Video Article

Dissection of *Xenopus laevis* Neural Crest for *in vitro* Explant Culture or *in vivo* Transplantation

Cecile Milet[1,2,3,4], Anne Helene Monsoro-Burq[1,2,3,4]

[1]Institut Curie, Centre Universitaire

[2]Université Paris Sud, Centre Universitaire

[3]CNRS UMR 3347, Centre Universitaire

[4]INSERM U1021, Centre Universitaire

Correspondence to: Anne Helene Monsoro-Burq at anne-helene.monsoro-burq@curie.fr

URL: https://www.jove.com/video/51118
DOI: doi:10.3791/51118

Keywords: Developmental Biology, Issue 85, Neural crest, *Xenopus laevis*, embryo, dissection, graft, fibronectin

Date Published: 3/4/2014

Citation: Milet, C., Monsoro-Burq, A.H. Dissection of *Xenopus laevis* Neural Crest for *in vitro* Explant Culture or *in vivo* Transplantation. *J. Vis. Exp.* (85), e51118, doi:10.3791/51118 (2014).

Abstract

The neural crest (NC) is a transient dorsal neural tube cell population that undergoes an epithelium-to-mesenchyme transition (EMT) at the end of neurulation, migrates extensively towards various organs, and differentiates into many types of derivatives (neurons, glia, cartilage and bone, pigmented and endocrine cells). In this protocol, we describe how to dissect the premigratory cranial NC from *Xenopus laevis* embryos, in order to study NC development *in vivo* and *in vitro*. The frog model offers many advantages to study early development; abundant batches are available, embryos develop rapidly, *in vivo* gain and loss of function strategies allow manipulation of gene expression prior to NC dissection in donor and/or host embryos. The NC explants can be plated on fibronectin and used for *in vitro* studies. They can be cultured for several days in a serum-free defined medium. We also describe how to graft NC explants back into host embryos for studying NC migration and differentiation *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/51118/

Introduction

The neural crest (NC) is a transient embryonic cell population that emerges from the neural tube at the end of neurulation in vertebrate embryos. The signaling and genetic events that control NC specification start as early as gastrulation. The NC is specified at the border between the neural and non-neural ectoderm by signals from surrounding dorsal tissues. At the end of neurulation, NC cells undergo an epithelium-to-mesenchyme transition (EMT) and migrate extensively in the embryo following stereotyped routes by responding to surrounding guiding cues. Once they have reached their final destination, they differentiate into a vast array of derivatives, *e.g.* neurons, glia, bone, cartilage, and pigmented cells[1-5]. Because of their contribution to many cell types and embryonic tissues, defects at any step of NC cells development, from induction to final differentiation, can cause congenital syndromes named neurocristopathies[6]. Experimental manipulation of the developing NC at different stages - specification, EMT, migration, and differentiation - will improve our understanding of neurocristopathies and allow design of potential therapeutic strategies.

The *Xenopus laevis* embryo is a model of choice to study NC development. Large numbers of embryos are easy to obtain, and external fertilization gives access to the very first steps of development. Many tools are available to experimentally manipulate *X. laevis* embryonic development. Gene gain-of-function and knockdown are easy to perform by microinjecting individual cells of early blastulas. Embryonic tissues can be cut for *in vitro* reassociation [7-11] or back-grafting assays[12,13].

In this protocol, we describe how to dissect out premigratory cranial NC in *X. laevis* late neurulas, prior to migration. These explants can be cultured on fibronectin-coated plates to study migration and differentiation in controlled *in vitro* experimental conditions. NC explants can also be grafted into normal or manipulated host embryos to study their migration and differentiation *in vivo*.

Protocol

Experiments comply with National and European regulation on the protection of animals used for scientific purposes and with internationally established principles of replacement, reduction and refinement.

1. Preparation of Fibronectin-coated Dishes[14]

1. Pipette 500 ml of 10 mg/ml culture grade fibronectin diluted in 1x Phosphate Buffered Saline (PBS) into a standard plastic Petri dish (Ø 40 x 11 mm). Incubate at 37 °C for 1 hr. Note: if using glass dishes or glass coverslips for subsequent imaging, use a 100 mg/ml fibronectin solution.
2. Rinse three times with 1x PBS.
3. Replace PBS with 500 ml of 1x PBS/0.1% Bovine Serum Albumin (BSA). Incubate at 37 °C for 30 min.
4. Rinse three times with 1x PBS.
5. Fill up with 1x PBS and keep at 4 °C until use (usually for 24-48 hr).

2. Dissection of the NC Explants

1. Prepare 3/4 Normal Amphibian Medium (NAM) (110 mM NaCl, 2 mM KCl, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.1 mM EDTA, 1 mM $NaHCO_3$, 0.2x PBS, 50 mg/ml gentamycin[10,15]). Do not keep more than 1 week for dissection, store at 4 °C. Older NAM can be used for preparing agarose-coated dissection dishes (below).
2. Prepare 60 mm Petri dishes for dissection, coated with 10 ml of 2% agarose in 3/4 NAM.
3. Collect dissection tools: plastic Pasteur pipettes, a finest insect pin mounted on pin holder (see materials table for reference), an "eyebrow knife" (made with eyebrow hair embedded in paraffin at the tip of a glass Pasteur pipette[16]), two fine dissection forceps, P1000 pipette and tips, a binocular stereoscope with magnification 8-40X to 50, a bright light source with optic fibers guides. All dissecting tools should be kept clean; after each experiment, rinse them twice with distilled water, once with 100% ethanol and let dry. Store away from dust.
4. Fill in the dissection dish with fresh NAM almost to the top.
5. Obtain *X. laevis* embryos according to standard procedures[10] and age them until they reach neurula stage 16 (according to Nieuwkoop and Faber developmental table[17]).
6. Place stage 16 embryos into the dissection dish using the plastic Pasteur pipette.
7. Carry out all subsequent steps under the binocular scope. Remove the vitelline membrane with two pairs of forceps. Be careful not to damage the dorsal part of the embryos.
8. Wait until the embryos have reached stage 17 to start the dissection. Stage 17 is appropriate since the neural crest is clearly set apart from the neural tube, in contrast to earlier stages. After stage 17, NC starts migrating and intermingling with the surrounding mesenchyme.
9. Dig a small hole in the agarose with forceps to make a niche for the embryos in order to maintain them during the dissection. Place one embryo dorsal side up. Note: modeling clay can be used to maintain the embryos instead digging a hole into the agarose.
10. Make an incision in the lateral part of the anterior neural fold with the eyebrow knife and the insect pin. Be careful not to go too deep into the embryo: aim to cut through 2-3 cell layers (never reach as deep as the archenteron!). During the whole dissection process, **never allow the dissected tissues to enter in contact with the air-liquid interface** since *X. laevis* embryonic tissues are lysed by surface tension.
11. Make a second parallel incision in the more dorsal part of the neural fold using the same instruments.
12. If needed, turn the dish around 90°. Make a third perpendicular incision with the eyebrow knife and the insect pin, at the posterior side of the first two incisions. The NC is a thick mass of cells underneath the pigmented outer layer of the ectoderm, lateral to the neural tube.
13. Use the tip of the eyebrow knife to detach the pigmented ectoderm layer from the underlying NC, starting from the third incision edge.
14. Use the side of the eyebrow knife to detach the NC tissue starting from the third incision. The NC tissue is rather greyish, while the underlying cephalic mesoderm is white. Carefully separate the NC from the mesoderm.
15. Detach the NC tissue anteriorly from the optic vesicle, and cut at this level to free the explant. As a control for first experiments, checking *snail2* expression (a canonical NC marker) in some explants by *in situ* hybridization is recommended.

3. Plating Explants on Fibronectin

1. Fill-in the fibronectin-coated dish with Modified Danilchick Medium (MDM, NaCl 53 mM, $Na2CO_3$ 5 mM, K Gluconate 4.5 mM, Na Gluconate 32 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, BSA 0.1 %, adjust pH to 8.3 with 1 M Bicine[18]).
2. Transfer the explants into the fibronectin-coated dish using a P1000 pipette. Never allow the explants to touch the air-liquid interface. First, pipette some liquid into the tip, then the explants, then some liquid again. Do not allow any air into the tip while transferring the explants into the fibronectin-coated dish. Once the tip is inside the liquid of the fibronectin-coated dish, let the explants slowly go down by gravity, or push very gently. **Avoid ejecting the explants too fast**.
3. Place 10-30 explants in the dish using the eyebrow knife. Avoid the sides of the dish, which are not adequate for subsequent imaging.
4. Try not to move the dish until the explants have attached on the fibronectin (it takes about 15-30 min). If needed, move the dish **very carefully**.
5. Place the dish in a refrigerated incubator (between 15-20 °C, for temperature chart see[17]). When placed at 15 °C, cells are usually migrating efficiently and scattering after 6 hr of incubation (this timing may vary between experiments).

4. Grafting Explants into Host Embryos

1. Cut a fine glass coverslip into very small pieces using coarse forceps. The size of the pieces should be approximately 1.5 mm^2. Immerse the pieces into a Petri dish containing 3/4 NAM or PBS using forceps.
2. Preparation of the host embryo: dissect out the host NC in 3/4 NAM as described in 3) with special care not to damage the host embryo while removing the vitelline membrane and dissecting the NC. The tissue tension tends to increase the size of the ablation: let the host embryo heal partially while dissecting the donor embryo.
3. In another agarose-coated dish or in another part of the same dish, dissect out the NC explant from the donor embryo as described in Protocol 3. Transfer the NC graft into the dish containing the host with care, as described in Protocol 3.

4. Immediately place the NC explant onto the host ablated area using forceps. The explant should attach partially. Put a piece of glass coverslip onto the grafted embryo to maintain the graft in place. Choose a piece of glass bigger than the embryo to avoid damaging it. The embryo will be a little bit flattened.
5. Wait for at least 15-20 min, then gently remove the coverslip. Let the embryo recover for another 10 min and carefully pipette it into a clean dish filled with 3/4 NAM, using the plastic Pasteur pipette.

Representative Results

When plated on fibronectin, the neural crest explants attach rapidly (15-30 min) and the explant spreads flat within 2 hr (**Figure 1A**). After 3-6 hr cells start to scatter. At 24 hr at 15 °C, many cells have started to migrate away from the explant (**Figures 1B** and **1C**). Yolk makes cells very bright under phase contrast (**Figure 1B**). Cell protrusions (filopodes and lamellipodes) are clearly visible after phalloidin staining of the actin cytoskeleton (**Figure 1C**).

For the orthotopic grafting experiment, the donor embryos were injected with GFP mRNA in order to follow the grafted cells in the unlabeled host embryo. The cranial NC was ablated on one side of the host; 30-60 min after surgery the graft has healed, replacing the ablated host cranial NC (**Figure 1D**, 2 hr after grafting). A few hours later, the GFP-positive cells have migrated out from the explant and followed the stereotyped migration routes of NC cells towards ventral craniofacial locations (**Figure 1E**). In tadpoles, NC-derived craniofacial structures have developed (**Figure 1G**). When cranial NC was ablated, these structures were missing, resulting in a shortened face with eye adjacent to the cement gland (**Figure 1H**, red arrows). In grafted tadpoles, the donor NC cells have contributed to the cranio-facial structures, resulting in a normal face morphology (**Figures 1F** and **1I**, green arrows).

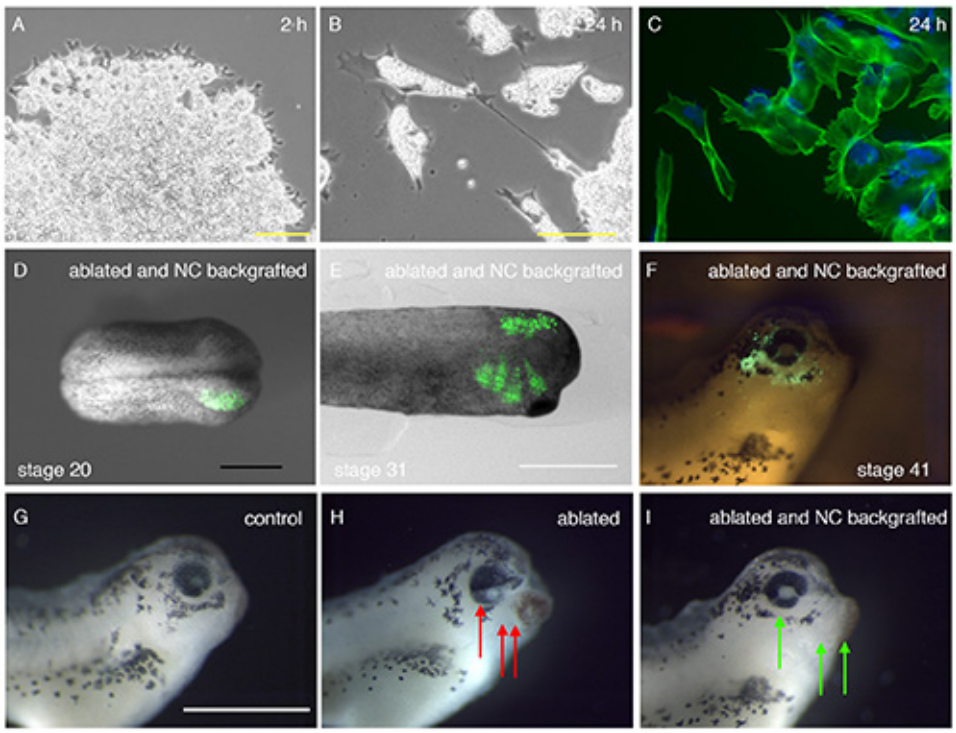

Figure 1. *In vitro* and *in vivo* behavior of explanted cranial NC. A-C) Premigratory NC was dissected out from a stage 17 neurula embryo and plated onto a fibronectin-coated dish. **A**) 2 hr after plating, the explant has attached and spread. **B**) 24 hr after plating, cells have efficiently migrated on the fibronectin. **C**) Lamellipodia and filopodia are clearly seen in migrating NC cells labeled with phalloidin (green). **D-I**) Neurula stage 17 GFP+ labelled premigratory NC was taken from a GFP-injected embryo and back-grafted into a host embryo, from which cranial NC was ablated at stage 17-18. **D**) Two hours after grafting, the explant has healed. **E-F**) Grafted NC cells have actively migrated out and populated the mandibular stream as shown at tadpole stage 31 and 41. **G**) Control stage 41 tadpole. **H**) Ablating the NC has resulted in a dramatic failure of face and eye development (**H**: note that the cement gland develops adjacent to the eye, due to the lack of NC cells populating the jaw areas, red arrows. Compare to control tadpole in **G**). **F, I**) In contrast, the grafted NC cells have restored eye and craniofacial structures development (**F**, **I**, green arrows). **A-C**, Scale bars = 100 mm; **D-I**, Scale bar = 1 mm. **D**, dorsal view. **E-I**, side views. This figure has been modified from Milet *et al.*[12] Please click here to view a larger version of this figure.

Discussion

This protocol describes an easy technique to explant premigratory cranial NC in *X. laevis* embryos. The embryos used for such experiments need to be robust and heal well. Discard any batch of unhealthy embryos. In addition, grow embryos at various temperatures (from 12 to 18-20 °C), in order to excise neural crest at stage 17 and not later. After stage 17, neural crest may be mixed with cephalic mesoderm and cannot be removed completely. *Xenopus* tissues heal quickly and then loose their adhesion to other tissues. It is important to work rapidly and place the neural crest explants into the host as soon as it is excised from the donor.

Concerning *in vitro* experiments, fibronectin culture dishes can get contaminated with bacteria or fungi after a couple of days. Use antibiotic and clean or sterile material and solutions as often as possible.

This procedure is optimized for cranial neural crest. Indeed, this cell population is well defined and localized in stage 17 *Xenopus* embryos. Unfortunately, this model is not appropriate to perform such experiments on trunk NC. Bird embryos are more suitable for those who are interested in manipulating such trunk NC cell population.

The explants described in this protocol can be used to study various aspects of NC behavior, both *in vivo* and *in vitro*. The NC molecular developmental program is largely conserved in vertebrates[19]. Frog development is a powerful model to study vertebrate NC EMT, migration and differentiation. *X. laevis* embryonic cells contain enough yolk to allow their survival *in vitro* for several days without addition of any external nutrient. Thus, *in vitro* studies can test the effects of various growth factors or chemical compounds, in a simple and fully controlled culture medium. Such experiments have been designed to analyze EMT and migration, including studying the effects of chemo-attractants or chemo-repellents, of activating or blocking signaling pathways, *etc.*[14,20-24] This approach can also be used to design improved directed NC differentiation protocols. In the context of developing protocols for regenerative medicine, obtaining diverse NC-derived cell types *in vitro* could be used in drug screening for understanding and treating neurocristopathies[25,26].

NC development is orchestrated by a complex interplay between cell autonomous regulations and signals from the surrounding tissues. Experimental manipulations of gene expression, either in the donor NC or in the host embryo, allow discriminating cell autonomous from noncell autonomous aspects of NC development. Combining gain-of-function and knock-down approaches to this protocol for *in vitro* culture and *in vivo* grafting has been successfully used[14,21,23,27-36].

This technique can be adapted to graft other tissues into host embryos and test for their migration and differentiation potentials. For example, we have used the pluripotent blastocoel roof ectoderm (the "animal cap") to look for a minimal transcriptional switch for NC development. We have studied the effect of two transcription factors, *pax3* and *zic1*, on NC commitment and subsequent development. Animal caps with *pax3* and *zic1* gain-of-function were plated on fibronectin *in vitro* or backgrafted into host embryos. We found that Pax3/Zic1-induced cells migrate and differentiate like *bona fide* NC cells both *in vitro* and in host embryos[12].

Numerous molecular mechanisms that govern NC EMT and migration are also involved in metastatic dissemination in cancer[37]. This protocol aims at providing a simple, cheap and efficient tool for developmental biologists and other researchers to explore the fundamental mechanisms of these key cell behaviors.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors thank Adeline Dolly for the pictures of explant on fibronectin, Eric Theveneau for helpful discussions and the Animal Facility of the Institut Curie. C.M. is a postdoctoral fellow of Region Ile de France (Domaine d'Interet Majeur Stem Pole), Universite Paris Sud (Attache Temporaire d'Enseignement et de Recherche), and Agence Nationale de la Recherche. This work was funded by the Universite Paris Sud (Attractivite 2011), the Centre National de la Recherche Scientifique (Action Thematique et Incitative sur Programme), Association pour la Recherche contre le Cancer Grant SFI20101201882, Ligue contre le Cancer, and Agence Nationale de la Recherche (Agence Nationale de la Recherche Programme Blanc).

References

1. Sauka-Spengler, T. & Bronner-Fraser, M. A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* **9**, (7) 557-568 (2008).
2. Theveneau, E. & Mayor, R. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. *Dev. Biol.* **366**, (1) 34-54 (2012).
3. Stuhlmiller, T. J. & García-Castro, M. I. Current perspectives of the signaling pathways directing neural crest induction. *Cell. Mol. Life Sci.* **69**, (22) 3715-3737 (2012).
4. Milet, C. & Monsoro-Burq, A. H. Neural crest induction at the neural plate border in vertebrates. *Dev. Biol.* **366**, (1) 22-33 (2012).
5. Pegoraro, C. & Monsoro-Burq, A. H. Signaling and transcriptional regulation in neural crest specification and migration: lessons from *Xenopus* embryos. *Dev. Biol.* **2**, (2) 247-259 (2013).
6. Etchevers, H. C., Amiel, J. & Lyonnet, S. Molecular Bases of Human Neurocristopathies. *Neural Crest Induct. Different.* **589**, 213-234 (2005).
7. Nieuwkoop, P. D. *et al.* Activation and organization of the central nervous system in amphibians. Part II. Differentiation and organization. *J. Exp. Zool.***120**, (1) 33-81 (1952).
8. Nieuwkoop, P. D. *et al.* Activation and organization of the central nervous system in amphibians. Part I. Induction and activation. *J. Exp. Zool.* **120**, (1) 1-31 (1952).
9. Sharpe, C. R., Fritz, A., De Robertis, E. M. & Gurdon, J. B. A homeobox-containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. *Cell.* **50**, (5) 749-758 (1987).
10. Sive, H. L., Grainger, R. M. & Harland, R. M. *Early development of Xenopus laevis: a laboratory manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y. (2000).
11. Monsoro-Burq, A.-H., Fletcher, R. B. & Harland, R. M. Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals. *Development.* **130**, (14) 3111-3124 (2003).
12. Milet, C., Maczkowiak, F., Roche, D. D. & Monsoro-Burq, A. H. Pax3 and Zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in *Xenopus* embryos. *Proc. Natl. Acad. Sci. U.S.A.* **110**, (14) 5528-5533 (2013).

13. Sadaghiani, B. & Thiébaud, C. H. Neural crest development in the *Xenopus laevis* embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev. Biol.* **124**, (1) 91-110 (1987).
14. Theveneau, E., *et al.* Collective chemotaxis requires contact-dependent cell polarity. *Dev. Cell.* **19**, (1) 39-53 (2010).
15. Slack, J. M. & Forman, D. An interaction between dorsal and ventral regions of the marginal zone in early amphibian embryos. *J. Embryol. Exp. Morphol.* **56**, 283-299 (1980).
16. Sive, H. L., Grainger, R. M. & Harland, R. M. Embryo dissection and micromanipulation tools. *CSH Protoc.* **2007**, pdb.top7 (2007).
17. Nieuwkoop, P. D. & Faber, J. *Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis*. Garland Pub.: New York (1994).
18. Theveneau, E. & Mayor, R. Beads on the run: beads as alternative tools for chemotaxis assays. *Methods Mol. Biol.* **769**, 449-460 (2011).
19. Bronner, M. E. & LeDouarin, N. M. Development and evolution of the neural crest: An overview. *Dev. Biol.* **366**, (1) 2-9 (2012).
20. Alfandari, D., Cousin, H., Gaultier, A., Hoffstrom, B. G. & DeSimone, D. W. Integrin α5β1 supports the migration of *Xenopus* cranial neural crest on fibronectin. *Dev. Biol.* **260**, (2) 449-464 (2003).
21. Fort, P., *et al.* Activity of the RhoU/Wrch1 GTPase is critical for cranial neural crest cell migration. *Dev. Biol.* **350**, (2) 451-463 (2011).
22. Matthews, H. K., *et al.* Directional migration of neural crest cells *in vivo* is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/ RhoA. *Development* .**135**, (10) 1771-1780 (2008).
23. Kashef, J., Köhler, A., Kuriyama, S., Alfandari, D., Mayor, R. & Wedlich, D. Cadherin-11 regulates protrusive activity in *Xenopus* cranial neural crest cells upstream of Trio and the small GTPases. *Genes Dev.* **23**, (12) 1393-1398 (2009).
24. Hwang, Y.-S., Luo, T., Xu, Y. & Sargent, T. D. Myosin-X is required for cranial neural crest cell migration in *Xenopus laevis*. *Dev. Dyn.* **238**, (10) 2522-2529 (2009).
25. Lee, G., *et al.* Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature.* **461**, (7262) 402-406 (2009).
26. Mica, Y., Lee, G., Chambers, S. M., Tomishima, M. J. & Studer, L. Modeling Neural Crest Induction, Melanocyte Specification, and Disease-Related Pigmentation Defects in hESCs and Patient-Specific iPSCs. *Cell Reports.* **3**, (4) 1140-1152 (2013).
27. Shnitsar, I. & Borchers, A. PTK7 recruits dsh to regulate neural crest migration. *Development.* **135**, (24) 4015-4024 (2008).
28. Matthews, H. K., *et al.* Directional migration of neural crest cells *in vivo* is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/ RhoA. *Development.* **135**, (10) 1771-1780 (2008).
29. Matthews, H. K., Broders-Bondon, F., Thiery, J. P. & Mayor, R. Wnt11r is required for cranial neural crest migration. *Dev. Dyn.* **237**, (11) 3404-3409 (2008).
30. Carmona-Fontaine, C., *et al.* Contact inhibition of locomotion *in vivo* controls neural crest directional migration. *Nature* **456**, (7224) 957—961 (2008).
31. Guiral, E. C., Faas, L. & Pownall, M. E. Neural crest migration requires the activity of the extracellular sulphatases XtSulf1 and XtSulf2. *Dev. Biol.* **341**, (2) 375-388 (2010).
32. Cousin, H., Abbruzzese, G., Kerdavid, E., Gaultier, A. & Alfandari, D. Translocation of the Cytoplasmic Domain of ADAM13 to the Nucleus Is Essential for Calpain8-a Expression and Cranial Neural Crest Cell Migration. *Dev. Cell.* **20**, (2) 256-263 (2011).
33. Borchers, A., David, R. & Wedlich, D. *Xenopus* cadherin-11 restrains cranial neural crest migration and influences neural crest specification. *Development.* **128**, (16) 3049-3060 (2001).
34. Borchers, A., Epperlein, H.-H. & Wedlich, D. An assay system to study migratory behavior of cranial neural crest cells in *Xenopus*. *Dev. Genes Evol.* **210**, (4) 217-222 (2000).
35. Kerney, R., Gross, J. B. & Hanken, J. Runx2 is essential for larval hyobranchial cartilage formation in *Xenopus laevis*. *Dev. Dyn.* **236**, (6) 1650-1662 (2007).
36. Yan, C. Y. I., Skourides, P., Chang, C. & Brivanlou, A. Samba, a *Xenopus* hnRNP expressed in neural and neural crest tissues. *Dev. Dyn.* **238**, (1) 204-209 (2009).
37. Acloque, H., Adams, M. S., Fishwick, K., Bronner-Fraser, M. & Nieto, M. A. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J. Clin. Invest.* **119**, (6) 1438-1449 (2009).

