SUPPLEMENTARY INFORMATION

Combined small molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors

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Supplementary Figures:

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Supplementary Tables:

Figure S1 - SM Chambers, et al.

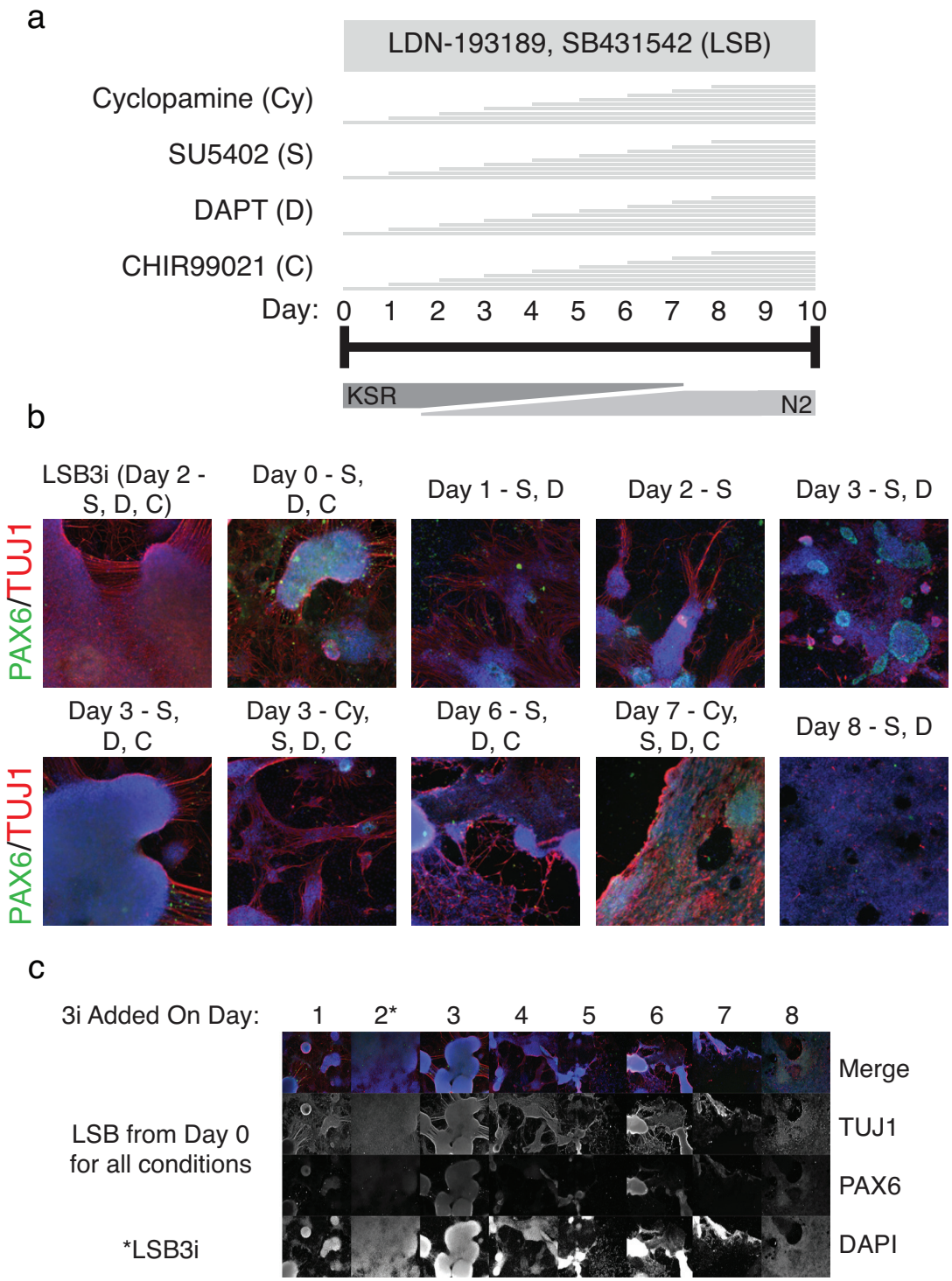

Figure S1 – LSB3i screen description. (a) Cyclopamine, SU5402, DAPT, and CHIR99021 were added on different days in approximately 400 different combinations. (b) Cells were fixed on day 10 and examined for the loss of PAX6 and acquisition of β3- tubulin (TUJ1) by immunofluorescence (examples shown). (c) Day 2 was determined to be the optimal day for addition of SU5402, DAPT, and CHIR99021 (3i).

Figure S2 - SM Chambers, et al.

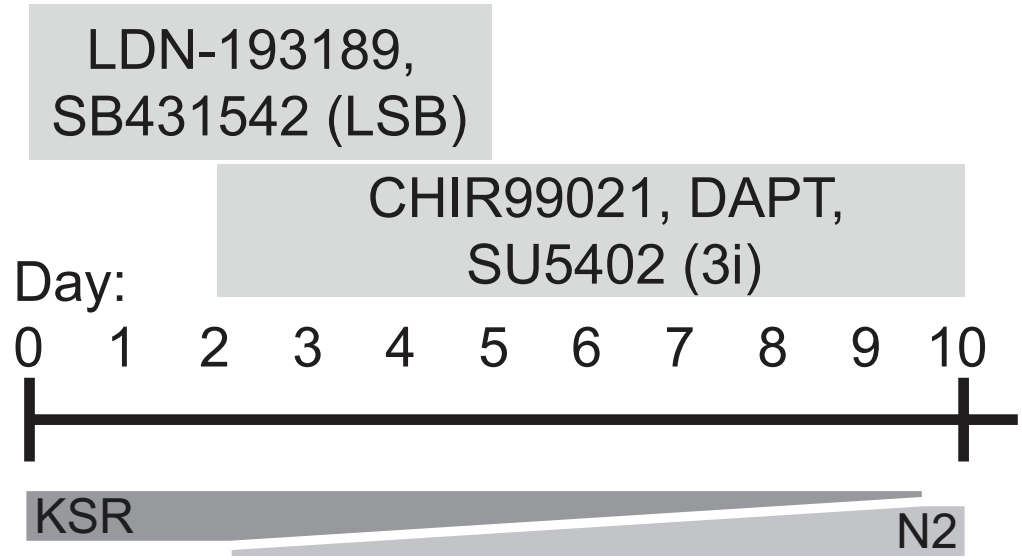

Figure S2 – LSB3i differentiation scheme. In the context of dual-SMAD inhibition using LDN-193189 and SB431542 (LSB), optimal neuronal differentiation was observed when CHIR99021, SU5402 and DAPT (3i) were added at day two of differentiation. Starting on day 4, N2 media was added in increasing 25% increments replacing KSR.

Figure S3 - SM Chambers, et al.

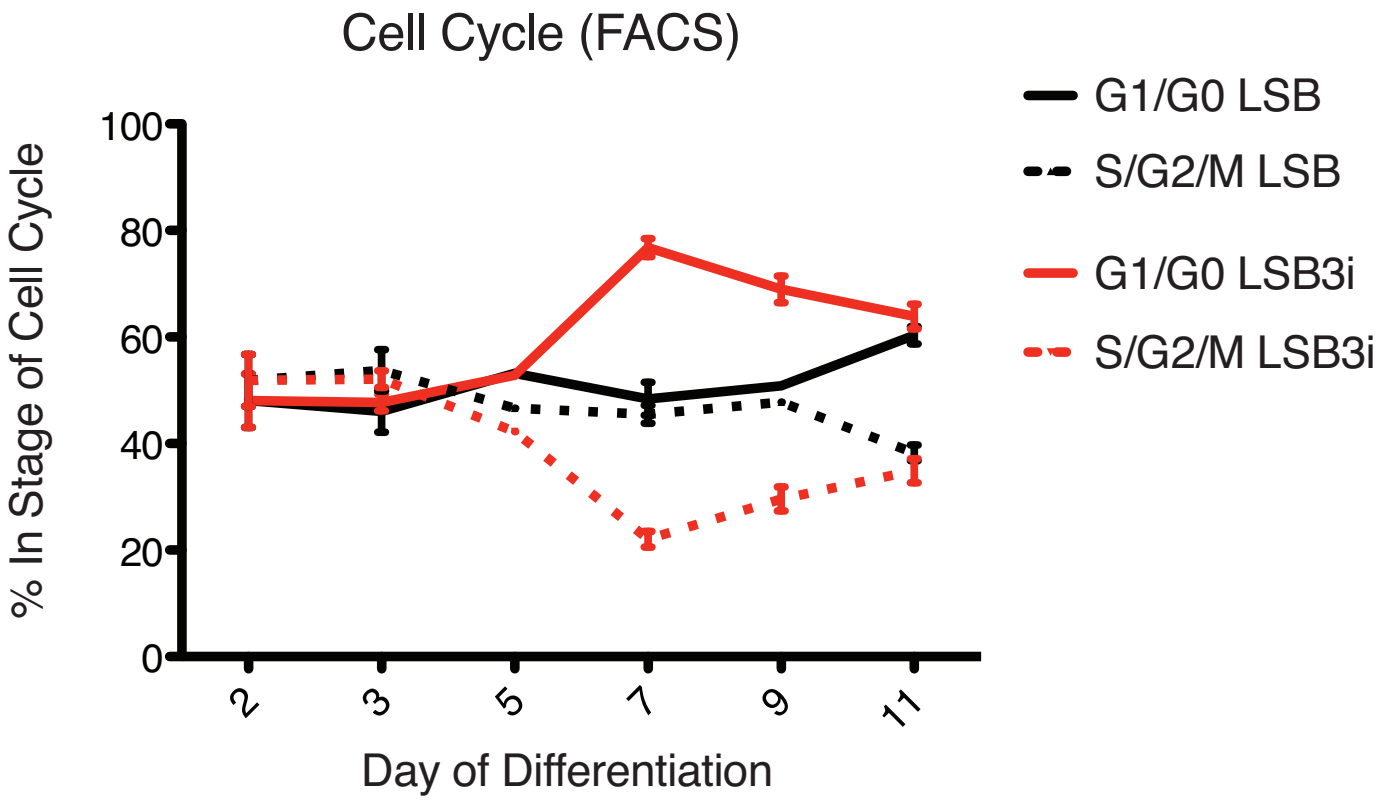

Figure S3 – Cell cycle analysis of LSB and LSB3i. By day 7 of differentiation, LSB3i treatment slows cell proliferation as measured by FACS.

Figure S4 - SM Chambers, et al.

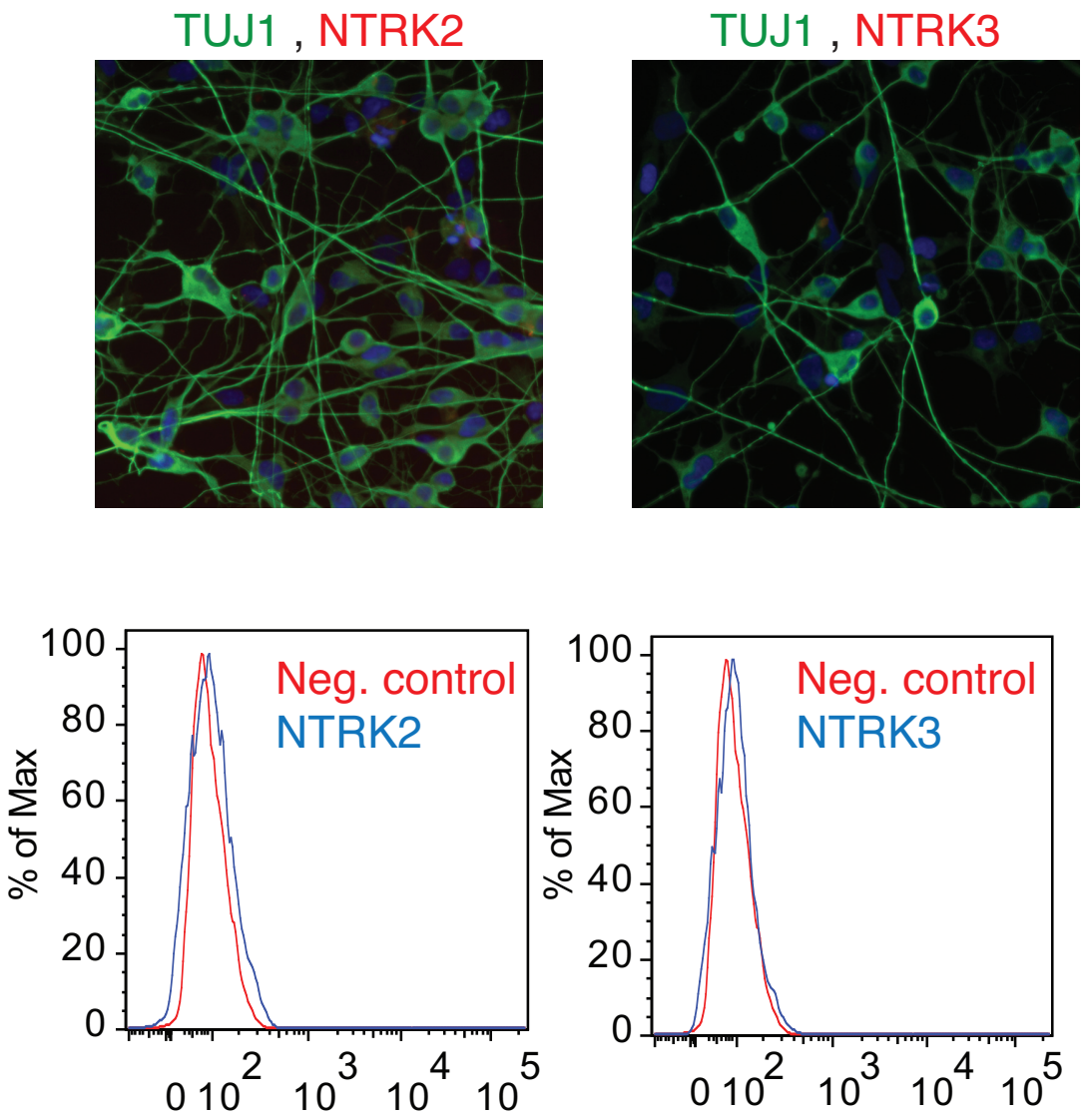

Figure S4 – Protein expression of NTRK2 and NTRK3. Little or no expression of NTRK2 and NTRK3 could be detected in LSB3i cells by immunofluorescence or FACS.

Figure S5 - SM Chambers, et al.

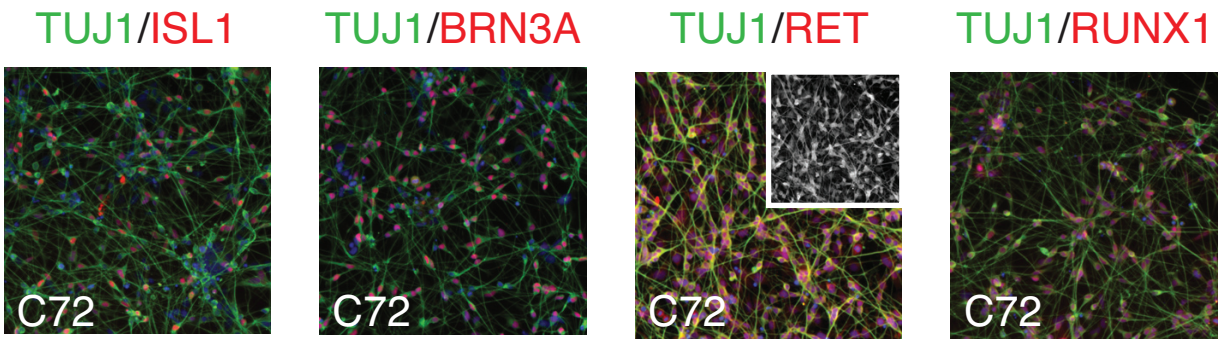

Figure S5 – LSB3i treated iPSC clone C72 rapidly acquires a nociceptor phenotype. TUJ1 positive neurons from LSB3i treated iPSC clone C72 expressISL1, BRN3A, RET, and RUNX1.

Figure S6 - SM Chambers, et al.

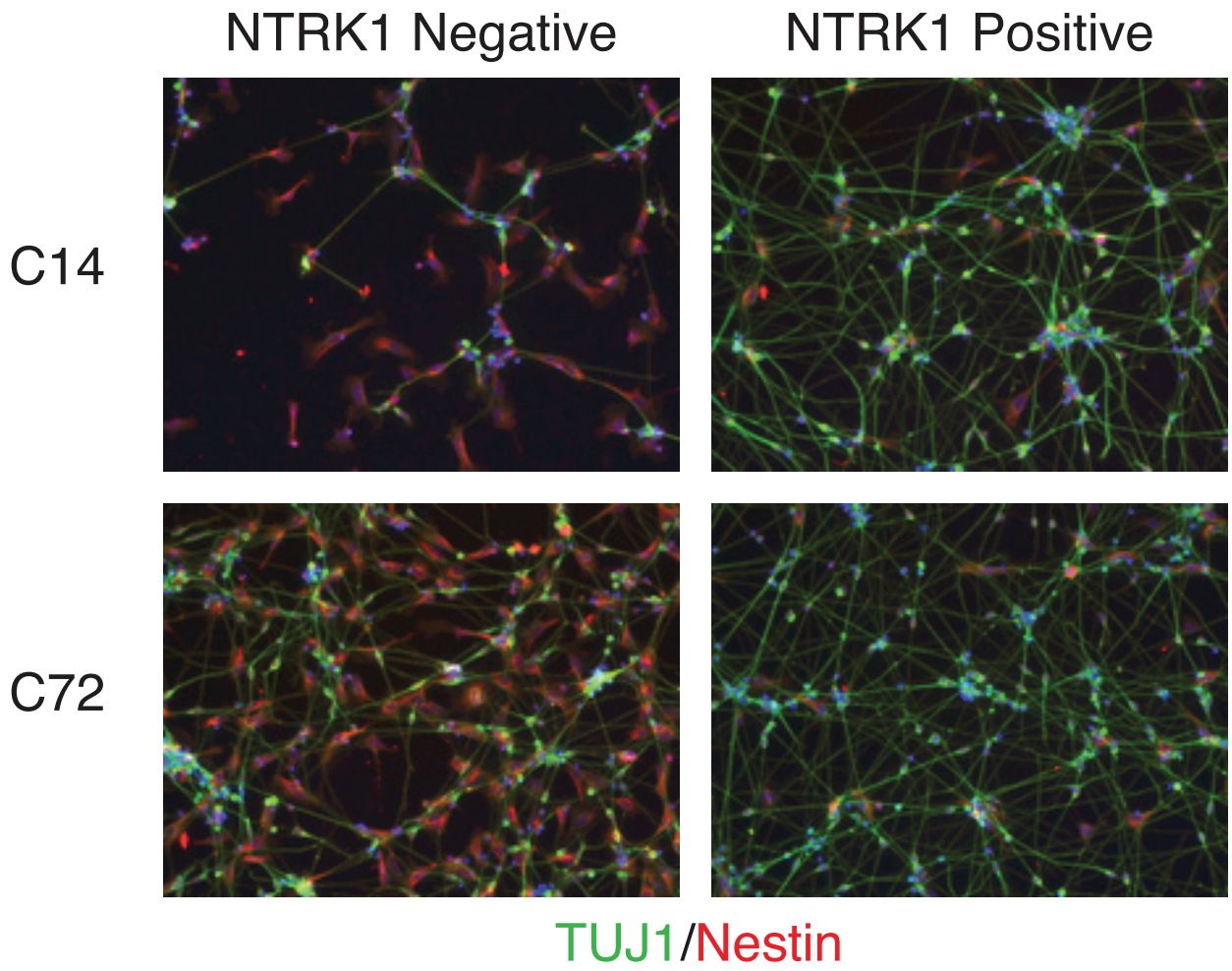

Figure S6 – NTRK1 FACS sorting enriches for hiPSC-derived LSB3i neurons. NTRK1 sorting on day 10 of differentiation can enrich for TUJ1 (green) positive neurons and remove nestin (red) positive progenitor cells.

Figure S7 - SM Chambers, et al.

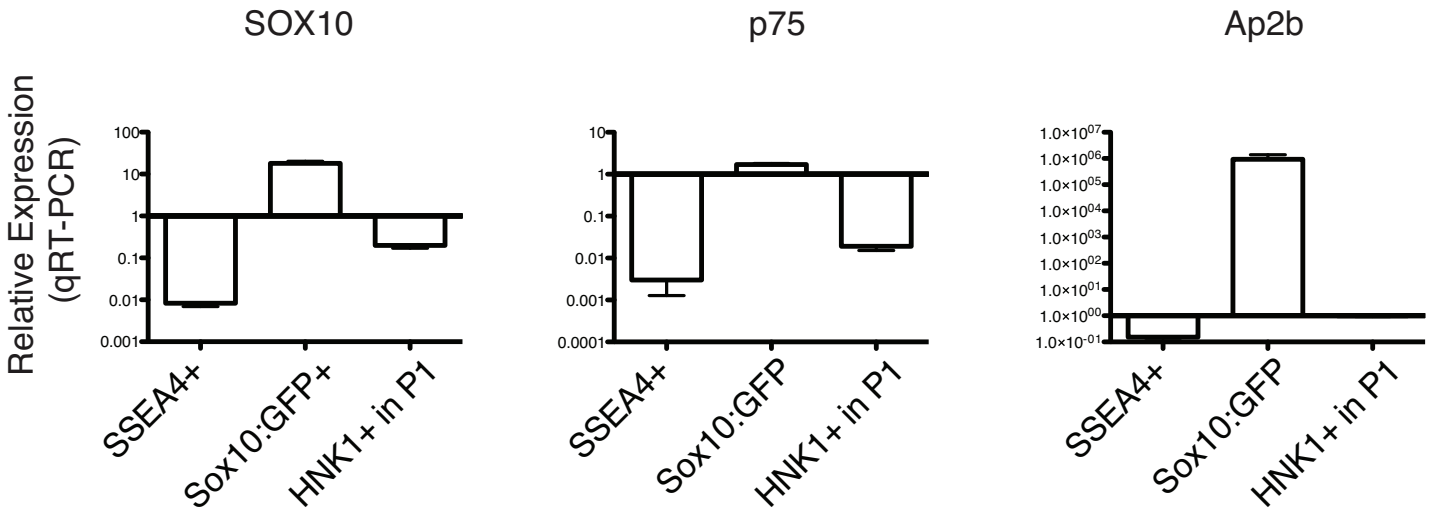

Figure S7 – qRT-PCR validation of SOX10::GFP BAC cell line. Compared to hPSCs sorted for SSEA4 and a previous method to enrich for neural crest stem cells by sorting for HNK1+ cells from neural cultures22, GFP+ cells sorted using the SOX10::GFP BAC are greatly enriched for the neural crest genes SOX10, p75, and AP2B measured by qRT-PCR. GFP+ cells under LSB3i were also negative for markers of other SOX10+ cell types (data not shown) such as oligodendrocytes (OLIG2) or otic placode precursors (SIX and FOXG1) confirming neural crest identity of the cells.

Figure S8 - SM Chambers, et al.

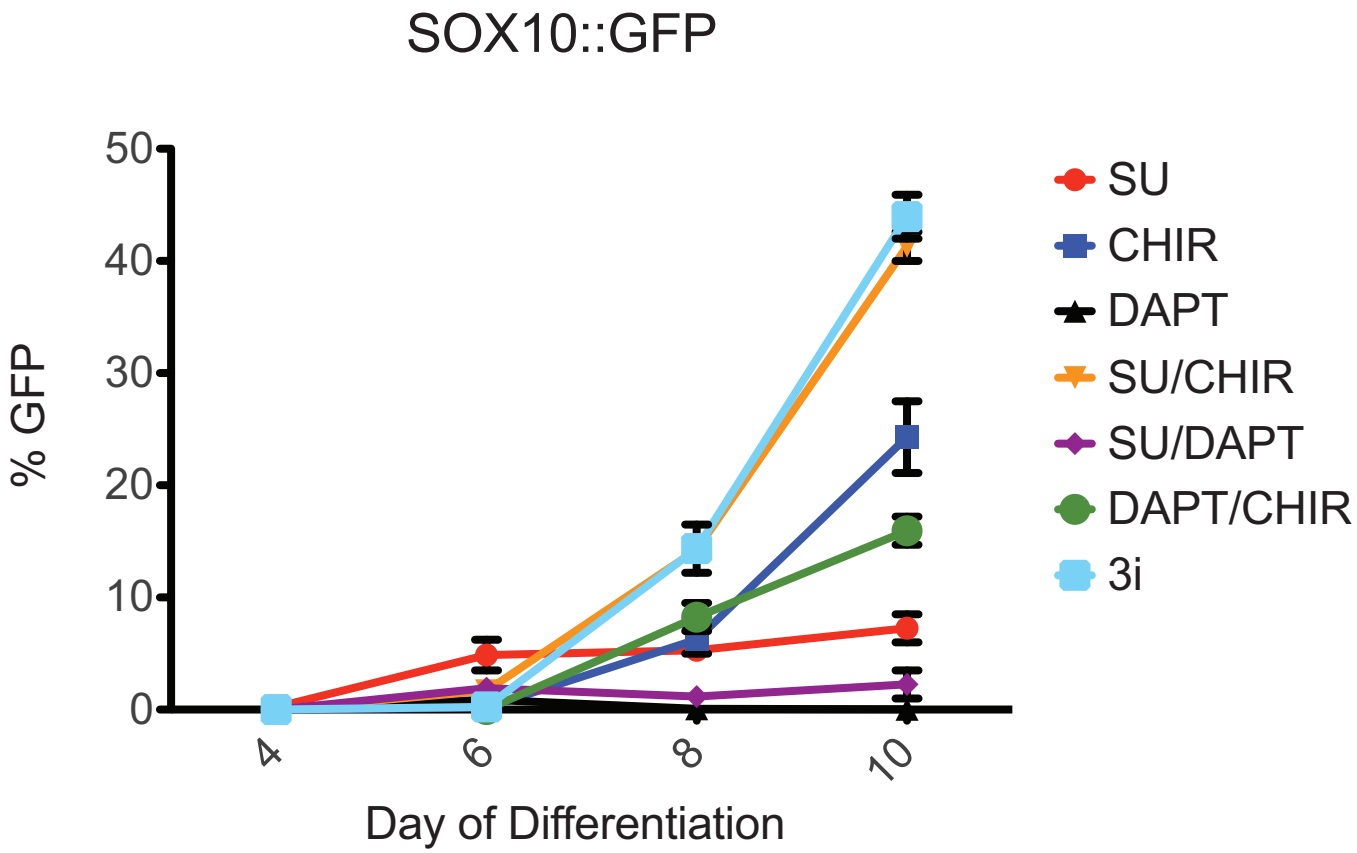

Figure S8 – SOX10::GFP expression for all combinations of 3i factors. Using the SOX10::GFP BAC hESC line, we examined the level of expression of SOX10 under different combinations of 3i factors to gain further mechanistic insight. Both 3i and SU/CHIR treatments displayed the fastest onset of SOX10 expression, suggesting both are critical for neural crest induction and DAPT acts primarily at the later stages of differentiation. When CHIR or DAPT/CHIR was added, moderate, yet delayed, levels of SOX10 was observed. In the absence of CHIR (DAPT, SU, SU/DAPT) little SOX10 expression was observed.

Figure S9 - SM Chambers, et al.

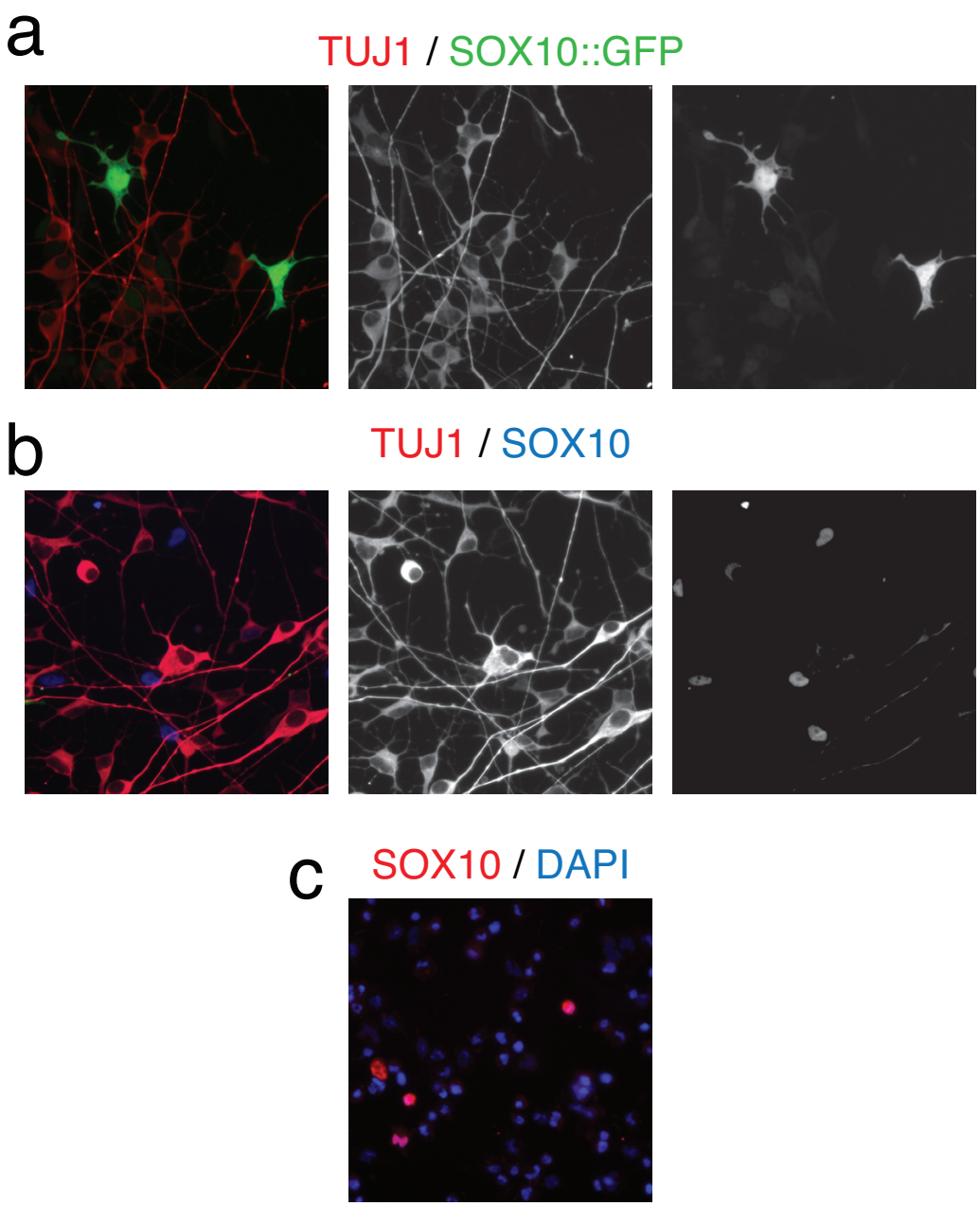

Figure S9 – SOX10 and neuronal β3-tubulin upon passage of LSB3i cells. Co-expression of markers neuronal β3-tubulin with SOX10 is never observed by (a) SOX10::GFP or (b) SOX10 antibody staining. (c) When passaged SOX10 expression decreases to < 5%.

Figure S10 - SM Chambers, et al.

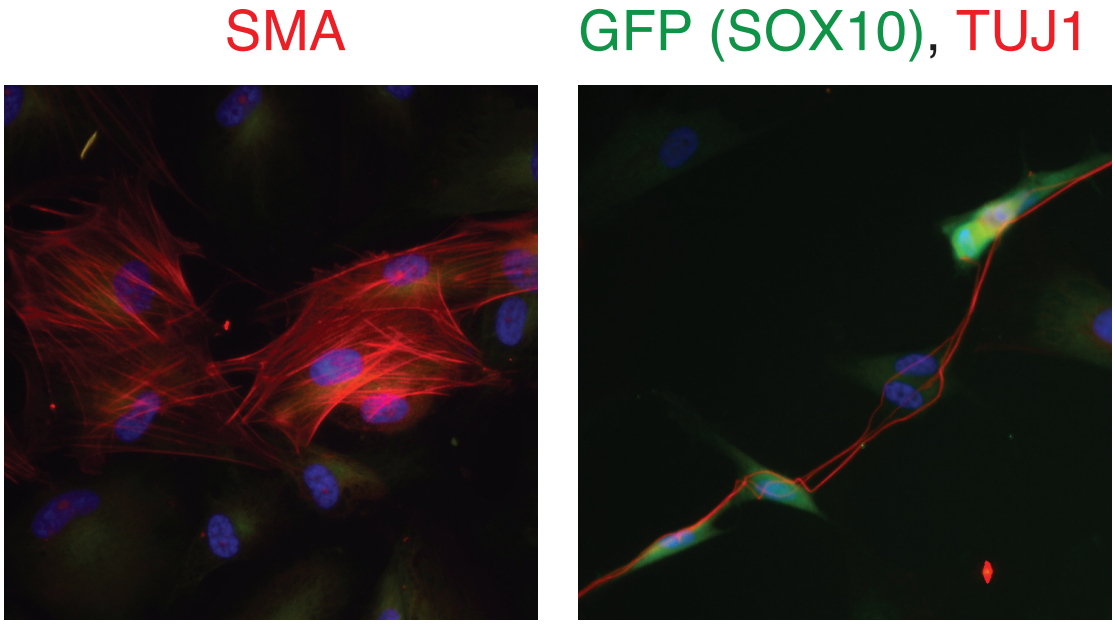

Figure S10 – Cell progeny from cells SOX10+ at day 15. SOX10::GFP cells sorted at late stages of LSB3i (day 15). Upon culturing the SOX10::GFP positive cells, neural crest progeny are observed such as smooth muscle cells marked by smooth muscle alpha actin (SMA), neurons expressing neuronal β3-tubulin (TUJ1), and putative Schwann cells expressing SOX10 in close proximity to neurons.

Figure S11 - SM Chambers, et al.

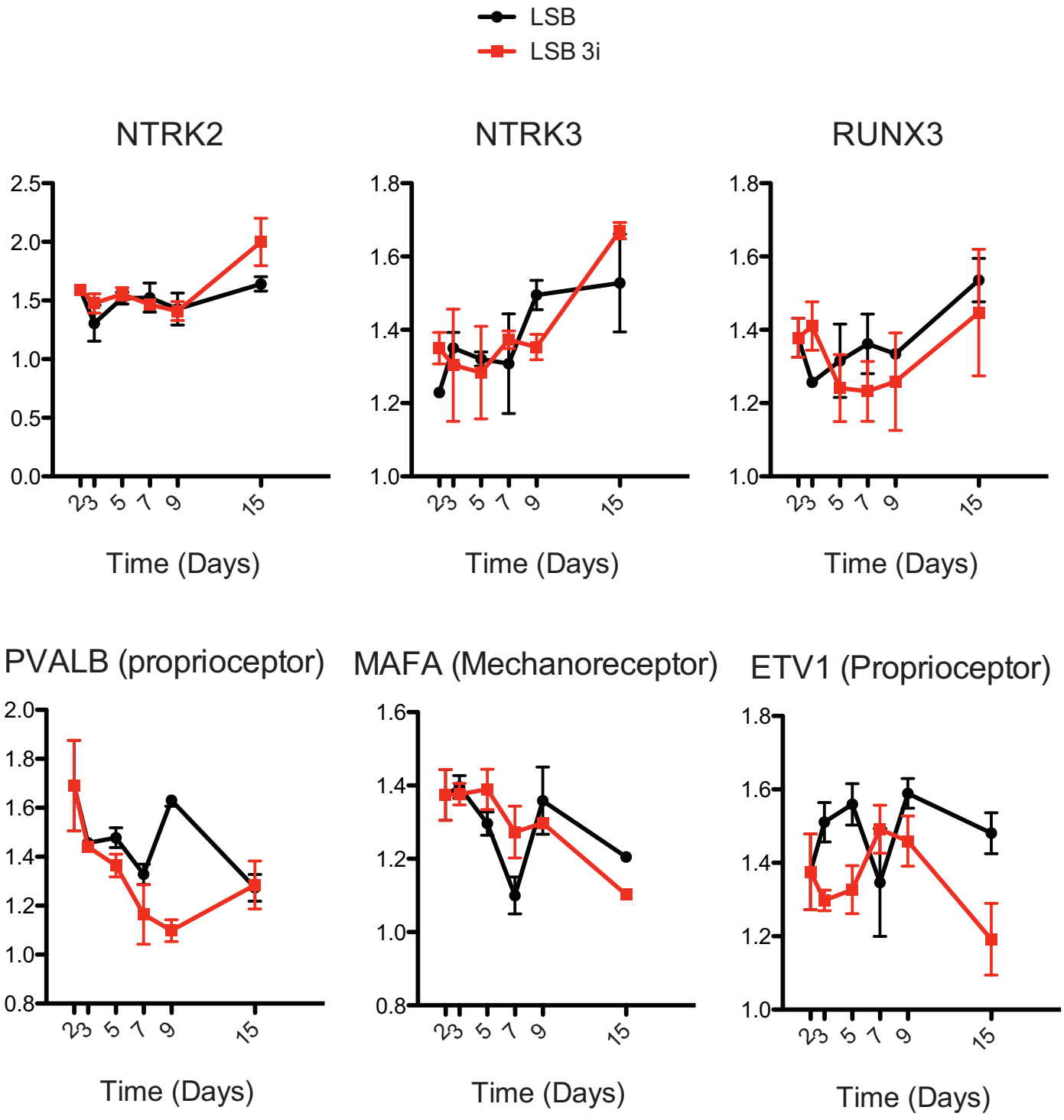

Figure S11 – Microarray gene expression for mechanoreceptor and proprioceptor markers. Minimal expression changes (< 2-fold) were found for markers of mechanoreceptors and proprioceptors including NTRK2, NTRK3, RUNX3, PVALB1, MAFA, and ETV1 when examined by microarray. Expression differences are normalized array values (log2).

Figure S12 - SM Chambers, et al.

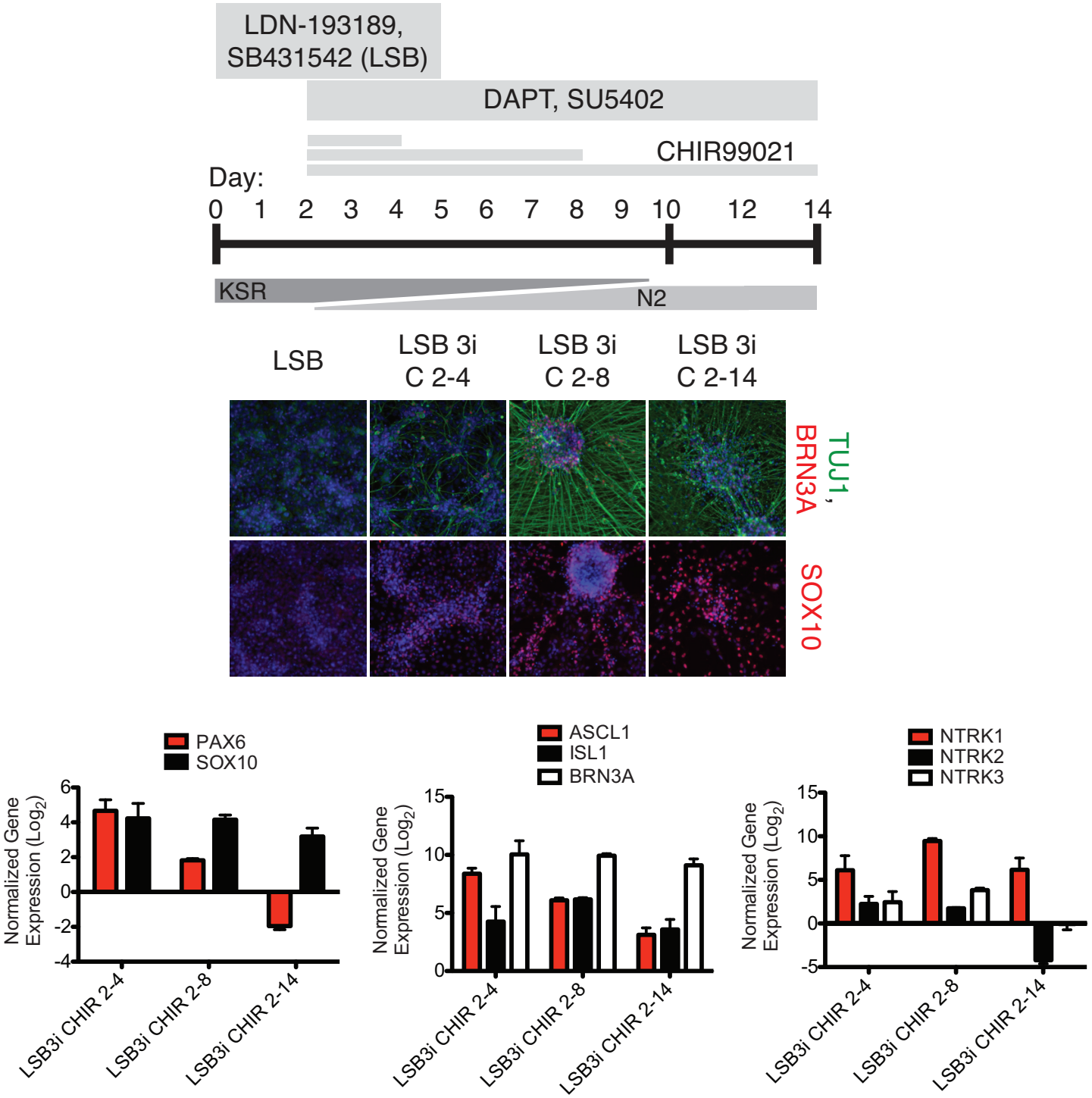

Figure S12 – Varying CHIR exposure indicates its requirement in both SOX10 expression and neuronogenesis. The length of time CHIR exposure was varied from 2 days (Days 2-4) up to 12 days (Days 2-14) and the resulting differentiations were examined for markers of neural crest and autonomic and sensory neuron populations. When CHIR is added for 2 days, SOX10 expression is robust and comparable to longer exposure. When CHIR is added for 6 days, the majority of the cells express β3-tubulin (TUJ1) and BRN3A indicating prolonged CHIR exposure promotes sensory neurogenesis. Continued exposure of CHIR further biases the cell fates towards nociceptors and away from other peripheral sensory neurons on the basis of NTRK1,2, and 3 gene expression measured by Real-Time PCR.

Figure S13 - SM Chambers, et al.

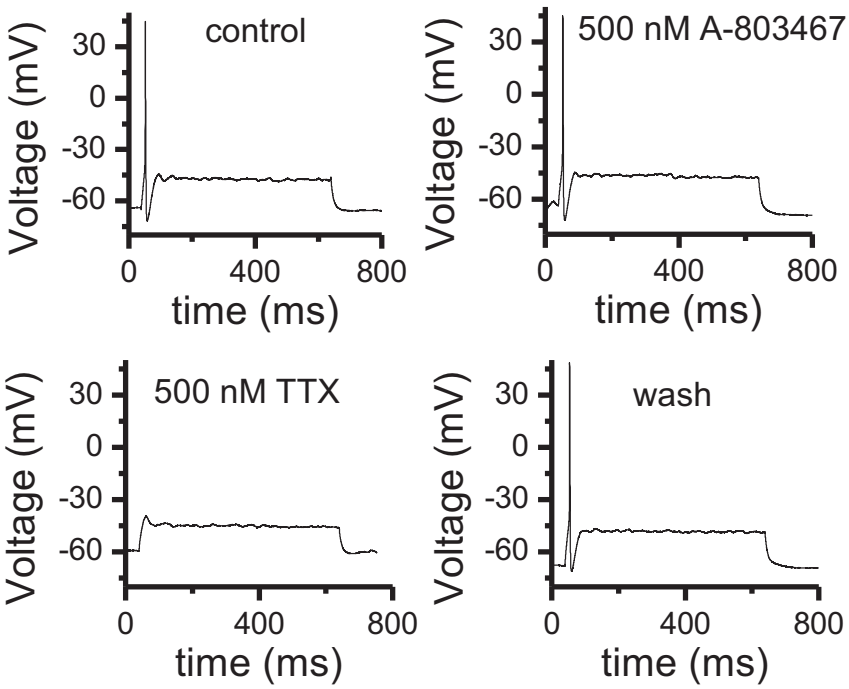

Figure S13 - A single action potential recorded following current injection of 75 pA. There was no significant effect upon application of 500 nM A-803467. Subsequent application of 500 nM TTX blocked the action potential with full recovery after wash.

Figure S14 - SM Chambers, et al.

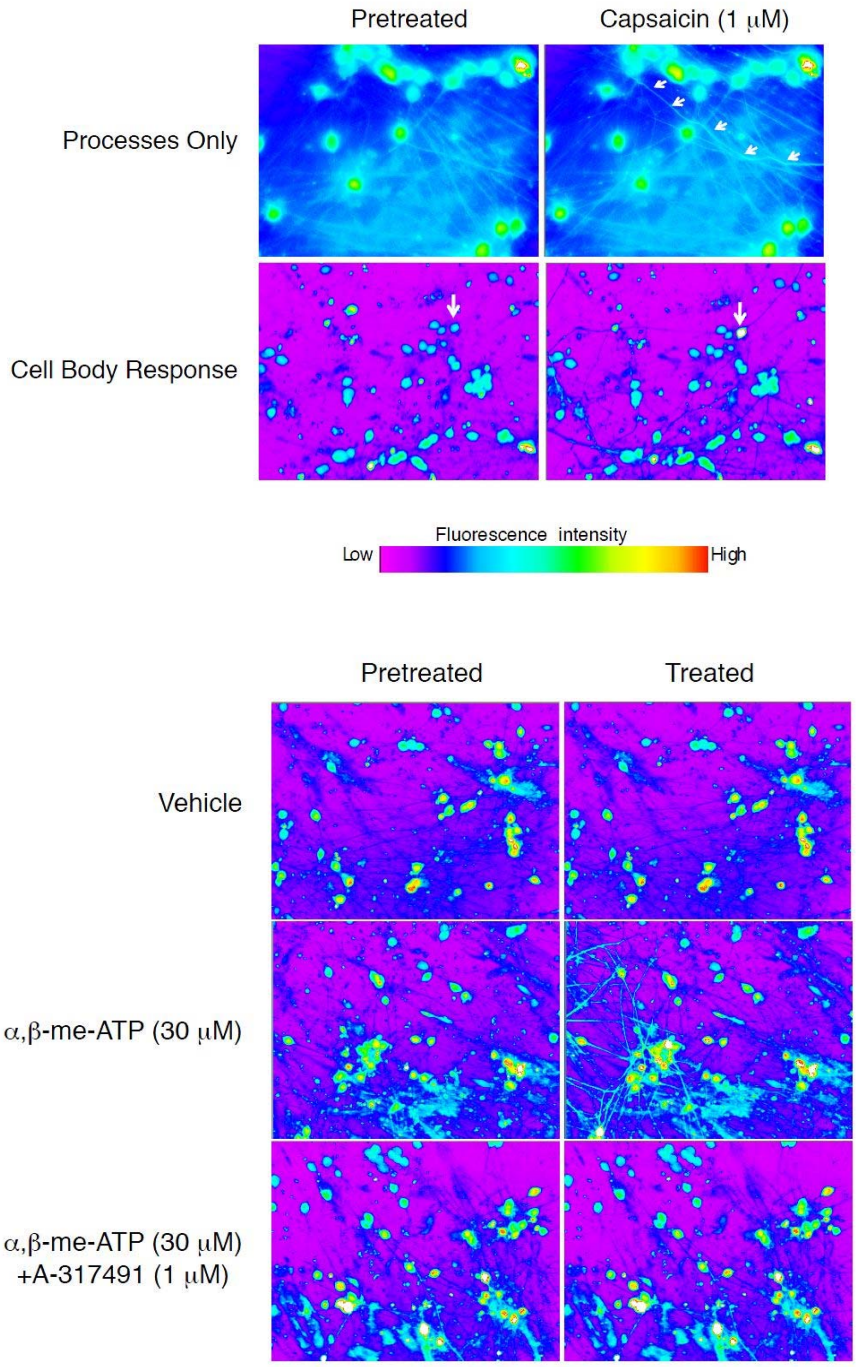

Figure S14 - Photomontage of calcium flux images of LSB3i induced hESC derived neurons. Top panel shows the response to α,β Methylene ATP and inhibition by A- 317491. Images on the left hand side are basal images prior to addition of agonist or vehicle. Images on the right hand side are post treatment. α,β Methylene ATP induced an increase in calcium flux, which was blocked by the antagonist. The lower panel shows examples of calcium flux induced by capsaicin. Capsaicin induced a response in cell bodies in relatively few neurons (arrow in lower images); in images where no cell body response was detected, responses in neurites were frequently observed (upper panel, arrows).

Figure S15 - SM Chambers, et al.

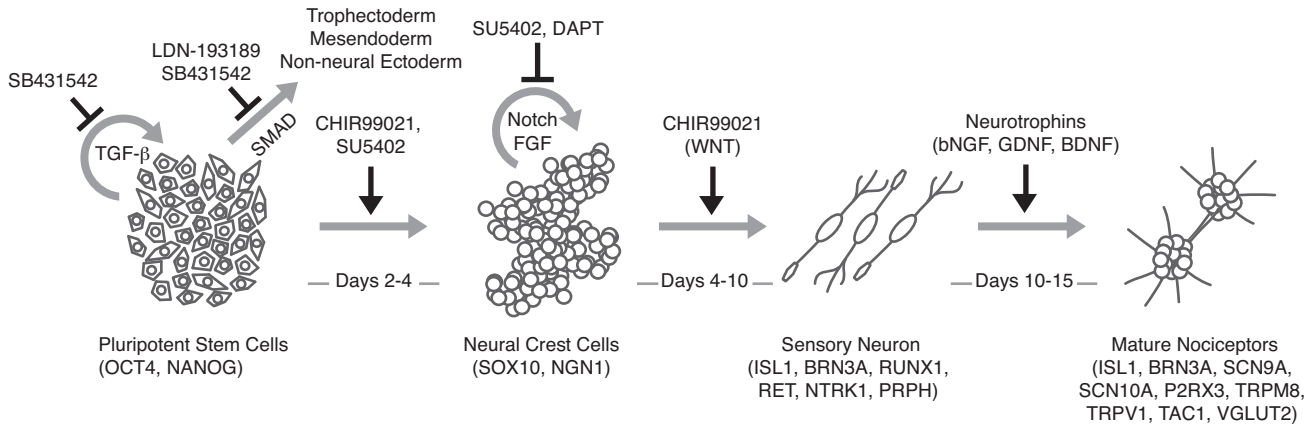

Figure S15 – LSB3i Differentiation model. Early LSB inhibits trophectoderm, mesendoderm, and non-neural ectoderm cell fates yielding neuroectoderm. CHIR99021, SU5402 and DAPT induce and accelerate neural crest stem cell identity by day 8 and promote rapid differentiation of the neural crest stem cells to nociceptors expressing peptidergic markers by day 10.

Table S1 - SM Chambers, et al.

Phases	**Genes**
Neurectoderm	PAX6, OTX2, DLK1, DKK1, CUZD1
Neural Crest	SOX10, MSX1, ID2, AP2B, ETS1, FOXD3
Neuron	NGN1, DCX, TUBB3, SYT4, STMN2, INA, GAP43, ISL1, POU4F1
Nociceptor	TAC1, VGLUT2, SLC15A3

Table S2 - SM Chambers, et al.

Marker	Determined By	Maximum Percent	Day of Expression	Cell Fate
SOX10	BAC GFP, Antibody	80%	Day 6-14	Neural Crest
Neuronal β3-Tubulin (TUJ1)	Antibody (FACS and IF)	75%	After Day 10	Neuron
Nestin	Antibody (FACS and IF)	25%	Day 10	Neural Progenitor
NTRK1	Antibody (FACS)	60%	Day 10	Nociceptor
BRN3A of TUJ1 neurons	Antibody (IF)	> 95%	After Day 11	Sensory Neuron
ISL1 of TUJ1 neurons	Antibody (IF)	> 95%	After Day 11	Sensory Neuron
RUNX1 (early)	Antibody (IF)	> 80%	Day 8	Nociceptor
RET	Antibody (IF)	> 80%	Day 14	Nociceptor
Functional SCN10A	Electrophysiology	20%	After Day 21	Nociceptor
TTX-R inhibited by A-803467	Electrophysiology	90%	After Day 21	Nociceptor
Functional P2RX3	Calcium flux	50%	After Day 21	Nociceptor
Functional TPVR1	Calcium flux	1-2%	After Day 21	Nociceptor