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# Mechanisms of cranial placode assembly

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**ABSTRACT Cranial placodes are transient ectodermal structures contributing to the paired sensory organs and ganglia of the vertebrate head. Placode progenitors are initially spread and intermixed within a continuous embryonic territory surrounding the anterior neural plate, the so-called pan-placodal region, which progressively breaks into distinct and compact placodal structures. The mechanisms driving the formation of these discrete placodes from the initial scattered distribution of their progenitors are poorly understood, and the implication of cell fate changes, local sorting out or massive cell movements is still a matter of debate. Here, we discuss different models that could account for placode assembly and review recent studies unraveling novel cellular and molecular aspects of this key event in the construction of the vertebrate head.**

KEY WORDS: *cranial placode, sensory organ, sorting-out, cell migration, coalescence*

## Introduction

Cranial placodes are transient embryonic structures from ectodermal origin that generate crucial parts of the vertebrate head. Anterior-most placodes give rise to the adenohypophysis, the olfactory sensory epithelium and the eye lens. More posterior placodes include the otic placode which generates the entire inner ear and the statoacoustic ganglion, trigeminal and epibranchial placodes that contribute to a set of ganglia relaying sensation from the face and viscera to the brain, a newly identified placode giving rise to the paratympanic organ and additional mechanosensory lateral line placodes in aquatic vertebrates (O'Neill *et al.*, 2012; Schlosser, 2010).

Despite diverse morphologies and functions, placodes and their derivatives arise from progenitors dispersed in a common horseshoe-shaped ectodermal domain called the pan-placodal or pre-placodal region (PPR), surrounding the anterior neural plate by the end of gastrulation (Fig. 1) (Bailey and Streit, 2006; Schlosser, 2010; Streit, 2008). The continuity of this PPR, revealed by fate maps and expression profiles, contrasts with the split pattern of placodal derivatives observed at later stages, in which discrete placode entities occupy specific positions along the anteroposterior axis, next to the neural tube (Fig. 1) (Bailey and Streit, 2006; Schlosser, 2010; Streit, 2008).

How the PPR field gets subdivided into individual placodes is still poorly understood, and the relative contribution of cell fate changes, apoptosis, local sorting out or large scale morphogenetic movements remains an open question. In this review, we summarise our current understanding of the cellular and molecular mechanisms involved in placode assembly, using examples from studies mostly performed with chick, *Xenopus*, and zebrafish embryos. We focus on the initial formation of discrete and individualised placodal structures. The long-distance migration of the lateral line placodes and the underlying mechanisms have been described elsewhere (Aman and Piotrowski, 2011; Ma and Raible, 2009) and will not be covered here, neither will the complex morphogenetic remodeling of sensory organs occuring at later stages, such as invagination of olfactory and otic placode epithelia.

Based on insights from fate mapping experiments, we propose that the assembly of placodes involves two sequential steps during development, initial segregation of their precursors and secondary coalescence, and we use this view as a framework for discussing the mechanisms underlying these two events.

## Fate mapping suggests a two-step placode assembly

### *Definition of the pre-placodal region*

The embryonic origin of cranial placodes was initially analysed by fate mapping studies based on transplantation experiments (reviewed in Schlosser, 2010). More recently, dye labelling lineage tracing was performed in fish (Kozlowski *et al.*, 1997; Whitlock and Westerfield, 2000; Dutta *et al.*, 2005), amphibians (Pieper *et al.*, 2011) and birds (Bhattacharyya *et al.*, 2004; Bhattacharyya and Bronner, 2013; Streit, 2002; Xu *et al.*, 2008). To generate these fate

*Abbreviations used in this paper:* PPR, pre-placodal region.

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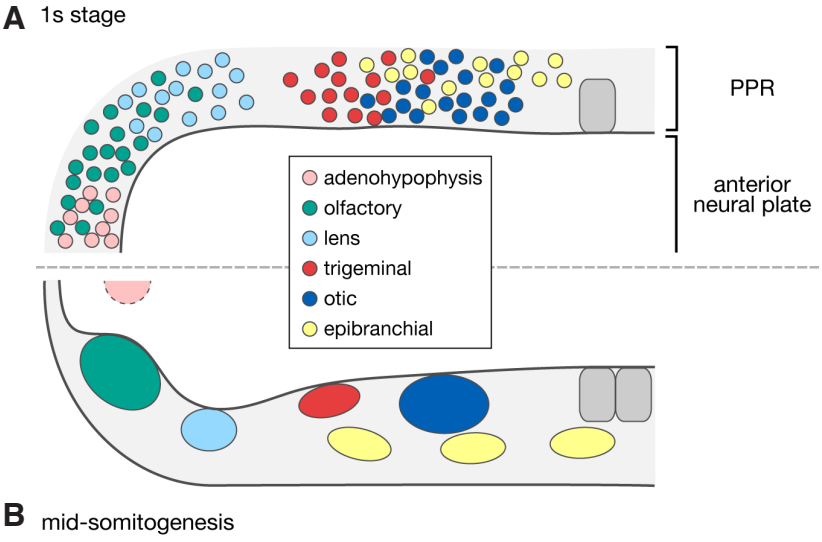



**Fig. 1. Position of placodal precursors and placodal structures before and after placode assembly. (A)** *Schematic view of placode progenitors scattered and intermixed within the pan-placodal region (PPR) surrounding the anterior neural plate at the 1s stage (dorsal view of a theoretical vertebrate).* **(B)** *Compact and individualised placodes occupying specific positions along the anteroposterior axis of the embryo from mid-somitogenesis stages onwards (dorsal view, the ventral position of the adenohypophysis is indicated by the dotted line surrounding the placode). Anterior to the left.*

maps, dyes were injected or uncaged in single cells or small patches of the head ectoderm at various developmental stages, ranging from gastrulation to late somitogenesis. The fate and location of the progeny was then assessed at later stages, when placodes can be identified by their position and morphology. These studies showed that sensory placodes arise from a crescent-shaped ectodermal territory surrounding the anterior neural plate at late gastrulation/early neurulation stages. This contiguous region coincides with the expression domain of transcription factors such as Eya1, Six1/2 and Six4/5, which are crucial for placode formation and are thought to establish a placodal bias in this domain (Lleras-Forero and Streit, 2012; Pieper *et al.*, 2011; Schlosser, 2010). These observations, together with unique properties shared by cells within this territory (Bailey *et al.*, 2006; Martin and Grooves, 2006), argue that it represents the common domain of origin for all placodes, the so-called pan-placodal or pre-placodal region (PPR) (Bailey and Streit, 2006; Schlosser, 2010; Streit, 2008).

### *Initial segregation and secondary coalescence*

Although information is still incomplete in some species about subsets of placodes, a common feature of these fate mapping studies is the initial overlap between the domains of origin of the different placodes (the extent of this overlap has been recently discussed and challenged, for details see Pieper *et al.*, 2011; Schlosser, 2010), which progressively decreases as development proceeds. That is, the precursors of a given placode appear scattered within the PPR domain and partially intermingled with the precursors of adjacent placodes as well as with other ectodermal cells such as epidermal, neural tube or neural crest cells (NCC), and undergo progressive segregation over time, becoming confined to a particular region (Fig. 1) (Kozlowski *et al.*, 1997; Bhattacharyya *et al.*, 2004; Bhattacharyya and Bronner, 2013; Dutta *et al.*, 2005; Pieper *et al.*, 2011; Streit, 2002; Whitlock and Westerfield, 2000; Xu *et al.*, 2008). This initial segregation leads to the formation of distinct placodal compartments that are still apposed to each other at early somitogenesis stages (Fig. 2). However, placode assembly is not yet fully completed at this stage. From mid/end of somitogenesis, placodal structures become more compact and clearly separated from each other by non-placodal areas (Figs. 1 and 2) (Schlosser, 2010; Schlosser and Ahrens, 2004; Streit, 2008). This split pattern and physical separation contrast with the close proximity of placodal domains observed shortly after their initial segregation (Fig. 2).

These observations therefore suggest a two-step sequence in placode formation: initial segregation of placodal precursors into adjacent placodal areas, followed by further compaction to form separated and discrete placodal structures (Fig. 2, the table indicates the developmental stages corresponding to these two steps reported in zebrafish, *Xenopus* and chick embryos).

The compaction step, referred to as secondary coalescence, is well illustrated by the early morphogenesis of the olfactory placode in zebrafish. Whitlock and Westerfield (2000) performed a fate map of the anterior neural plate at the 4-5 somites (4-5s) stage, which shows that the paired olfactory placodes arise from two elongated stripe-shaped cell fields flanking the neural plate. Importantly, no lens precursors were found to be intermixed with olfactory placode progenitors at 4-5s, suggesting that the segregation step is already achieved at this stage for these two placodes in zebrafish. Comparison of the shape of the two initial domains at 4-5s with that of the two spherical olfactory placodes seen from 20s reveals that a coalescence process takes place between these two stages, after segregation with lens progenitors, and before further tissue remodeling by invagination of the epithelium (Whitlock and Westerfield, 2000). This is consistent with the fate mapping of olfactory and lens precursors in chick embryos, which reveals intermingling until 4-5s stage, when the two placodal domains are still juxtaposed to each other, followed by subsequent spatial separation of the two cell populations at later stages (Bhattacharyya *et al.*, 2004; Bhattacharyya and Bronner, 2013).

The driving forces that control initial segregation and secondary coalescence of placodes are still poorly characterised. Both steps could be regulated by similar mechanisms (differential adhesion or chemotactism could for instance mediate cell segregation and further compaction of placodes) or involve distinct cellular processes. Recent studies uncovered novel aspects of the secondary coalescence phase (see part C). However, the initial segregation is still an unsolved issue, and the underlying mechanisms are currently under vigorous debate in the field.

## Mechanisms driving the initial segregation of placodal precursors

How do placodal precursors form segregated embryonic domains from their scattered and intermixed distribution within the continuous PPR? After briefly presenting the two main models that could account for this initial segregation, we discuss old and novel evidence supporting each scenario.

***Two possible scenarios***

*Model 1: Large-scale sorting out of early-specified intermingled placodal precursors*

According to this model, specification of the progenitors of distinct placodes would precede segregation and provide them with properties allowing them to sort out from each other, leading to the formation of immiscible adjacent pladodal areas. Possible mechanisms to mediate this cell sorting include differential intercellular adhesion, in which randomly moving cells specifically adhere to and aggregate with alike placodal cells (Model 1a, Fig. 3), and active directional migration guided by local sources of secreted or matrix-bound cues that attract or repel distinct placodal cell subpopulations (Model 1b, Fig. 3).

*Model 2: Random movements preceding specification of placodal identities by surrounding signalling centers*

In this scenario, multipotent precursors would be exposed to local gradients of environmental signals triggering their specification into particular placodal identities along the anteroposterior axis. Downstream of this signalling, patterning mechanisms such as cross-repression of transcription factors, as seen in the central nervous system, would ensure the formation of a sharp border between placodal domains. The initial intermingling observed in fate maps implies random movements and mixing of the multipotent progenitors within the PPR territory before their specification by extrinsic signals (Model 2, Fig. 3).

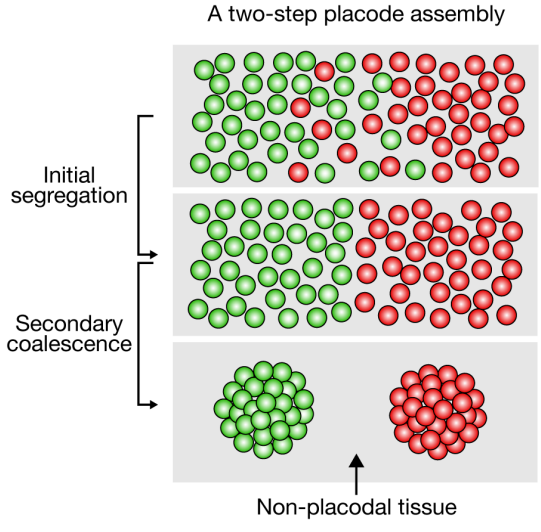
***Confrontation of the models with experimental evidence***

*Are placodal precursors specified before their segregation?*

To sort out from each other, intermingled precursors of future adjacent placodes need to be different, i.e. to have undergone an early specification event that confers them with characteristics allowing their segregation. What is the evidence for such an early specification?

In the fate mapping experiments described above, single labelled cells most often generated progeny in only one specific placode, even when the labelling occured before segregation (Bhattacharyya and Bronner, 2013; Dutta *et al.*, 2005; Xu *et al.*, 2008). This observation suggests that placodal progenitors are lineage-restricted before their spatial segregation (Bhattacharyya and Bronner, 2013; Dutta *et al.*, 2005; Toro and Varga, 2007), although this needs to be more directly proven. The classic assay to estimate the timing of specification for a given cell population consists in dissecting small pieces of tissue at different stages and culturing them in isolation, in order to assess whether cells can differentiate according to their normal fate in a neutral environment. The general outcome of these experiments performed for sub-regions of the PPR is that the onset of placode specification is approximately concomitant with the decrease in the overlap between placodal precursors, which makes it difficult to conclude about the precise temporal sequence linking the two events (Baker *et al.*, 1999; Groves and Broner-Fraser, 2000; Baker and Bronner-Fraser, 2001; Bhattacharyya and Bronner-Fraser, 2008).

Intermingling of early-specified placodal precursors should be reflected by salt-and-pepper expression profiles of placode-specific markers. The formation of segregated placode primordia correlates in time with upregulation of transcription factors specifically expressed in individual or groups of placodes (Bailey and Streit, 2006; Pieper *et al.*, 2011; Schlosser, 2010; Streit, 2008). Pax2 and Pax8, expressed in the otic and epibranchial placode progenitors (and in lateral line placodes in fish and amphibians), show salt-and-pepper expression in chick (Groves and Bronner-Fraser, 2000; Streit, 2002), *Xenopus* (Pieper *et al.*, 2011; Schlosser and Ahrens, 2004), zebrafish (Bhat *et al.*, 2013; Bhat and Riley, 2011; Hans and Westerfield, 2007; McCarroll *et al.*, 2012; Padanad *et*




| | Developmental stages | | |
|---|---|---|---|
| | zebrafish | xenopus | chick |
| Mixing | Bud stage<br>lens - olfactory | Stage 14<br>all placodes | HH5 to 2s<br>otic - epibranchial<br>HH6/7 (0/1s)<br>lens - olfactory<br>otic - trigeminal |
| Segregated domains | 4/5s<br>lens - olfactory | Stage 21<br>all placodes | 4s<br>otic - epibranchia<br>6/7s<br>lens - olfactory<br>8/9s<br>otic - trigeminal |
| Compact placodes | 10s trigeminal<br>18/20s<br>olfactory, pLLP, lens | Stage 27<br>all placodes | 10/11s<br>lens - olfactory |

**Fig. 2. Placode assembly seen as a two-step developmental process.** *Fate map studies suggest two sequential steps in placode formation: segregation of intermingled placodal precursors into immiscible but still juxtaposed placodal domains (initial segregation), and further compaction resulting in discrete and condensed placodes separated by non-placodal tissues (secondary coalescence). The table indicates the corresponding developmental stages reported in studies using zebrafish,* Xenopus *and chick embryos (Data compiled from Kozlowski* et al.*, 1997; Bhattacharyya and Bronner, 2013; Bhattacharyya and Bronner-Fraser, 2004; Breau* et al.*, 2012; Dutta* et al.*, 2005; Harden* et al.*, 2012; Knaut* et al.*, 2005; Kwan* et al.*, 2011; Pieper* et al.*, 2011; Streit, 2002; Whitlock and Westerfield, 2000; Xu* et al.*, 2008). In the upper panel, the colored dots represent progenitors of adjacent placodes, whether or not they are specified. HH: Hamburger Hamilton. pLLP: posterior lateral line placode. s: somite.*

*al.*, 2012) and mice (Ohyama and Groves, 2004). Pax6, a transcription factor expressed in the lens placode, exhibit mosaic expression in zebrafish (Dutta *et al.*, 2005; Hans and Westerfield, 2007). It will be important to understand the mechanisms able to generate these mosaic patterns, such as stochastic activation of gene expression, threshold effects or lateral inhibition within the PPR. However, salt-and-pepper expression could also reflect the progressive up or down-regulation of a placode-specific marker within a contiguous placodal domain, and does not constitute a direct proof of the early specification and intermingling of placodal precursors. Double *in situ* hybridisations for markers of adjacent placodes would represent stronger evidence, but are almost absent from the literature, except for a double *in situ* hybridisation performed on zebrafish embryos at bud stage, which suggests intermingling between Pitx3-positive adenohypophysis precursors and Dlx3-expressing olfactory precursors (Dutta *et al.*, 2005). Thus, existing data do not resolve the question of when placodal fates are specified.

*Do placodal precursors undergo directional movements during segregation?*

If the initial segregation step is driven by directional cell movements, these should be detected by live imaging. The first attempts to monitor the dynamic behaviour of placodal progenitors during segregation were made in chick embryos labelled with dye crystal spots in the otic/epibranchial (Streit, 2002) and lens/olfactory (Bhattacharyya *et al.*, 2004) presumptive regions. In both reports, the movies start at stages when placodal precursors are still intermingled, according to fate maps performed in the same studies (Bhattacharyya and Bronner-Fraser, 2004; Streit, 2002). In the first hours of the time-lapse sequence shown in Streit, 2002, coordinated convergence movements directed towards the midline and affecting the whole embryo can be observed, during which dye-labelled cells remain together. Strikingly, the labelled cells start to spread and migrate away from their initial cluster only when the first 6/7 somites have formed, i.e. when initial segregation of the precursors is already achieved (Fig. 2). Similar observations can be made for the second report: overall convergence towards the midline is followed by splitting of presumptive olfactory and lens cells from stage 5s, which corresponds to the timing of the secondary coalescence phase, according to fate maps performed in the same study (Bhattacharyya *et al.*, 2004). Thus, in both studies, the documented cell movements likely represent the secondary coalescence process which occurs at later stages, rather than directional migration driving the early segregation of placodal populations.

A recent live imaging study of placodal cell behaviour during segregation stages was performed in *Xenopus* (Pieper *et al.*, 2011). The authors performed a detailed analysis of cell movements and neighbour exchange, and found no evidence supporting the notion of directional movements. This work argues against extensive long-distance directed cell migration, but does not excude the possibility of local directional rearrangements that would sharpen the boundaries between adjacent placodal domains.

*Is the initial segregation driven by adhesion-mediated sorting-out?*

Live imaging analysis does not support the notion of large-scale directional movements mediating the initial segregation of placodal precursors. One explanation could be that it rather relies on differential adhesion-mediated sorting-out, which can occur without directional movements (Model 1a, Fig. 3). This is supported by functional studies of Pax6 and Dlx5 transcription factors, respectively markers for lens and olfactory placode precursors.

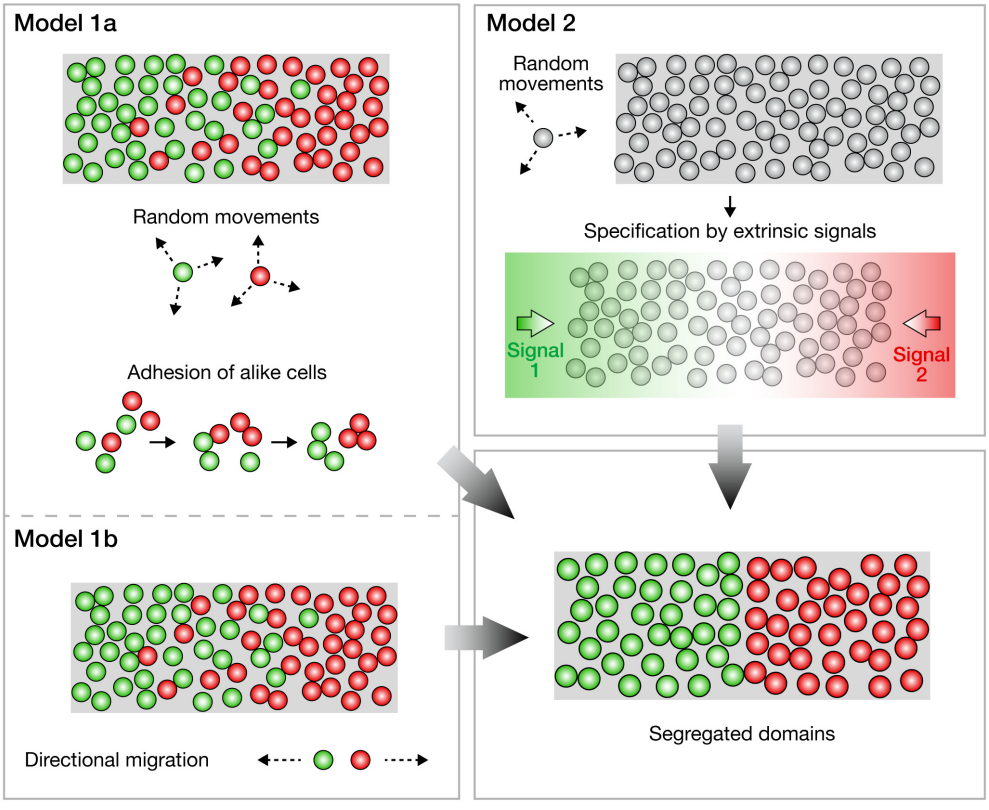



**Fig. 3. Two scenarios for the initial segregation of placodal precursors. (Model 1).** *Large-scale sorting out of early-specified intermingled placodal precursors. Specified placodal precursors initially present a high degree of intermixing and actively sort-out from each other by differential adhesion (Model 1a) or directional migration (Model 1b).* **(Model 2).** *Random movements preceding specification of placodal identities by surrounding signalling centers. Unspecified progenitors undergo random movements within the PPR before being specified by environmental signals. Further patterning mechanisms (cross-repression of transcription factors) lead to the formation of a sharp boundary between placodal domains. Grey and colored dots represent unspecified and specified placodal progenitors, respectively.*

In mouse chimaeras containing a mixture of *Pax6*$^{-/-}$ and wild type cells, *Pax6* mutant cells are excluded from the lens (Collinson *et al.*, 2000). This suggests a capacity of Pax6-positive and Pax6-negative populations to segregate from each other, although loss of *Pax6*$^{-/-}$ cells by apoptosis in the lens tissue has not been ruled out. It has been hypothesised that Pax6 regulates the expression of intercellular adhesion molecules responsible for this sorting out process (Collinson *et al.*, 2000), but a more recent study in mouse rather suggests that Pax6 controls lens formation by regulating the expression of extracellular matrix (ECM) components, including fribronectin (Huang *et al.*, 2011). In zebrafish, forced expression of Dlx5, normally present in a domain anterior and adjacent to the Pax6$^{+}$ region (the presumptive olfactory placode domain), leads to the formation of clusters of Dlx5-overexpressing cells and their exclusion from the lens placode. Despite the lack of loss-of-function data, this suggests that Dlx5 could control cell sorting by regulating adhesive properties of placodal cells (Bhattacharyya *et al.*, 2004).

*Towards a reconciliation of the two models*

The real situation could correspond to an hybrid scenario that reconciles Models 1 and 2, in which extrinsic signals specify adjacent placodal territories with fuzzy boundaries that are further refined and sharpened by local, small-scale active rearrangements, as recently described for the dorso-ventral patterning in the zebrafish spinal cord (Xiong *et al.*, 2013). In this situation, the extent of intermixing is limited, consistent with the idea that this overlap was overestimated due to artefacts in fate mapping experiments (Pieper *et al.*, 2011; Schlosser, 2010).

The hybrid model is supported by the investigation of the local segregation between otic and epibranchial precursors carried out in zebrafish (McCarroll *et al.*, 2012). The authors use Kaede fate-mapping to show that otic and a subset of epibranchial progenitors initially occupy adjacent regions within a Pax2 expression domain lateral to the hindbrain, the so-called PPA (Posterior Placodal Area). The main source of epibranchial precursors resides outside of the PPA, in the adjacent, more lateral non-neural ectoderm (Bhat and Riley, 2011; Padanad and Riley, 2011). The authors observe heterogeneous Pax2 expression within the PPA, and ask whether differences in Pax2 expression levels could drive or influence the formation of otic versus epibranchial placodes. Cells overexpressing Pax2 preferentially incorporated into the otic placode and contributed less to epibranchial placodes as compared with control cells. Moreover, they were seen moving towards the otic field in live imaging experiments. Loss-of-function was more complex to achieve due to the requirement of Pax2 for normal epibranchial placode development (Padanad and Riley, 2011), and functional redundancy between Pax2 and Pax8 in the PPA domain. Nevertheless, partial double knockdown of Pax2 and Pax8 led to an increased number of epibranchial placodal cells, combined with a reduction (although not statistically significant) of otic vesicle size. Together, these results suggest that otic and the subset of epibranchial precursors found in the PPA locally segregate from each other according to Pax2/8 expression levels, with high and low expressing cells preferentially contributing to the otic and epibranchial placodes, respectively. The authors further show that Wnt signalling induces high expression of Pax2 within the PPA and promotes otic placode formation. They propose a model in which specification by an extrinsic signal (Wnt secreted by the neural tube) leads to the presence of two adjacent cell populations (otic and epibranchial precursors) separated by a fuzzy border, which locally segregate from each other to contribute to distinct placodes (McCarroll *et al.*, 2012). This model will need to be confirmed by an non-equivocal visualisation of the local intermixing between the two cell populations within the PPA, and the detailed characterisation of their behaviour and fate during the segregation of otic and epibranchial placodes.

Thus, despite important progress, the issue of initial segregation still deserves further investigation. To tackle this fundamental question, the ability to follow single cell fates over and after segregation, and to correlate cell movements with potential changes in gene expression, will be decisive. This could be achieved with transgenic lines specifically labelling adjacent populations of placodal precursors with different colors. The construction of such tools is now feasible by knocking-in with engineered nucleases techniques (Auer *et al.*, 2013). A potential difficulty is that placode segregation occurs during overall convergence movements towards the midline of the embryo (Bhattacharyya and Bronner-Fraser, 2004; Streit, 2002). It will be challenging to capture potential short-distance directional cell displacements within these converging fields of cells, but this can be done by a careful analysis of neighbour exchange.

## Morphogenetic movements involved in secondary placode coalescence

Once placodal cells occupy defined areas with no or poor intermingling, how is the physical separation of placodes achieved?

Many mechanisms could account for placode condensation and physical separation, including cell fate transitions, cell death, changes in cell shapes, and passive or active cell movements. To our knowledge, no experimental data support the contribution of cell fate changes in the secondary coalescence phase. Apoptotic cells were observed at interplacodal boundaries during the separation between otic and epibranchial areas, and later during the resolution of the epibranchial placode into discrete clusters in mammals (Knabe *et al.*, 2009; Washausen and Knabe, 2012; Washausen *et al.*, 2005), but their functional importance in placode separation has not been assessed. Cell shape changes such as a transition from flattened to epithelial morphology (mesenchyme to epithelium transition), or a reduction in placodal cell volume, could both result in overall compaction of placodal areas, but such morphological transitions have not been described so far. Placodal cells could be passively pushed or tracted to their final location by large-scale movements, folding or growth of surrounding tissues. For example, the outgrowth of the retina may contribute to the separation of olfactory and lens progenitors, although this needs to be experimentally tested.

Here, we focus on recent reports that point to a significant contribution of active morphogenetic movements to the secondary placode coalescence, and summarize our current knowledge of the cell behaviours and mechanisms involved.

### *Diverse behaviours and movements*

*Delamination*

Trigeminal and epibranchial (geniculate, petrosal and nodose) placodes are neurogenic patches embedded in the surface ectoderm, which produce neurons that meet and coalesce with NCC to form the cranial ganglia in the underlying mesenchyme (D'Amico-Martel and Noden, 1983). The ganglia thus condense

at a distance from the placodal ectoderm, suggesting the implication of detachment from the ectoderm (delamination) and inward migration of placodal cells. We consider these processes as taking part in the secondary coalescence of placodes, as they start shortly after the end of placodal precursor segregation (Xu *et al.*, 2008; Stark *et al.*, 1997), and contribute to generate discrete and condensed placodal-derived ganglia by the end of somitogenesis. In the otic placode, another neurogenic placode, neuroblasts also detach from the epithelium to form the statoacoutstic ganglion, but this delamination step affects only a fraction of otic placodal cells (Schneider-Maunoury and Pujades, 2007).

The delamination process of trigeminal and epibranchial placodes was initially observed on sections of fixed tissues in mammals and in chick embryos (for example, see Blentic *et al.*, 2011; Graham *et al.*, 2007; Knabe *et al.*, 2009; McCabe *et al.*, 2009; Shiau *et al.*, 2008; Xu *et al.*, 2008), and by DiI labelling of the surface ectoderm in chick (Stark *et al.*, 1997; Begbie, 2001a; McCabe *et al.*, 2009). However, delamination of trigeminal or epibranchial cells from the ectoderm has to our knowledge not yet been directly observed in zebrafish or amphibian embryos, and its timing and modalities remain unclear in these species. Lately, a transverse slice culture system was developed to follow dynamic cell behaviours during delamination of trigeminal neuroblasts in chick (Shiau *et al.*, 2011). The movies show neurons detaching from the surface ectoderm as individual cells or as chains of cells connected by axon-like protrusions. The delamination of trigeminal/epibranchial cells, which occurs after the initiation of neurogenesis in the placode areas (Begbie, 2002; McCabe *et al.*, 2009), appears to be distinct from the canonical epithelium-to-mesenchyme transition (EMT) undergone by NCC emerging from the neural tube, since placodal cells seem to leave the ectoderm as neurons and, at least in the epibranchial placode, do not express the Snail transcription factor known to control EMT (Fig. 4) (Graham *et al.*, 2007).

*Directional cell movements*

As we mentioned earlier, the extensive movements observed in live imaging studies performed in chick embryos most likely correspond to the coalescence phase (Bhattacharyya and Bronner-Fraser, 2004; Streit, 2002). However, in the absence of single cell resolution, it is difficult to conclude whether the movements of the dye spots reflect true directional cell migration or passive movements following the morphogenesis of surrounding tissues.

Single cell resolution of lens cell behaviour has been obtained in a dynamic analysis of optic cup morphogenesis in zebrafish (Kwan *et al.*, 2011), in which the eye region was imaged fom 6s onwards, after lens/olfactory segregation. 3D manual cell tracking shows that lens precursors converge towards the top of the retina by directed antero-posterior and postero-anterior movements, before undergoing shape changes resulting in the invagination of the lens placode. Interestingly, antero-posterior migrating lens cells move in coordination with underlying retinal cells, suggesting a possible interaction between the two cell populations (Kwan *et al.*, 2011). Two other recent reports uncovered convergence movements during olfactory and otic placode coalescence in zebrafish. Overall antero-posterior convergence movements of placode precursors accompany olfactory placode coalescence between 4s and 18s stages (Harden *et al.*, 2012), whereas combined convergence movements along three directions (latero-medial, antero-posterior and postero-anterior) lead to the formation of the otic placode next to the hindbrain between 4s and 11s (Bhat and Riley, 2011).

As described above, the shapes and behaviour of trigeminal and epibranchial cells have been nicely captured in chick embryos during and shortly after delamination of cells into the mesenchyme, but their behaviour during migration and ganglia aggregation has not been documented. A likely explanation is that ganglion coalescence requires movements along the antero-posterior axis that cannot be visualised on transverse sections or with a slice culture assay. Indeed, live imaging on dorsal views of whole-mount zebrafish embryos uncovered a striking antero-posterior component in the migration of trigeminal placodal cells before their condensation into ganglia. In zebrafish, trigeminal neuron precursors appear initially scattered within two elongated domains along the brain (Bhat and Riley, 2011; Knaut *et al.*, 2005). Time-lapse imaging revealed that between 4s and 10s stages, anterior trigeminal neuronal progenitors migrate directionally as isolated cells or with a chain-like pattern towards less motile posterior cells and fuse with them to form a compact cluster next to the midbrain/hindbrain boundary (Fig. 4) (Bhat and Riley, 2011; Knaut *et al.*, 2005).

Altogether, these live imaging studies clearly show that the secondary coalescence of several cranial placodes implicates directional cell movements. The link between cell migration and delamination remains however elusive in several cases. Whereas the migration of trigeminal/epibranchial cells towards the site of ganglion aggregation clearly follows delamination in mammals and chick, it remains unclear whether placodal cells migrate freely in the mesenchyme underlying the ectoderm, or crawl on or even move within the ectodermal layer in all the other situations described above. Moreover, in mammals and chick, whether trigeminal/epibranchial placodal cells undergo coalescence movements within the surface ectoderm before the delamination process needs to be clarified. This will require a careful examination of the position of placodal cells in relation to the ectoderm during their coalescence movements.

*Cell retention*

Another cellular process involved in placode coalescence is cell retention, in which placodal cells are trapped at the site of placode formation and prevented from moving away by active mechanisms such as chemotactism or cell/matrix adhesion.

This scenario has for instance been described for the coalescence of the posterior lateral line placode in zebrafish. This placode, found in aquatic vertebrates, generates a group of cells (the primordium) which migrates from head to tail along the somitic myoseptum to deposit superficial sensory organs called neuromasts (Aman and Piotrowski, 2011; Ma and Raible, 2009). We observed (Breau *et al.*, 2012) that prior to the onset of this antero-posterior migration, the compact state of the primordium is not established, as isolated cells with lateral line placode identity are present ahead of the primordium. Although these isolated cells appear static, we showed that they are actively maintained in position by a chemotactic mechanism, in the absence of which they migrate away from the primordium (Fig. 4). This cell retention facilitates the fusion of the isolated placodal cells with the primordium as it advances (Breau *et al.*, 2012). In a similar way, trigeminal neuroblasts also appear to be actively retained by chemotactism at the site of ganglion aggregation in zebrafish (Knaut *et al.*, 2005).

Studies in chick and mouse revealed that lens placode formation is correlated with an increase in cell density in the ectoderm

overlying the retina, while the contact area between the retina and the surrounding ectoderm does not change. During this process, lens cells change their shape from cuboidal to columnar, but do not proliferate more or become smaller in volume than pre-placodal ectodermal cells (Huang *et al.*, 2011). Huang and co-workers attributed this local increase in cell density to continued proliferation of lens cells combined with their anchoring to the underlying ECM, a retention mechanism that would work against the expansion of the placodal ectoderm. However, cell crowding at the site of lens placode assembly could also result from incoming migrating cells, as reported in zebrafish (Kwan *et al.*, 2011).

Thus, placode coalescence can involve various types of movements and behaviours, including delamination, directional individual and chain migration, collective convergence or active cell retention. What are the underlying molecular mechanisms?

### *Cellular interactions and molecular cues*

*Interaction between placodal and neural crest cells*

An accumulating body of evidence shows that interactions between placodal and cranial NCC guide trigeminal/epibranchial placodal cells during their delamination, inward migration and coalescence. Close proximity between the two cell types has been shown in a number of studies at several stages of gangliogenesis (for instance, see Begbie, 2001; Shiau *et al.*, 2008; Shigetani *et al.*, 2008; Theveneau *et al.*, 2013), the most recent one reporting the intriguing observation that NCC form corridors that wrap around migrating epibranchial neuroblasts in chick and mouse (Freter *et al.*, 2013). Contradictory results were obtained from cell ablation experiments attempting to unravel a functional interaction between the two cell populations. Whereas NCC depletion had no consequence on the development of the facial (geniculate) ganglion in mice (Coppola *et al.*, 2010), NCC ablation in chick perturbed the coalescence of cranial ganglia without affecting delamination of placodal cells (Shiau *et al.*, 2008), or led to early delamination defects in another study (Begbie, 2001). A recent report demonstrated with genetic ablation of subtypes of NCC that chondrogenic NCC, but not gliogenic NCC, are required for initial epibranchial ganglia formation in zebrafish (Culbertson *et al.*, 2011). This suggests that the conflicting results obtained in various contexts of NCC depletion might come from differences in the nature of NCC subpopulations affected by each ablation procedure (Culbertson *et al.*, 2011).

In chick, the Slit/Robo system mediates at least partly the interaction between NCC and trigeminal placodal cells. NCC express the Slit1 ligand, whereas placodal cells express its cognate receptor Robo2, and functional perturbation of this ligand-receptor pair leads to disorganised trigeminal ganglia (Shiau *et al.*, 2008). The same authors further showed that N-Cadherin acts in cooperation with Slit1/Robo2 signalling to mediate coalescence of the trigeminal ganglion (Shiau and Bronner-Fraser, 2009).

The coalescence of discrete epibranchial placodes from the initial epibranchial placodal domain has recently been shown to involve an original mechanism of reciprocal interactions between NCC and placodal cells in *Xenopus* (Theveneau *et al.*, 2013). This study clearly demonstrates that epibranchial placodal cells migrate

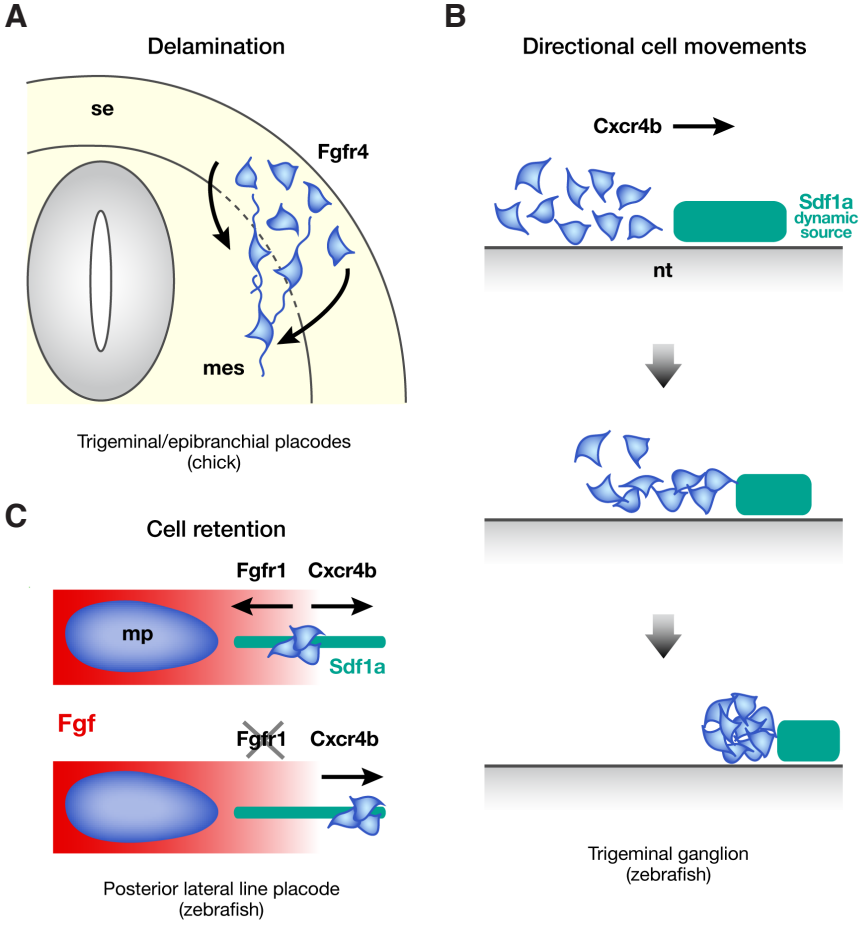



**Fig. 4. Examples illustrating active morphogenetic mechanisms that drive secondary placode coalescence. (A)** *Delamination. Trigeminal and epibranchial placodal cells delaminate from the surface ectoderm (se) as neurons before migrating in the underlying mesenchyme (mes) and coalescing into sensory ganglia. In chick, this process depends on the function of the Fgfr4 receptor expressed by placodal cells (Lassiter* et al., *2009). Dorsal to the top. nt: neural tube.* **(B)** *Directional cell movements. In zebrafish, Cxcr4b-expressing trigeminal neurons undergo an antero-posterior migration before condensing into ganglia. They move as isolated cells or with a chain-like pattern towards a dynamic source of Sdf1a cue (Knaut* et al., *2005; Lewellis* et al., *2013). Anterior to the left. nt: neural tube.* **(C)** *Cell retention. In zebrafish, before the migration onset of the posterior lateral line primordium, the compact state of the primordium is not fully established, as isolated cells with lateral line placode identity are present ahead of the main primordium (mp). The static position of these cells results from two opposing chemotactic signals. Cxcr4b/Sdf1a signalling attracts the cells away from the primordium, whereas Fgf ligands secreted by the main primordium attract them in the opposite direction, thereby retaining them in the vicinity of the placode. Thus, upon inhibition of Fgf signalling, the cells move away in a Cxcr4b/Sdf1a-dependent manner. This chemotactic cell retention mechanism facilitates the coalescence of the isolated cells with the main primordium into a compact group of migrating cells (Breau* et al., *2012). Anterior to the left.*

ventrally and separate into distinct streams once they are contacted by the adjacent and more dorsal cranial NCC. This results from a chase-and-run behaviour, in which NCC are attracted by placodal cells, and placodal cells are in turn repelled by NCC (Theveneau *et al.*, 2013).

*Cell/matrix adhesion*

Morphogenetic movements most often rely on interactions between cells and ECM components, which are mainly mediated by integrin receptors. Bhat and Riley performed a functional analysis in zebrafish of *itga5,* a gene encoding an alpha integrin subunit expressed in the PPR domain from 1s stage and in the placodes during coalescence (Bhat and Riley, 2011). Morpholino-mediated knockdown leads to disorganised posterior placodes (trigeminal, epibranchial and otic placodes), and live imaging shows that cell behaviours are more erratic during convergence movements accompanying otic placode coalescence and the antero-posterior migration of trigeminal neuroblasts (Bhat and Riley, 2011). Itga5-containing integrins are thus required for proper coalescence of posterior placodes, and it will be interesting to identify the ligands that mediate this effect. Moreover, anterior placodes are not affected in itga5-deficient embryos, suggesting that anterior placode assembly relies on other integrin receptors or other adhesion systems to be identified.

In mouse embryos, the increase in cell density observed during lens formation correlates with the accumulation of fibronectin, a major ECM component, between the retina and the overlying placodal ectoderm (Huang *et al.*, 2011). Knocking-out the fibronectin gene disrupts cell crowding in the lens placode, which appears larger than in controls (Huang *et al.*, 2011). This result suggests that fibronectin works as a retention/anchoring cue for lens cells located above the retina (as proposed by the authors). Alternatively, fibronectin could act as a cue guiding the directional migration of lens precursors towards the site of placode formation.

*Chemokine signalling*

The Cxcr4b/Sdf1a(Cxcl12a) chemokine pathway, which guides the movements of many cell types during development, adult life and in pathologic conditions such as cancer, controls the assembly of olfactory, trigeminal and epibranchial placodes.

In zebrafish, the Cxcr4b receptor is expressed in olfactory placode progenitors surrounding the Sdf1a-producing telencephalon. In *cxcr4b* mutants, a subset of olfactory placode cells occuppy aberrant anterior positions by the end of the coalescence process (Miyasaka *et al.*, 2007). It has been suggested that Cxcr4b/Sdf1a works as a retention signal against the overall anterior morphogenetic stream that takes place in the head region at these stages (Miyasaka *et al.*, 2007). However, live imaging recently revealed that olfactory placode cells, instead of being retained in position, move towards the posterior zone of the olfactory field to form the two compact placodes (Harden *et al.*, 2012), suggesting that their anterior mis-positionning in *cxcr4b* mutants could result from their impaired antero-posterior movements. This will have to be confirmed by time lapse imaging. Moreover, as placode assembly is only partially affected in *cxcr4b* mutants (Miyasaka *et al.*, 2007), other molecular mechanisms must be at work and remain to be identified.

Cxcr4b/Sdf1a chemokine signalling also guides the antero-posterior migration and coalescence of trigeminal neurons in zebrafish. Cxcr4b is expressed by trigeminal neurons, whereas the Sdf1a cue is produced by surrounding cells of unknown identity, posterior to the sensory neurons, near the site of ganglion assembly (Fig. 4). In Cxcr4b/Sdf1a-deficient embryos, anterior neurons fail to properly migrate towards and coalesce with more posterior cells, and neurons are seen escaping from small clusters, suggesting that the Cxcr4b/Sdf1a pathway not only guides antero-posterior migration of trigeminal neurons, but also maintains them in the ganglion cluster, acting as a retention signal. Importantly, this attraction and retention mechanisms seem to work against anterior morphogenetic movements of the surrounding tissues, as shown by labelling of cells in the environment of the placodal cells (Knaut *et al.*, 2005). A second study further showed that another receptor for Sdf1a, Cxcr7b, is required for the migration of trigeminal neurons (Lewellis *et al.*, 2013). Consitent with its expression in the close environment of the migrating neurons, Cxcr7b is required in the vicinity of the neurons, rather in the neurons themselves (Lewellis *et al.*, 2013), unlike Cxcr4b which acts cell-autonomously (Knaut *et al.*, 2005). Double Cxcr4b/Cxcr7b mutants show migration defects that are not more drastic than in single mutants, suggesting that the two receptors work in the same pathway. Overexpression of Cxcr7b in the vicinity of the neurons disturbs their mirgation. Moreover, using internalisation of a Cxcr4b-GFP fusion protein as a readout for active Sdf1/Cxcr4b signalling, the authors demonstrate that Cxcr7b decreases this signalling. These results suggest that Cxcr7b inactivates Sdf1 by acting as a sink for this chemokine, as shown in other systems (Boldajipour *et al.*, 2008; Donà *et al.*, 2013; Venkiteswaran *et al.*, 2013). The authors propose a model in which Cxcr7b works in synergy with a Sdf1 mRNA clearance system mediated by a microRNA, miR-430; together they dynamically sculpt the source of Sdf1 in such a way that it is always closely associated with and ahead of migrating trigeminal neurons, therefore guiding them along the antero-posterior axis (Fig. 4, Lewellis *et al.*, 2013). Thus, if Cxcr7b and Cxcr4b likely compete for Sdf1a ligand binding at the molecular level, they act together to promote the migration of trigeminal neurons. The tight spatiotemporal control of the chemokine source would prevent trigeminal neurons from responding to other nearby sources of Sdf1a attractant such as the telencephalon in between the two olfactory placodes (Lewellis *et al.*, 2013).

Finally, Cxcr4b/Sdf1a chemokine signalling mediates the chase behaviour in the reciprocal interactions of NCC and epibranchial placodes described by Theveneau and colleagues (Theveneau *et al.*, 2013). In this situation, placodal cells play the role of the Sfd1a source that attracts Cxcr4b-expressing NCC. In turn, contacts between NCC and placodes repel placodal cells, a process that requires Wnt-PCP and N-cadherin signalling. This mechanism ensures a constant displacement of the Sdf1a source and coordinated movements of the two adjacent cell populations, which results in the separation of the epibranchial placodal domain into distinct digit-shaped streams of cells (Theveneau *et al.*, 2013).

*Fgf signalling*

Beside its known role in placode induction, specification and differentiation (Bailey *et al.*, 2006; Freter *et al.*, 2008; Ladher, 2005; Maier *et al.*, 2010; Martin, 2006; Nechiporuk *et al.*, 2006; Nikaido *et al.*, 2007; Sun *et al.*, 2007), Fgf signalling controls some aspects of the movements and behaviours driving placode coalescence.

The Fgfr4 receptor is transiently expressed in cells of the trigeminal placode during the delamination process in chick em-

bryos. Expression of dominant negative forms of Fgfr4 prevents the delamination of placodal cells, which eventually loose their trigeminal identity and their capacity to differentiate into sensory neurons (Fig. 4) (Lassiter *et al.*, 2009). The nature and localisation of the Fgf cue controlling this process remain to be identified.

We mentioned earlier that in zebrafish, isolated placodal cells are located ahead of the posterior lateral line primordium before its migration onset. We showed that upon inhibition of Fgf signalling, these isolated cells move away from the primordium, demonstrating that Fgf activity is required for their maintenance in the vicinity of the placode. We further showed that this retention is mediated by Fgf chemotactic ligands secreted by the primordium (Fig. 4) (Breau *et al.*, 2012). This mechanism facilitates the coalescence of the placode into a compact group of migrating cells. The forward migration of the isolated cells observed in the absence of Fgf signalling is due to Cxcr4b/Sdf1a-mediated attraction, which later controls the antero-posterior migration of the primordium (Fig. 4) (Aman and Piotrowski, 2011; Ma and Raible, 2009). Thus, while Cxcr4b/Sdf1a signalling is necessary for the coalescence of several placodes, it works against the initial compaction of the posterior lateral line placode, which requires the counteracting effect of Fgf cues.

## Conclusions and future directions

In conclusion, we propose to see placode assembly as a sequence of two events, the early segregation of placodal precursors and their subsequent coalescence into discrete placodal derivatives. This view provides a comprehensive framework that facilitates our interpretation and understanding of old and novel experimental data obtained in the field of cranial placode development.

A better knowledge of the mechanisms underpinning both steps of placode assembly will require to revisit fate map studies with state-of-the-art lineage tracing tools, such as those available in the zebrafish model (Cre/Lox technology, local protein photoactivation), combined with high-resolution live imaging of behaviour, polarity/shape changes, and intracellular dynamics of placodal cells.

There is increasing evidence supporting the importance of cell movements and active retention during the secondary placode coalescence phase. Cxcr4b/Sdf1a chemokine signalling appears to be commonly used by different placodal cell types during coalescence. It will be interesting to determine whether the Fgf pathway, mostly known for its implication in early placode induction/specification or late remodelling of placodal-derived tissues (Lecaudey *et al.*, 2008; Sai and Ladher, 2008), also plays a common role in the morphogenetic mechanisms driving placode assembly. A major challenge will be to identify the intracellular regulators that translate these signals into cell shape changes and migratory behaviours, as the diversity of placode morphologies may come from differences in their way of interpreting and transducing these common cues. The discovery of new molecular players will benefit from techniques such as RNA sequencing, chemical and genetic screens, and emerging genome editing approaches using engineered endonucleases.

Finally, during neurogenic placode formation, placodal-derived neurons not only have to find their proper location, but also face the challenge of establishing appropriate connections with their peripheral and central target tissues to form functional neuronal circuits relaying sensory information to the brain. How the assembly of placodal-derived structures is coordinated with the initiation of this innervation represents another fascinating, yet unexplored question in the developmental biology of sensory organs and ganglia.

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