) at 8 a.m. under conditions to minimize stress. Plasma was isolated by centrifugation for 20 min at 2,000 g using a refrigerated centrifuge. The supernatant was collected for ELISA to measure hormone levels. GH levels were measured similarly both *in vitro* and *in vivo* after transplantation using the Human Growth Hormone ELISA kit (Calbiotech).

SUPPLEMENTARY REFERENCES:

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