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Supplemental information

Neuroblastoma differentiation *in vivo* excludes cranial tumors

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Supplemental Figures

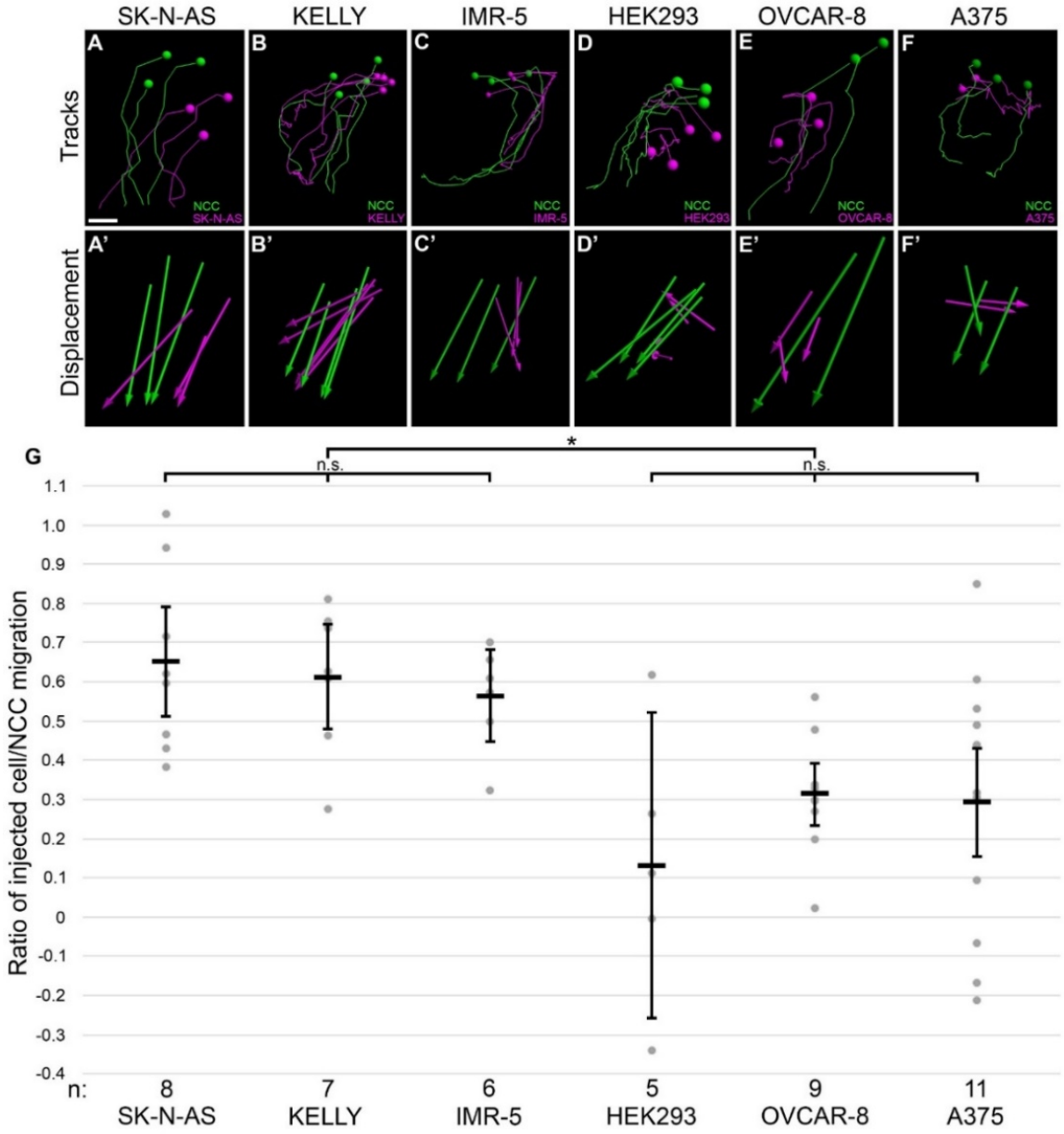

Figure S1. NB cells comigrate with cranial NCCs in zebrafish embryos while non-NB cells do not. Related to Figure 1.
(**A**, **B**, **C**, **D**, **E**, **F**) Migration tracks for injected cells (purple) and NCCs (green). (**A'**, **B'**, **C'**, **D'**, **E'**, **F'**) Total displacement of injected cells and NCCs from 2-14 hpi. (**A** and **A'**) SK-N-AS NB cells (duplicated from Figure 1); (**B** and **B'**) KELLY NB cells; (**C** and **C'**) IMR-5 NB cells; (**D** and **D'**) HEK293 human embryonic kidney cells; (**E** and **E'**) OVCAR-8 ovarian cancer cells; (**F** and **F'**) A375 melanoma cells. (**G**) Ratio of injected cells' displacement to NCCs' displacement (negative ratios indicate migration in the opposite direction of NCCs). Horizontal bars denote mean values, with 95% confidence intervals indicated. No statistically significant difference was found between the migration ratios of SK-N-AS, KELLY, and IMR-5 NB cells (n.s., $p>0.05$), and all three NB cell lines demonstrated significantly increased migration ratios in comparison to all tested non-NB cell lines (HEK293, OVCAR-8, A375). *, $p<0.05$. Scale bar (**A** to **F'**): 30 μm.

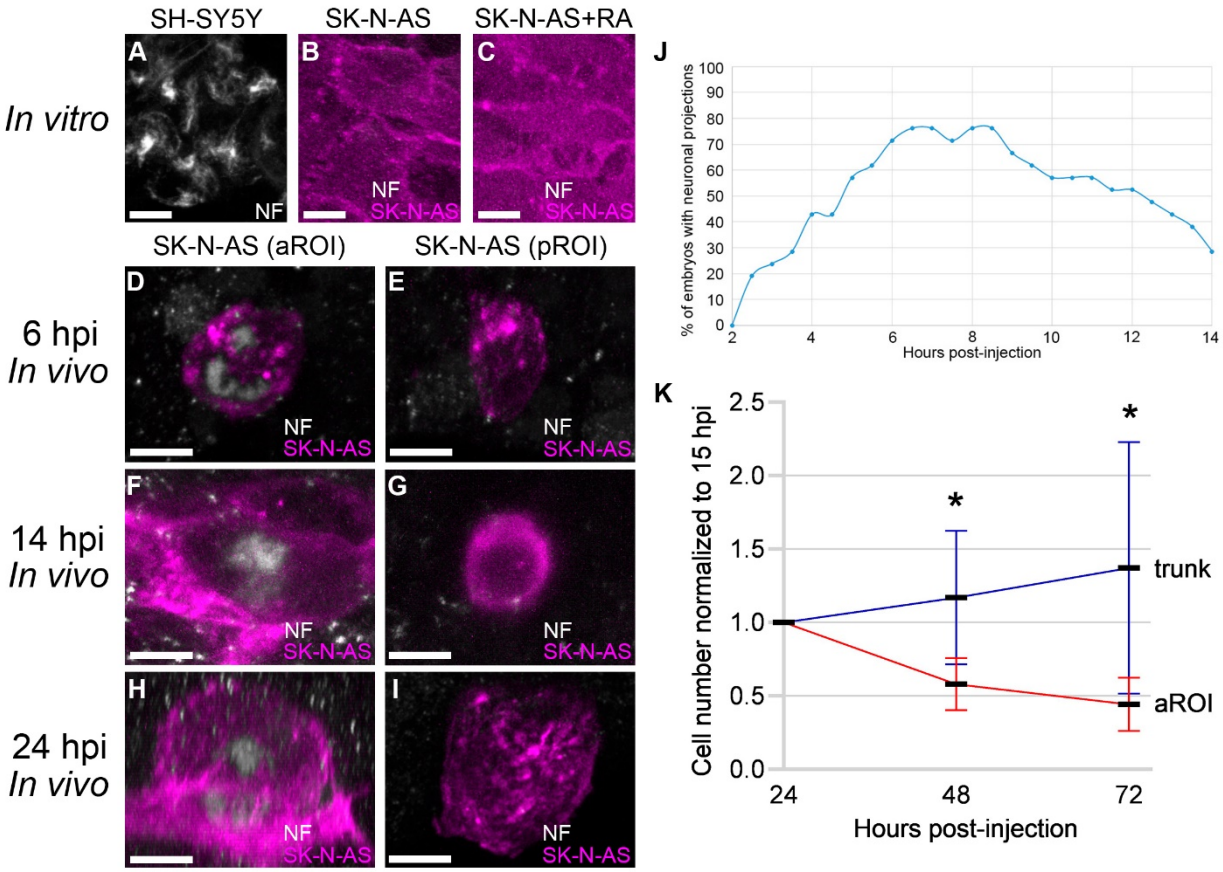

Figure S2. SK-N-AS NB cells do not express neurofilament protein in cell culture even after RA treatment but do express it upon differentiation in zebrafish embryos; neuronal projections peak at 6-8 hours post-injection; SK-N-AS cell numbers decrease in the zebrafish aROI but not the trunk. Related to Figure 2.

(**A**) *In vitro* immunolabeling with anti-neurofilament (NF; white) for SH-SY5Y NB cells (positive control). (**B** and **C**) Membrane-localized mCherry-expressing SK-N-AS cells (purple) without (**B**) and with (**C**) 24 hour 5 μM RA treatment are negative for NF. (**D** to **I**) 8 μm thick optical slices of immunolabeling of representative NF-positive cells within the zebrafish aROI at 6 hpi (**D**), 14 hpi (**F**), and 24 hpi (**H**) (n=14/17 embryos across all time points; 74%) and representative NF-negative cells within the zebrafish pROI at 6 hpi (**E**), 14 hpi (**G**), and 24 hpi (**I**) (n=7/7 embryos across all time points; 100%). Scale bar (**A** to **I**): 10 μm. (**J**) Neuronal projections peak at 6-8 hours post-injection. Percentage of control zebrafish embryos (n=21) harboring neuronal projections over time post-injection. Variation occurs due to the formation of new projections and the collapse of existing projections. (K) SK-N-AS cell numbers decrease in the zebrafish aROI but not the trunk. Ratio of number of SK-N-AS cells in the aROI versus the trunk at 48 hpi and 72 hpi in comparison to 15 hpi (n=21 embryos for the aROI and n=7 embryos for the trunk). aROI data are duplicated from Figure 2J for clarity. * $p<0.05$.

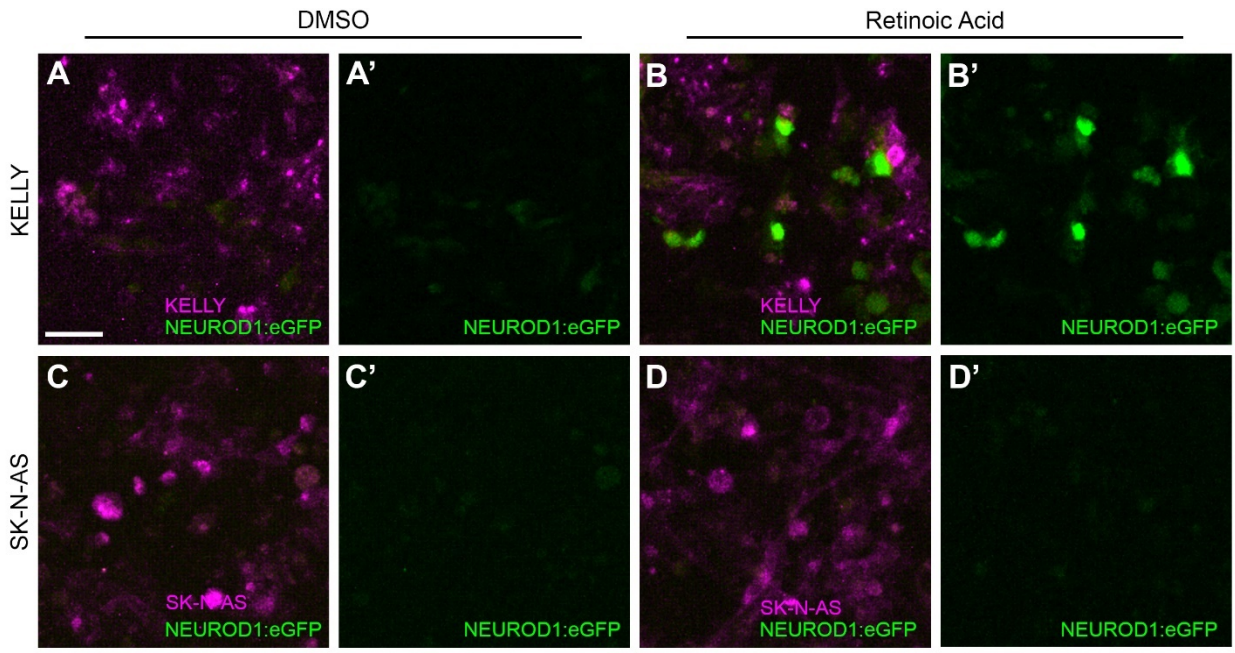

Figure S3. NEUROD1:eGFP expression presents and increases with RA treatment in KELLY cells but not in SK-N-AS cells *in vitro*. Related to Figure 2.
51.2 µm thick optical slices of *in vitro* cultured KELLY (**A** to **B'**) or SK-N-AS (**C** to **D'**) cells transfected with NEUROD1:eGFP and treated with DMSO or 10 µM RA for 96 hours to induce KELLY cell differentiation. Scale bar: 50 µm.

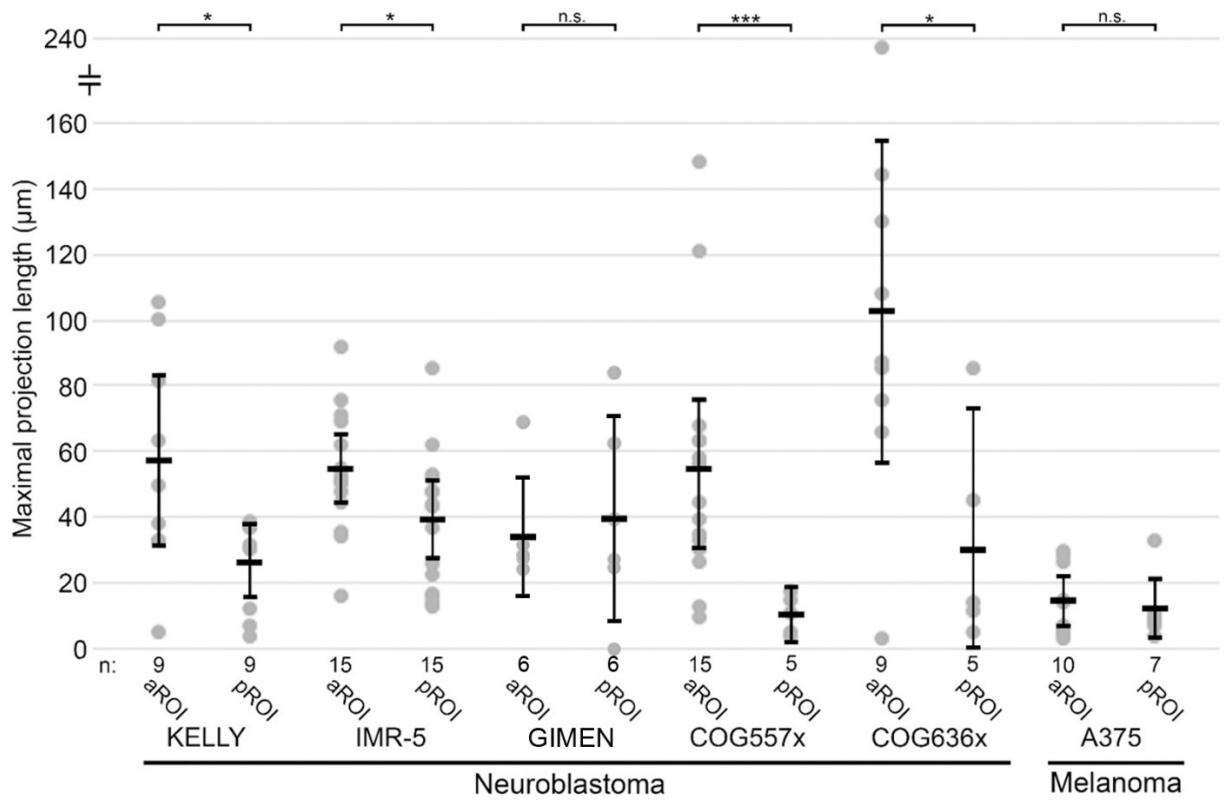

Figure S4. Multiple injected NB cell lines and PDX samples exhibit the same differentiation phenotype as SK-N-AS cells in zebrafish embryos while A375 melanoma cells do not. Related to Figure 2.

Scatter plot of maximal projection lengths segregated into aROI and pROI groups for injected NB cell lines KELLY, IMR-5, and GIMEN, NB PDX samples COG557x and COG636x, and melanoma cell line A375. Horizontal bars denote mean values, with 95% confidence intervals indicated. n.s. $p>0.05$; * $p<0.05$; *** $p<0.001$.

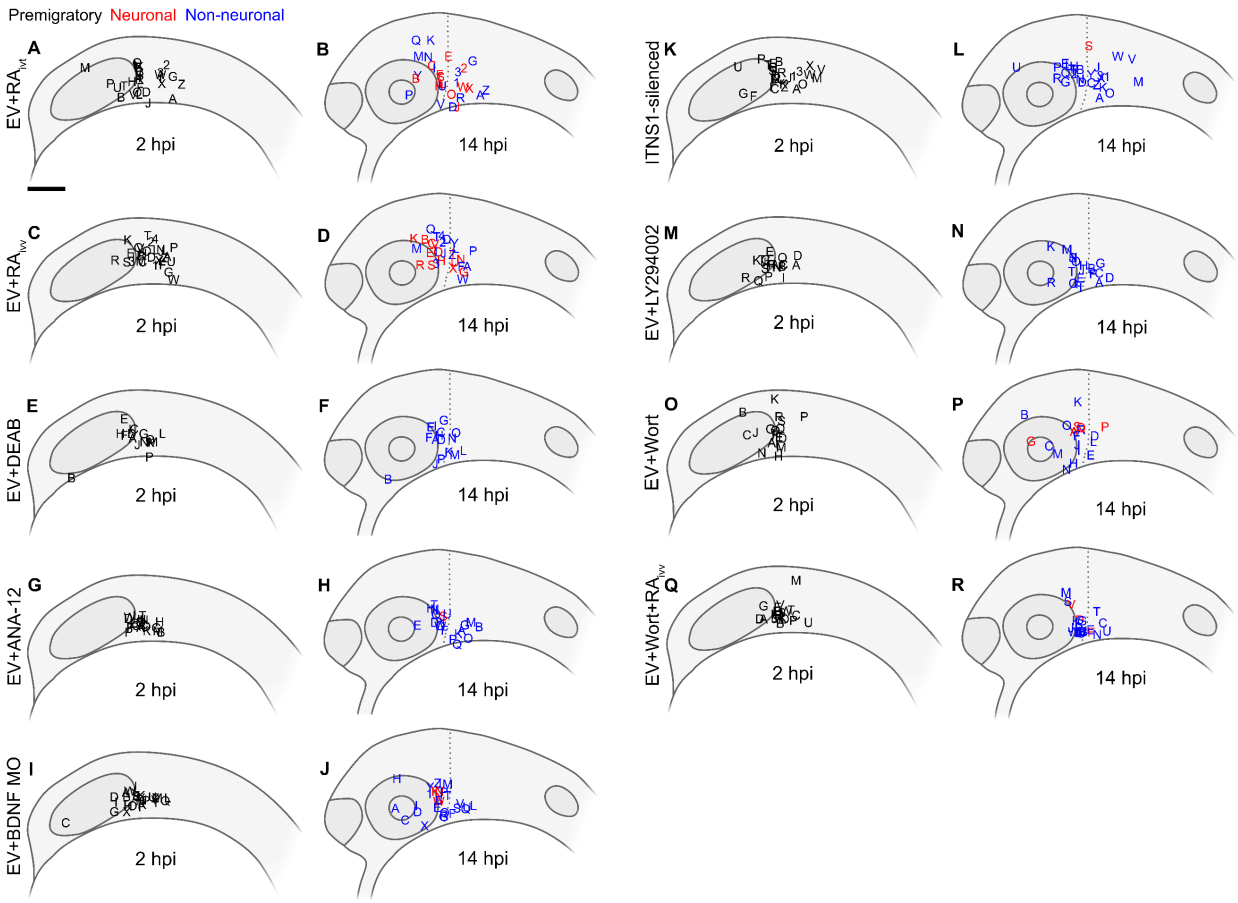

Figure S5. Scaled models of treatment groups from Figures 3-5 including premigratory locations. Related to Figure 4.

(**A** to **R**) Lateral view schematics of zebrafish embryos at 2 hpi and 14 hpi that were injected with membrane-localized mCherry-expressing SK-N-AS NB cells. Black labels indicate sites of injection; red and blue labels indicate final locations of neuronal and non-neuronal cells, respectively. (**A** and **B**) Empty vector (EV) + 5 μM RA on cultured cells for 24 hours pre-injection (n=29 embryos) at 2 hpi (**A**) and 14 hpi (**B**). **B** is duplicated from Figure 3B. (**C** and **D**) EV + 1 μM RA on host embryos (n=30 embryos) at 2 hpi (**C**) and 14 hpi (**D**). **D** is duplicated from Figure 3C. (**E** and **F**) EV + 50 μM DEAB on host embryos (n=16 embryos) at 2 hpi (**E**) and 14 hpi (**F**). **F** is duplicated from Figure 3D. (**G** and **H**) EV + 10 μM ANA-12 on cultured cells for 30 minutes pre-injection and on host embryos post-injection (n=23 embryos) at 2 hpi (**G**) and 14 hpi (**H**). **H** is duplicated from Figure S7B. (**I** and **J**) EV + previous injection at the 1-cell stage of 2 ng BDNF translation-blocking morpholino (MO) (n=27 embryos) at 2 hpi (**I**) and 14 hpi (**J**). **J** is duplicated from Figure 4D. (**K** and **L**) ITSN-1-silenced (sh1 or sh2) SK-N-AS cells (n=29 embryos) at 2 hpi (**K**) and 14 hpi (**L**). **L** is duplicated from Figure 5B. (**M** and **N**) EV + 100 μM LY294002 on cultured cells for 2 hours pre-injection (n=20 embryos) at 2 hpi (**M**) and 14 hpi (**N**). **N** is duplicated from Figure 5C. (**O** and **P**) EV + 100 nM wortmannin on cultured cells for 24 hours pre-injection (n=19 embryos) at 2 hpi (**O**) and 14 hpi (**P**). **P** is duplicated from figure 5D. (**Q** and **R**) EV + 1 μM wortmannin on cultured cells for 24 hours pre-injection and 1 μM RA on host embryos (n=23 embryos) at 2 hpi (**Q**) and 14 hpi (**R**). **R** is duplicated from figure 5E. Scale bar (**A** to **R**): 100 μm.

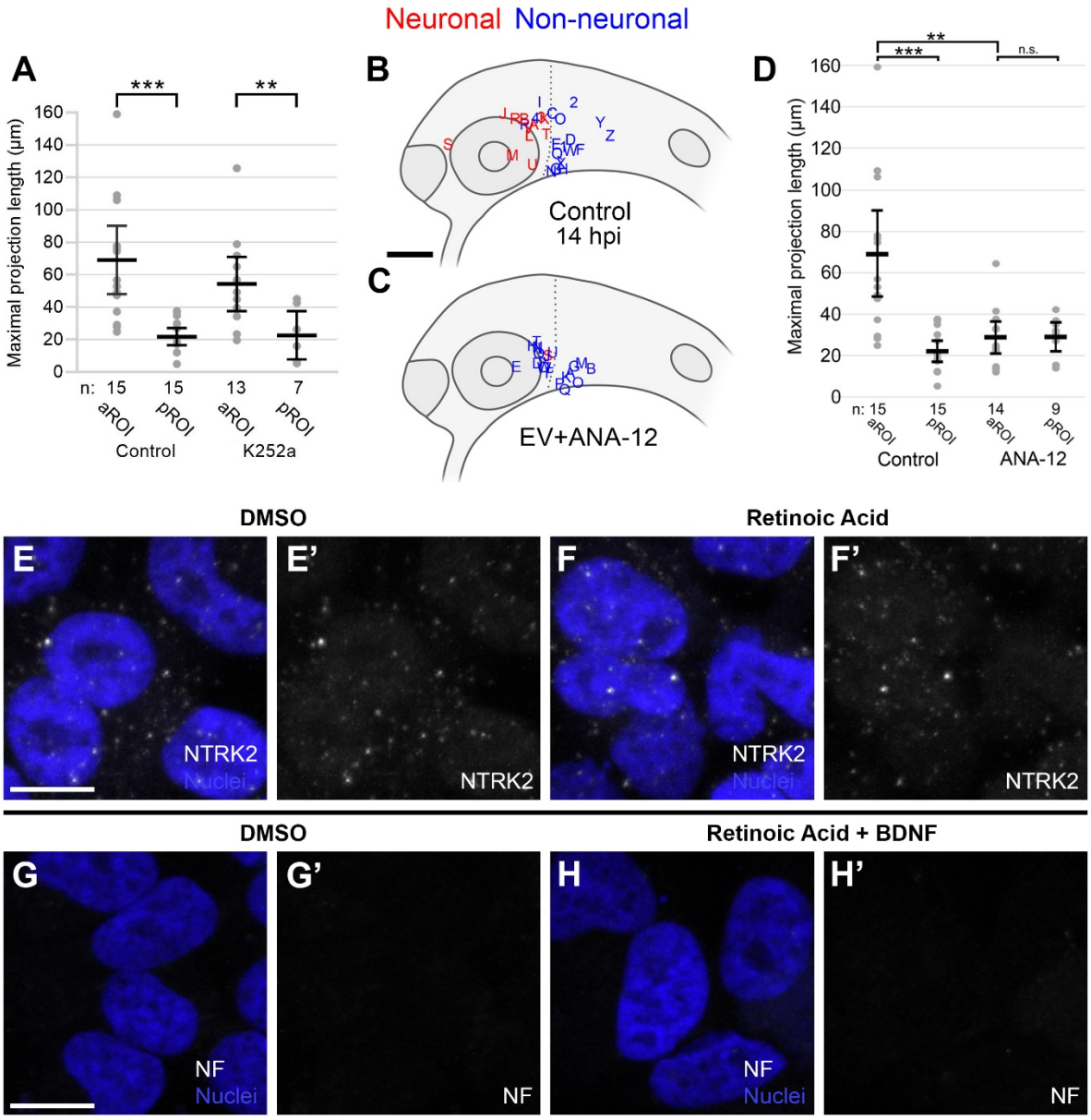

Figure S6. BDNF receptor TrkB, but not NGF receptor TrkA, is required for NB differentiation. RA treatment does not alter TrkB expression *in vitro*, and RA/BDNF treatment does not induce NB differentiation *in vitro*. Related to Figure 5.
(**A**) Scatter plot of maximal projection lengths segregated into zebrafish aROI and pROI groups for control and TrkA antagonist K252a-treated SK-N-AS cells. (**B** and **C**) Lateral view schematics of zebrafish embryos at 14 hpi that were injected with membrane-localized mCherry-expressing SK-N-AS NB cells that either differentiated into neurons (red) or not (blue) during time-lapse imaging. (**B**) empty vector (EV) or scrambled vector (n=30 embryos), duplicated from Figure 2B for clarity; (**C**) EV + 10 µM ANA-12 (TrkB inhibitor) on cultured cells for 30 minutes pre-injection and on host embryos post-injection through time-lapse (n=23 embryos). (**D**) Scatter plot of maximal projection lengths and their segregation into aROI and pROI groups stratified by tested conditions. Control aROI and pROI columns in (**A**) and (**D**) are duplicated from Figure 2I for clarity and are composed of scrambled and empty vector data

points. Horizontal bars in (**A**) and (**D**) denote mean values, with 95% confidence intervals indicated. n.s. $p>0.05$; ** $p<0.01$; *** $p<0.001$. Scale bar (**B** and **C**): 100 µm. (**E** to **H'**) RA treatment does not alter TrkB expression *in vitro*, and RA/BDNF treatment does not induce NB differentiation *in vitro*. (**E** to **F'**) Optical projections of hybridization chain reaction (HCR) against TrkB mRNA (NTRK2; white) in cultured SK-N-AS NB cells after 24 hours of DMSO (control) treatment (**E** and **E'**) or 10 µM RA/DMSO treatment (**F** and **F'**). (**G** to **H'**) Optical projections of immunolabeling with anti-neurofilament (NF; white) in cultured SK-N-AS NB cells after 24 hours of DMSO (control) treatment (**G** and **G'**) or 10 µM RA+200ng/ml BDNF/DMSO treatment (**H** and **H'**). Nuclear blue: DAPI; White: NTRK2 mRNA (**E** to **F'**); NF immunostaining (**G** to **H'**). Scale bar (**E** to **H'**): 15 µm.

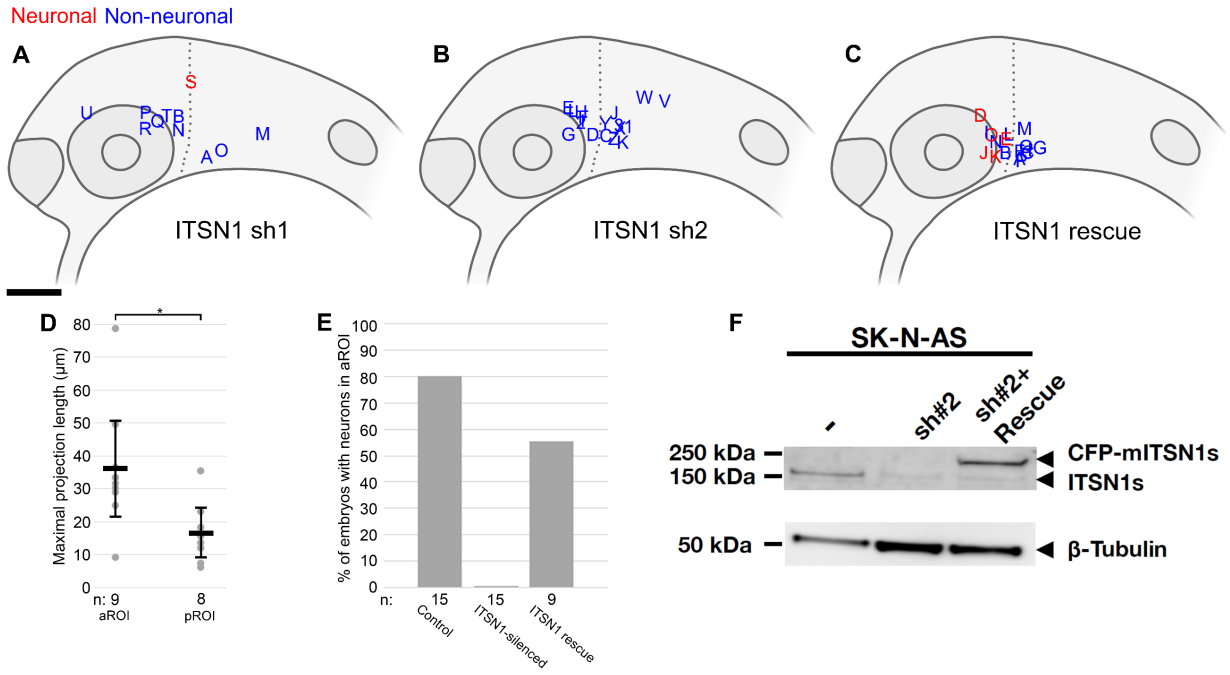

Figure S7. Stable expression of murine ITSN1 rescues neuronal differentiation in ITSN1-silenced SK-N-AS NB cells. Related to Figure 6.

(**A** to **C**) Lateral view schematics of zebrafish embryos at 14 hpi that were injected with membrane-localized mCherry-expressing SK-N-AS cells also stably expressing (**A**) ITSN1-sh1 (**B**) ITSN1-sh2 or (**C**) ITSN1-sh2 + murine ITSN1s-CFP. Red and blue labels indicate final locations of neuronal and non-neuronal cells, respectively. (**D**) Scatter plot shows maximal projection lengths segregated into aROI and pROI from the ITSN1-sh2 + murine ITSN1s-CFP rescue in (**C**). Horizontal bars denote mean values, with 95% confidence intervals indicated. * $p<0.05$. (**E**) Percentage of embryos in which SK-N-AS cells differentiated into neurons in the aROI. Control group was injected with cells containing empty or scrambled vector (Fig. 2B); ITSN1-silenced with cells containing sh1 or sh2; and ITSN1 rescue with cells containing sh2 + murine ITSN1s-CFP. (**F**) Western blot of cell lysates from parental SK-N-AS cells (lane 1), ITSN1-silenced SK-N-AS cells (sh2, lane 2), and ITSN1-silenced SK-N-AS cells (sh2) stably transfected with a CFP-tagged mouse ITSN1s (short isoform) expression construct (lane 3). Probed with anti-ITSN1 polyclonal antibody (Russo and O'Bryan, 2012) and anti-β-tubulin antibody (Sigma #T4026) as a loading control. Scale bar (**A** to **C**): 100 µm.