Supplementary Online Information for

Robo2-Slit1–dependent cell-cell interactions mediate assembly of the trigeminal ganglion

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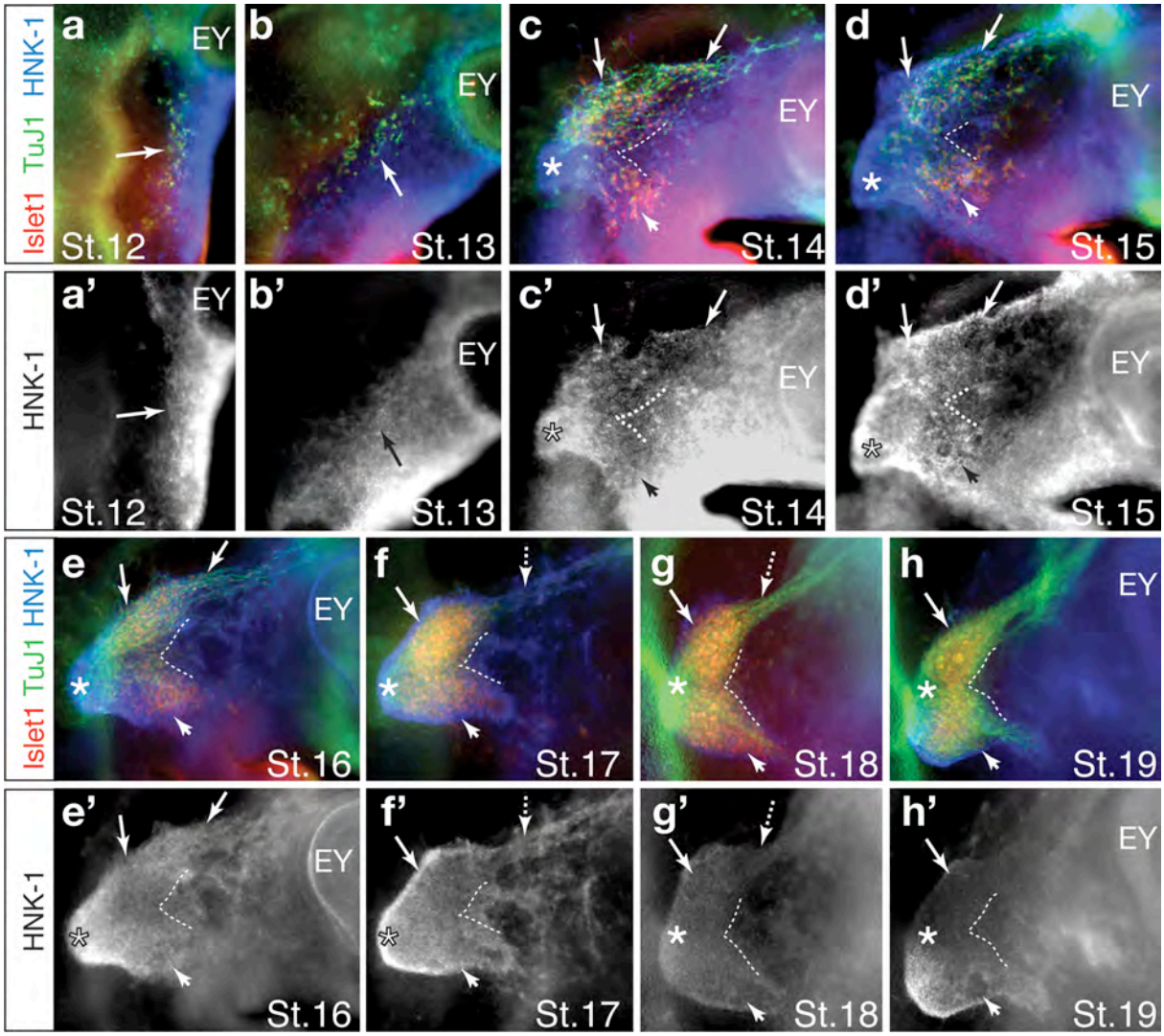
Supplementary Methods

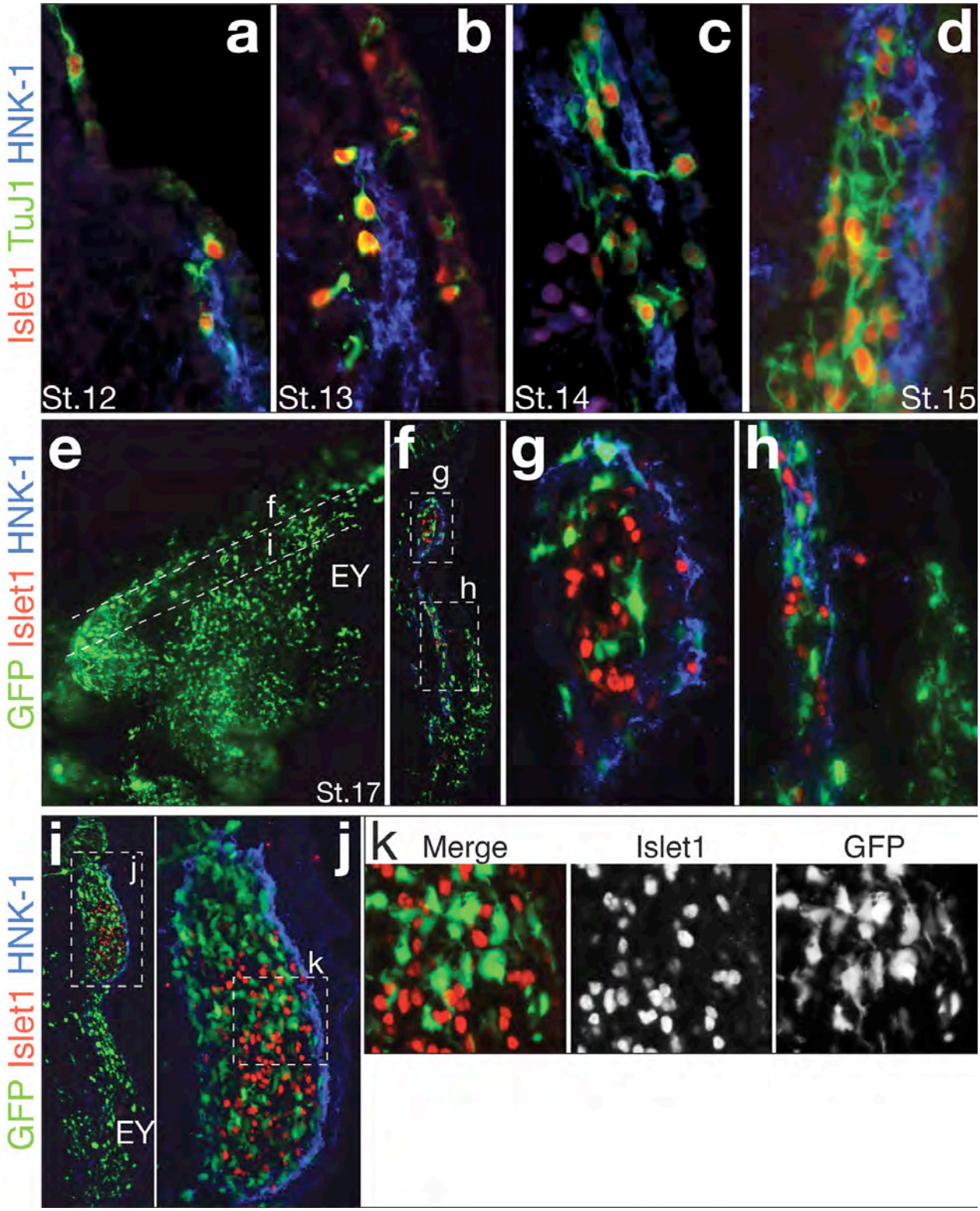
Supplementary Figure 1. Successive stages of trigeminal ganglion formation.



(**a**–**h**) Chick embryos at sequential stages between 12 and 19 show progressive development of the trigeminal ganglion. During these stages, there is extensive overlap and intermingling of placode and neural crest populations, as visualized using antibodies against Islet1 and TuJ1 for placode cells and HNK-1 for neural crest cells (also shown in **a'**–**h'**). By stage 12, (**a**) several placode cells can be detected diffusely distributed along the dorsal edge of the (**a'**) neural crest stream (arrow) between the levels of the developing eye and anterior hindbrain. From stages 12–15, the OpV placode neurons (arrows) appear first and are already

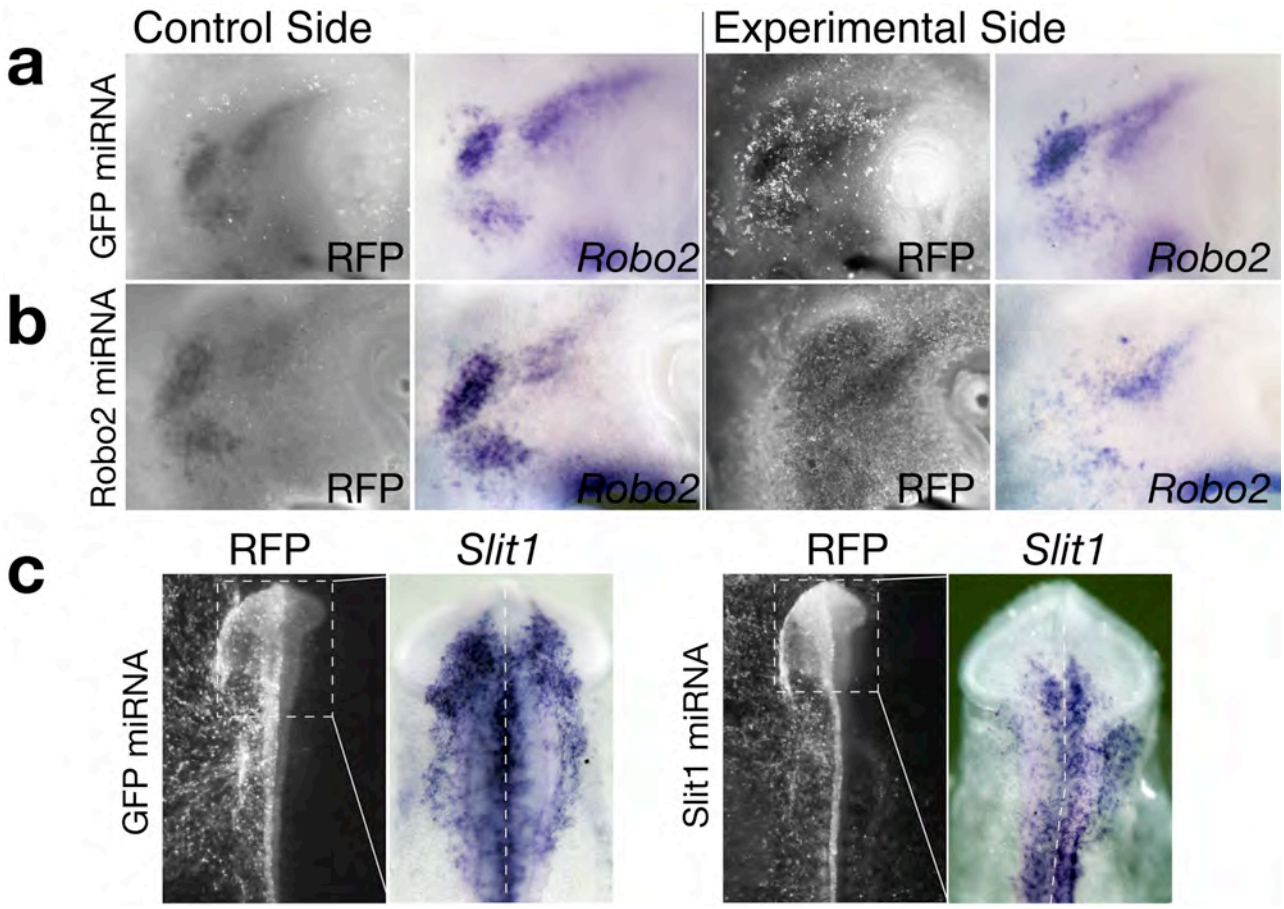
located more dorsally than the MmV placode cells (arrowheads). Though diffuse, placode cells already prefigure the morphology of the future bi-lobed ganglion. (**a'**–**h'**) Assembly of trigeminal placode cells takes place within the domain of the trigeminal neural crest stream throughout development; by stage 14 the most proximal region (asterisk) already appears to be occupied by only neural crest cells. By stage 16, the interlobic region (demarcated by the white dotted lines) that lies between the OpV and MmV lobes is mostly devoid of placode neurons, and the trigeminal neural crest has begun to segregate from the periocular neural crest. This results in an increasingly more defined trigeminal morphology. By stages 17/18, placode and neural crest cells are well condensed into the ganglion and few placodal cell bodies are found along the OpV projection (dotted arrow). EY, eye; *, most proximal region to hindbrain.

Supplementary Figure 2. Placode neurons and neural crest cells intermingle intimately to form the ganglion *in vivo*.



(**a**–**d**) Transverse sections of chick embryos during early times of cell-cell intermixing from successive stages 12 to 15. Placode neurons (Islet1+ and TuJ1+) begin to ingress into the ectoderm by stage 12 and continue to be generated in the ectoderm prior to undergoing an epithelial to mesenchymal transition to migrate into the same region as the HNK-1 expressing neural crest cells as shown in **c** and **d.** Placode cells make abundant cell contacts among themselves as well as with the neural crest cells. (**e**–**k**) Placode and neural crest cells continue to exhibit intimate contacts throughout gangliogenesis, as visualized by GFP-labeled neural crest cells closely intermixing with Islet1+ placode cells in the condensed ganglion at stage 17. (**e**) At stages of placode condensation into the ganglion, the trigeminal neural crest cells also condense into the ganglion and have separated from the other neural crest populations surrounding the eye and migrating into the first branchial arch. (**f**–**k**) Neural crest cells labeled by GFP condense wherever the Islet1+ placode cells are present, indicating intermixing of neural crest and placode cells (**g**, **i**–**k**) in the ganglion lobe and (**h**) along the OpV projection. EY, eye.

Supplementary Figure 3. Robo2 and Slit1 RNAi constructs deplete *Robo2* and *Slit1* transcripts.



(**a, b**) Embryos were electroporated with the RNAi vectors in the trigeminal ectoderm at stages 8–10 and then assessed for *Robo2* depletion by in situ hybridization in the trigeminal ganglion at stages 15–18. Control (which lacks RNAi) and experimental sides (with RNAi, RFP+) were compared in each embryo. In contrast to the control GFP miRNA embryo in **a**, a marked depletion of *Robo2* is detected in the Robo2 miRNA embryo in **b**. (**c**) Embryos were electroporated with the RNAi vectors on the left side at stages 4–6 and then assessed for *Slit1* knockdown in the premigratory and migratory neural crest cells at stages 8–10. Control GFP miRNA electroporated embryo displays normal *Slit1* expression whereas the Slit1 miRNA electroporated embryo shows reduction of *Slit1* expression on the side with the RNAi vector expression (RFP+).

Supplementary Methods

Immunohistochemistry

All primary and secondary antibodies were diluted in blocking solution (PBS containing 5% sheep serum and 0.1% or 0.2% Triton X-100 or Tween-20). Primary antibodies used were anti-GFP (Molecular Probes; 1:1000 or 1:2000), anti-HNK-1 (American Type Culture; 1:3, 1:5, 1:20 or 1:50), anti-QCPN (Developmental Studies Hybridoma Bank; 1:1), anti-Islet1 (clone 40.2D6, Developmental Studies Hybridoma Bank; 1:250 or 1:500), and anti-TuJ1 (Covance; 1:250 or 1:500). The following secondary antibodies were obtained from Molecular Probes and used at 1:1000 or 1:2000 dilutions: Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 350 goat anti-mouse IgM, Alexa Fluor 568 goat anti-mouse IgM, Alexa Fluor 568 goat anti-mouse IgG1, Alexa Fluor 488 goat anti-mouse IgG2A, and Alexa Fluor 568 goat anti-mouse IgG2A. Staining was performed on whole chick embryos or 12 μm sections.

In ovo electroporation of trigeminal ectoderm or presumptive neural crest

DNA injection by air pressure using a glass micropipette was targeted on the trigeminal ectoderm for ectoderm electroporation and in the neural folds at stages 7–9 or presumptive neural crest region at stages 4–6 for neural crest electroporation. Platinum electrodes were placed vertically across the chick embryo for ectoderm electroporation (delivering 5 × 8 V in 50 ms at 100 ms intervals) and for neural crest electroporation at stages 4–6 (5 × 4–7 V in 50 ms at 100 ms intervals), or placed horizontally across the forming neural tube of the chick embryo for neural crest electroporation at stages 7–9 (5 × 12 V in 50 ms at 100 ms intervals). Electroporated eggs were re-sealed and re-incubated at 37˚C to reach the desired stages. For short term analysis of RNAi constructs in neural crest at stages 8–10, electroporations were conducted on whole chick embryo explants at stages 4–6 placed ventral side up on filter paper rings. DNA was injected on the left side of the embryo between the vitelline membrane and the epiblast. Platinum electrodes were placed vertically across the embryo and a

current of 5 × 7 V in 50 ms duration at 100 ms intervals were delivered. Embryos were cultured in 1 ml of albumen in tissue culture dishes (35 × 10 mm, NUNC) for 6–16 hours. *cyto-pCIG* is a modified version of pCIG[1] that drives cytoplasmic GFP expression where 3×NLS is digested out at the NcoI site and re-ligated at the blunted NcoI sites.

Neural fold and ectoderm tissue ablations

For neural crest ablation, neural folds were bilaterally removed from stage 8 (3–4 somites) chick embryos (to eliminate any cross-over of neural crest migration from one side to the other) at the axial level corresponding to the region between caudal diencephalon and the first pair of somites. Eggs were re-sealed and collected after 40 hours of further incubation at 37ºC to reach approximately stage 18. Neural crest ablated embryos did not have neural tubes that healed completely in the region of ablation, however, at these stages normal development seemed to occur in the rest of the head. For placode ablation, the dorsolateral ectoderm adjacent to the neural tube in the region between the optic and the otic vesicles was removed from one side of the chick embryo at stages 11/12, with the unoperated side as control. Eggs were re-sealed and re-incubated at 37ºC for about 24 hours to reach stage 18. Placode ablated embryos appeared to develop normally.

GFP labeling of the ectoderm combined with quail-chick grafts

In ovo electroporation of the trigeminal ectoderm with *cyto-pCIG* was performed on stage 9 (6–7 somites) chick embryos followed by a quail-chick neural fold graft. Dorsal neural tube from the region of caudal diencephalon to rhombomere 2 of a quail embryo was isotopically grafted into a stage-matched electroporated chick host from which this region had been ablated. Embryos were re-incubated at 37ºC for 36–40 hours, collected at stages 17/18 then processed further for immunohistochemistry as described previously.

Analysis of Islet1+ placode cells during ingression

Chick embryos at stages 9–10 (6–10 somites) were electroporated with control GFP (*cyto-pCIG* or *pCAβ-IRES-mGFP*) or *Robo2Δ-GFP* expression vectors in the trigeminal ectoderm. Electroporated eggs were incubated for 20–24 hours at 37ºC to obtain stage 14 embryos. Embryos with broad and high GFP expression in the trigeminal region of the head ectoderm in at least one side of the head were collected for analysis and cryosectioned at 12 µm along the frontal plane through the head encompassing the entire presumptive trigeminal area. Each side of the embryo head that was well transfected was individually analyzed. Sections were immunostained using antibodies against GFP, HNK-1, and Islet1 as aforementioned. All sections were imaged and all Islet1 positive cells in the surface ectoderm, associated with the basal margin of the ectoderm, and in the mesenchyme of the head anterior to the otic placode were counted. In the region of analysis, uniformly ubiquitous, and sometimes weak, Islet1 signals were observed in the ventral and/or pharyngeal ectoderm, as previously described[2]. These signals were not counted, as they appeared to be non-specific to trigeminal placode cells. Average and standard deviation of the percentages of Islet1+ placode cells associated with the ectoderm and in the mesenchyme were obtained.

RNAi

The RNAi vector backbone was pRFPRNAiC in which the *Afl*II site was replaced with an *Mlu*I site and the outer 3' oligoneucleotide sequence for amplifying the miRNA hairpins also incorporated the *Mlu*I restriction site sequence for cloning.

1. Megason, S.G. & McMahon, A.P. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-98 (2002).
2. Begbie, J., Ballivet, M. & Graham, A. Early steps in the production of sensory neurons by the neurogenic placodes. *Mol Cell Neurosci* **21**, 502-11 (2002).