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## Cellular organization and boundary formation in craniofacial development

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### Abstract

Craniofacial morphogenesis is a highly dynamic process that requires changes in the behaviors and physical properties of cells in order to achieve the proper organization of different craniofacial structures. Boundary formation is a critical process in cellular organization, patterning, and ultimately tissue separation. There are several recurring cellular mechanisms through which boundary formation and cellular organization occur including, transcriptional patterning, cell segregation, cell adhesion and migratory guidance. Disruption of normal boundary formation has dramatic morphological consequences, and can result in human craniofacial congenital anomalies. In this review we discuss boundary formation during craniofacial development, specifically focusing on the cellular behaviors and mechanisms underlying the self-organizing properties that are critical for craniofacial morphogenesis.

### General introduction

Craniofacial morphogenesis is a highly dynamic and complex physical process. It requires the establishment of transcriptional identity and differentiation of cells, but also precise signaling control that organizes cells into distinct populations with boundaries between them, ultimately forming distinct craniofacial structures. A critical component of craniofacial development is the specification and migration of the cranial neural crest cells (CNCCs). These cells are multipotent progenitors that originate at the border between the neural ectoderm and non-neural ectoderm. CNCCs undergo epithelial to mesenchymal transition (EMT), enabling delamination and migration from the rhombomeres of the hindbrain to populate the branchial arches and extensively contribute to structures of the head and face. The CNCCs differentiate to form bones, cartilage, peripheral nervous system, muscles, and pigment cells. The facial prominences, populated by the CNCCs, undergo complex morphogenetic changes that require continual, tightly regulated rearrangement of cells to ensure appropriate development of the craniofacial complex. The mechanisms by which cellular organization is achieved during craniofacial development are varied and complicated and we are just beginning to understand them. *In this review, we focus on cell behaviors that organize the craniofacial complex, including cell migration, segregation, and boundary formation, and discuss what is known about the underlying mechanisms that drive these behaviors.* Ultimately, elucidating the fundamental cellular principles that give rise to

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craniofacial structure is critical to understanding the vertebrate craniofacial form and how common defects of craniofacial structure arise.

## Cell segregation and boundary formation in craniofacial development

Boundary formation, a critical organizing process for embryonic cell populations, commences from the earliest stages of craniofacial development and often occurs through cell segregation, in which cells with distinct identities or properties segregate, or sort, from each other. In the embryonic hindbrain, inter-rhombomeric boundaries partition the neural ectoderm into a series of segments that act as organizing centers along the rostrocaudal axis of the embryo neuroectoderm, organizing hindbrain development, as well as, impacting NCC organization and development. Rhombomere segmentation sets the stage for the organization of distinct populations of NCCs as they migrate to populate the craniofacial primordia. While attractive guidance directs NCCs toward the craniofacial primordia, repulsive migratory guidance maintains the stereotyped segmentation of discrete NCC streams throughout migration. The cellular mechanisms of repulsive NCC guidance and boundary formation by cell sorting have many similarities, particularly in cell behavioral mechanisms. Upon arrival of the NCCs in the pharyngeal arches (PA), continued regulation of movement or flow of NCC-derived mesenchyme culminates in distinct populations that will give rise to neuronal and glial cell types and undergo condensation to give rise to cartilage and bone. Segmental NCC migration from the developing forebrain and midbrain to populate the frontonasal region and first PA also contribute to formation of the head and face. The PAs are composed of an internal core of mesoderm surrounded by NCC-derived mesenchyme and bound externally by ectoderm and internally by endoderm. Between the PAs are the ectodermally-derived pharyngeal grooves externally, and endodermally-derived pharyngeal pouches; which also exhibit segmental organization (Frisdal & Trainor, 2014). As differentiation of NCCs to mesenchymal derivatives begins to occur, the proper organization of cells must still be maintained; for example, suture boundaries in the skull are required to prevent ectopic bone formation. Below we will discuss the developmental and cellular mechanisms of boundary formation in craniofacial morphogenesis including rhombomere boundaries, neural crest migratory guidance, pharyngeal segmentation, suture boundaries, and aberrant boundary formation. These events require highly coordinated regulation of cell fate specification, cell adhesion, actomyosin contractility and cell polarization that exhibit both similarities and differences between these distinct processes.

## Introduction to cell segregation

From early studies of developmental biology, it has been recognized that embryonic development requires the self-organization of cells into discrete regions, leading to the formation and maintenance of embryonic boundaries, preventing the intermixing of distinct cell populations. Embryonic boundaries are critical for patterning, organization, and tissue separation. Various hypotheses of the cellular mechanisms that drive cell segregation and boundary formation in different organisms and tissues have been proposed. The main cell behaviors hypothesized to underlie cell organization and boundary formation includes changes to cell adhesion, repulsion, migration and cytoskeletal dynamics such as actomyosin contractility.

The first predominant hypothesis for how cell segregation and boundary formation occurs arose from seminal work by Townes and Holtfreter in which different embryonic tissues from *Xenopus* were dissociated and subsequently mixed. These studies revealed that the cells did not remain intermixed but instead segregated into aggregates of their tissue of origin (Townes & Holtfreter, 1955). Townes and Holtfretter also observed a hierarchy of cell contact strength between cell types, where some cell types were consistently surrounded by others that sorted to the periphery (Townes & Holtfreter, 1955). These experiments gave rise to the idea of tissue affinity, describing the property of cells to recognize the identity of neighboring cells and preferentially contact “like” cells in order to re-aggregate (Townes & Holtfreter, 1955). Following the discovery of cell adhesion molecules, this idea gave way to the differential adhesion hypothesis (DAH), which proposes that cell segregation is driven by differences in adhesion between populations, achieved through the types of cadherin expressed, termed selective adhesion, or through differing levels of cadherin expression, termed differential adhesion (Fig. 1A) (Duguay, Foty, & Steinberg, 2003; Steinberg & Takeichi, 1994). This hypothesis predicts that cells will maximize their adhesive contacts to cluster hierarchically based on adhesion differences; the most adhesive cell population will cluster internally and be surrounded by less adhesive populations (Fig. 1A). Support for the differential adhesion hypothesis comes from studies demonstrating that differential cadherin expression is able to predict cell aggregation *in vitro*. L-cells, which lack endogenous cadherins, can be engineered to express different types and levels of cadherins and mixed, resulting in the aggregation of the cells expressing higher levels of cadherin in the center, while the cells with lower cadherin expression segregate to the outside of these clusters (Duguay et al., 2003; Foty & Steinberg, 2005; Steinberg & Takeichi, 1994). Indeed, differences in cadherin expression occur across embryonic boundaries relevant to craniofacial morphogenesis, such as the inter-rhombomeric boundaries (Ganzler & Redies, 1995; Inoue, Chisaka, Matsunami, & Takeichi, 1997; Matsunami & Takeichi, 1995; Nakagawa & Takeichi, 1995). However, there are very few *in vivo* examples of cell segregation and boundary formation that can be clearly attributed to differential adhesion, partly because manipulations of cadherin expression often cause catastrophic loss of tissue cohesion (Kintner, 1992; Lee & Gumbiner, 1995; Levine, Lee, Kintner, & Gumbiner, 1994). Interestingly, in several studies of *in vitro* cell segregation in which cadherin expression is disrupted, primarily through shRNA knockdown of cadherins or their regulators, segregation is either unaffected or only partially abolished (Cortina et al., 2007; Taylor et al., 2017). This suggests that while cell adhesion is capable of regulating segregation and boundary formation, it is likely not the sole driver of segregation in all systems.

Cell-cell repulsion, the collapse of cellular processes followed by migration away from the repulsive source, has also been hypothesized to drive cell segregation and boundary formation (Fig 1B) (Mellitzer, Xu, & Wilkinson, 1999; Poliakov, Cotrina, Pasini, & Wilkinson, 2008). In this model, segregation is driven by local repulsive cues, which trigger retraction and repulsive migration (Fig. 1B). Several different signaling pathways important for craniofacial development, including Eph/ephrin and neuropilin/semaphorin signaling, can mediate cellular repulsion. Eph receptor tyrosine kinases and their ephrin signaling partners are expressed throughout the development of the vertebrate craniofacial complex and often act to restrict intermingling between Eph-expressing and ephrin-expressing cells

(Bush & Soriano, 2010; O'Neill et al., 2016; Risley, Garrod, Henkemeyer, & McLean, 2009; Smith, Robinson, Patel, & Wilkinson, 1997). Eph/ephrin mediated repulsive migration is observed in cells in culture, where upon contact with an ephrin-expressing cell, the Eph-expressing cell will collapse and move away from the ephrin-expressing cell source (Astin et al., 2010; O'Neill et al., 2016; Poliakov et al., 2008). Ephs and ephrins can signal bidirectionally, with forward signaling occurring through the Eph receptor and reverse signaling occurring through the ephrin, suggesting the possibility of simultaneous bidirectional guidance (Mellitzer et al., 1999; Qiling Xu, Mellitzer, Robinson, & Wilkinson, 1999). To give rise to boundary formation by this mechanism repeated repulsion and migration of cells away from heterotypic contacts would ultimately result in the segregation of these two cell types (Fig. 1B). Semaphorin guidance molecules can be membrane-bound or tethered, providing the ability to regulate cellular guidance locally, or at a distance. Semaphorin signaling through plexin receptors and neuropilin co-receptors mediates cellular guidance through both repulsive and attractive functions mediated by changes in the cytoskeleton and cell adhesion (Tran, Kolodkin, & Bharadwaj, 2007).

A third and more recently proposed mechanism for cell segregation is the differential interfacial tension hypothesis (DITH) (Fig 1C). In addition to differential adhesion, this hypothesis incorporates differential cortical tension generated by cytoskeletal contractility, stating that both factors contribute to differences in the ability of cells to make stable contacts (Brodland, 2002; Krieg et al., 2008). Interfacial tension, or the balance of forces acting at a given interface, is frequently thought of in the context of a cell-cell interface or a cell-media interface. Whereas *in vitro* cell-media interactions involve all of the cell-non-cell interactions (e.g. substrate and liquid medium), *in vivo*, cell-medium interactions are constituted by whatever surrounds the cells that are organizing (this can be extracellular matrix (ECM), fluid, yolk, or other cells) (Cerchiari et al., 2015; Krieg et al., 2008; Maitre et al., 2012). Cell-cell interfacial tension, the force with which cells contact each other, arises through the contractile cell cortex, which is coupled to cell adhesion molecules, linking neighboring cells and resulting in modulation of cell contact at the cellular interface (Lecuit & Lenne, 2007). Thus, if a population of cells has a high cortical tension, it will minimize high-tension interactions by aggregating together, resulting in segregation of populations (Fig 1C). There is increasing evidence to support a critical role of actomyosin contractility in cellular organization by driving boundary formation. For example, differential cortical tension has been shown to drive cell segregation in zebrafish germ layer separation and mammary epithelium organization (Cerchiari et al., 2015; Krieg et al., 2008; Maitre et al., 2012). In both of these systems, adhesion alone was not predictive of cell sorting patterns, but rather the cells with the highest cell-medium interfacial tension aggregated at the center, thereby minimizing unfavorable, or high interfacial tension, interactions of cells with their surrounding media (Fig. 1C) (Cerchiari et al., 2015; Krieg et al., 2008). Additionally, in various organisms and boundary systems, including at rhombomere boundaries and aberrant boundaries in the craniofacial mesenchyme, actomyosin enrichment is observed, suggesting actomyosin contractility and differential interfacial tension may be playing a role at many boundaries in the developing embryo (Calzolari, Terriente, & Pujades, 2014; Cooke et al., 2001; O'Neill et al., 2016). Given the complexity of actomyosin cytoskeletal regulation, many of these studies have employed overexpression, pharmacological inhibition, or

dominant-negative disruption of pleiotropic factors, and many questions remain as to how these mechanisms may contribute to cellular organization *in vivo* and in what contexts these cell behaviors are contributing to cellular organization and boundary formation.

## Rhombomere organization and mechanisms of segregation

One of the best-studied examples of boundary formation, the separation between rhombomeres of the hindbrain, is particularly relevant to craniofacial development. The vertebrate hindbrain is organized into a series of 7 morphologically distinct segments, the rhombomeres, with compartment boundaries between them; this process is critical for establishing the regional identity of the hindbrain that will eventually form distinct adult brain structures (Lumsden & Krumlauf, 1996). Rhombomere boundaries also establish the NCC-segmentation patterns that will ultimately determine craniofacial organization by giving rise to skeletal elements with correct position and identity, as well as the periodic organization of neurons that innervate different facial and pharyngeal regions. The patterned induction of hindbrain positional identity by retinoic acid and Fgf signaling results in the expression of transcription factors including *Egr2/Krox20*, *Maib*, and *Hox* genes with overlapping and initially imprecisely delimited domains that presage rhombomere formation (Fig. 2A) (Tümpel, Wiedemann, & Krumlauf, 2009). Two major mechanisms contribute to rhombomere compartmentalization and boundary formation; the first involves changes in gene expression to match positional identity; the second is based on the spatial segregation of cells with distinct identities (Addison & Wilkinson, 2016). Initially, upon the generation of patterned stripes, cell intermixing persists and changes in rhombomere identity can occur through regulation of gene expression (Fraser, Keynes, & Lumsden, 1990). For example, in the mouse hindbrain, the rhombomere 3 (r3) *Egr2* expression domain normally expands anteriorly and posteriorly at the expense of neighboring territories. In the absence of *Egr2*, r3 cells acquire the identity of neighboring r2 and r4 rhombomeres instead (Voiculescu et al., 2001). Interestingly, whereas individual cells change their identity when they move between rhombomere domains, groups of cells do not, despite the change in anterior-posterior (A-P) positional information (Addison, Xu, Cayuso, & Wilkinson, 2018; Trainor & Krumlauf, 2000). *Egr2* is a critical regulator of local cell identity switching, as mosaic expression of *Egr2* within even-numbered rhombomeres causes a non-cell-autonomous increase in *Egr2* expression in neighboring cells in chick and zebrafish (Addison et al., 2018; Giudicelli, Taillebourg, Charnay, & Gilardi-Hebenstreit, 2001). A recent study has shown that this effect depends on *Egr2* repression of *Cyp26b1* and *Cyp26c1*, enzymes that degrade retinoic acid, within r3 and r5, presumably leading to a local increase in local retinoic acid levels (Fig. 2A) (Addison et al., 2018). This suggests that the gradient generated by the posterior retinoic acid source may be somewhat discontinuous across even-odd rhombomere boundaries, though such discontinuities have not yet been observed with existing reporters (Shimozono, Imura, Kitaguchi, Higashijima, & Miyawaki, 2013; White, Nie, Lander, & Schilling, 2007). Nevertheless, these findings provide important insights into a mechanism of early boundary establishment and sharpening.

In addition to the establishment of rhombomere cell identity by transcriptional regulation, cell sorting is critical for rhombomere boundary straightening and the formation of a physical barrier to cell crossing (Fig. 2A). Using clonal analysis in chick, zebrafish, and

mouse embryos, it has been shown that clones of cells do not cross rhombomere boundaries after developmental time points when regional identity has been established (Fraser et al., 1990; Jimenez-Guri et al., 2010; Qiling Xu et al., 1999). The cellular mechanisms by which rhombomere boundary segregation occurs are still under active investigation. Differential adhesion was the first mechanism proposed to drive rhombomere organization, based on studies in which cells from quail rhombomeres were transplanted into the rhombomeres of a chick host (Guthrie & Lumsden, 1991; Guthrie, Prince, & Lumsden, 1993). In these experiments, it was observed that no cell mixing occurred when cells were transplanted into an adjacent rhombomere segment, while cells from the same segment did mix (Guthrie et al., 1993). These transplant experiments demonstrated that adjacent rhombomeres are immiscible, raising the possibility that these affinities were based on a fundamental property of cells specific to each individual rhombomere such as the strength or specificity of adhesion (Guthrie et al., 1993). The degree of cell mixing varied between cells derived from rhombomeres from different A-P positions, suggesting that an adhesive code may parallel the Hox code of the hindbrain (Fig. 2A) (Redies & Takeichi, 1996). This hypothesis was consistent with studies of cadherin expression in the hindbrain showing that different rhombomere segments expressed different cadherins throughout development (Inoue et al., 1997; Nakagawa & Takeichi, 1995). R-cadherin (*Cdh4*) was shown to be expressed in a subset of rhombomeres in both chick and mouse embryos (Fig. 2A) (Ganzler & Redies, 1995; Matsunami & Takeichi, 1995). Similarly, cadherin-6 is expressed in restricted rhombomere domains and segmentally-migrating NCCs (Fig. 2A) (Inoue et al., 1997; Nakagawa & Takeichi, 1995). Further, when cells from different rhombomeres were mixed *in vitro*, they preferentially re-aggregated with cells from the same rhombomere, and upon depletion of cadherin mediated adhesion, this region-specific cell segregation was no longer observed (Wizenmann & Lumsden, 1997). Despite this evidence suggesting a role of cadherin-mediated differential cell adhesion in rhombomere segmentation, phenotypic support for this mechanism as a major driver of hindbrain segmentation is lacking.

In contrast, several studies, primarily in zebrafish, have revealed that the Eph/ephrin signaling pathway is a key regulator of rhombomere segregation. Several Eph receptor tyrosine kinases and ephrin binding partners exhibit a reciprocal pattern of expression in odd-numbered and even-numbered rhombomeres respectively (Fig. 2A) (Becker et al., 1994; Bergemann, Cheng, Brambilla, Klein, & Flanagan, 1995; Cooke et al., 2001; Xu, Allodus, Holder, & Wilkinson, 1995). EphA4 expression in r3 and r5 is directly promoted by *Egr2*, and EphB4a expression in r5 and r6 is driven by the transcription factor Val/Mafba (Fig. 2A) (Cooke et al., 2001; Theil et al., 1998). Complementary to this expression, ephrin-B3 is expressed in even-numbered rhombomeres (r2/r4/r6), ephrin-B2a (*Efnb2a*) is expressed in r2/r4/r7, and ephrin-B2b is expressed in r1 and r4 (Fig. 2A) (Addison & Wilkinson, 2016; Cooke & Moens, 2002; Cooke et al., 2001; Xu et al., 1995; Xu et al., 1999). Disruption of EphA4 signaling by expression of a truncated dominant-negative receptor resulted in cells with r3/r5 identity mislocalized within neighboring even-numbered rhombomeres. Mosaic overexpression of a cytoplasmic truncated ephrin-B2 lacking reverse signaling function resulted in exclusion of these cells from r3 and r5, and mosaic expression of truncated EphA4 lacking intracellular forward signaling function resulted in exclusion of those cells from r2/r4/r6, suggesting that bidirectional signaling is involved in their segregation (Xu et

al., 1995; Xu et al., 1999). In mouse, though ephrin-B1 does not exhibit restricted rhombomere localization, mosaic disruption of ephrin-B1 expression in the headfold at these stages resulted in cell segregation in the hindbrain, a process for which forward signaling, and not reverse, is required (O'Neill et al., 2016). In zebrafish, morpholino knockdown of EphA4 or ephrin-B2a resulted in increased intermixing of r3 and r5 cells into their neighboring rhombomeres (Cooke, Kemp, & Moens, 2005). Interestingly, simultaneous knockdown of EphA4 and ephrin-B2a resulted in a disruption of rhombomere boundaries far greater in severity than in either EphA4 or ephrin-B2a knockdowns alone, consistent with the existence of additional signaling partners for both in these rhombomere boundaries, or with the existence of receptor-ligand interaction-independent roles for these molecules (Cooke et al., 2005; Kemp, Cooke, & Moens, 2009). As it has been demonstrated that Eph receptors can hetero-oligomerize (Janes et al., 2011), it will be interesting to determine whether the formation of distinct receptor/ligand complexes in different rhombomeres and at rhombomere boundaries further increases the complexity of the Eph/ephrin code.

Based on the role of Eph/ephrin signaling in repulsive axon guidance as well as time-lapse imaging of cell movement in zebrafish rhombomeres, it was hypothesized that Eph/ephrin signaling drives repulsive interactions between adjacent Eph-expressing and ephrin-expressing cells, leading to formation and maintenance of the rhombomere compartments (Fig. 1B) (Xu et al., 1999). However, when *EphA4* knockdown cells were transplanted into wild-type zebrafish embryos, or when wild-type cells were transplanted into *EphA4* knockdown embryos, *EphA4* expressing and non-expressing cells segregated within r3 and r5, suggesting that *EphA4* expression may confer a selective adhesive property that drives boundary formation (Cooke et al., 2005). Similarly, the transplantation of *Efnb2a* knockdown cells into wild-type embryos, or *vice versa*, resulted in segregation of *Efnb2a* expressing and non-expressing cells (Kemp et al., 2009). *EphA4*- and *Efnb2a*-mediated selective adhesion functioned independent of each other (Kemp et al., 2009). This intra-rhombomeric segregation would not be predicted by mechanisms of repulsion alone, leading to the conclusion that *EphA4* and *Efnb2a* promote adhesion in addition to repulsion during rhombomere boundary formation (Cooke et al., 2005; Kemp et al., 2009).

Most recently, actomyosin contractility has also been proposed to play an important role at inter-rhombomeric boundaries. Actomyosin contractility has specifically been shown to be important in inter-rhombomeric boundary straightening and maintenance rather than in initial organization and boundary formation. Following rhombomere patterning, actin and myosin II begin to accumulate at inter-rhombomeric boundaries forming actomyosin cables (Calzolari et al., 2014). Disruption of actin or myosin II using ROCK inhibitors or blebbistatin treatment caused actomyosin cables to be dismantled, which led to jagged boundaries between rhombomeres (Calzolari et al., 2014). Conversely, calyculin A treatment, which maintains phosphorylated myosin and therefore enhances actomyosin contractility, resulted in stabilization of these rhombomere boundaries (Calzolari et al., 2014). These data suggest a critical role for actomyosin contractility in the maintenance of rhombomere compartment boundaries. It will be important to test these hypotheses using specific genetic perturbations of actomyosin contractility as this study employed only pharmacologic inhibition of actomyosin, which could lead to indirect effects. If and how actomyosin contractility plays a role in the establishment of these boundaries remains



unclear. Additionally, the relative contributions of differential adhesion, cellular repulsion, and/or actomyosin contractility to cell segregation in the establishment of the rhombomere boundaries remain unknown.

In addition to serving as physical boundaries, in many cases developmental boundaries also act as signaling centers that couple cell behaviors with patterning and cell fate specification. Specialized boundary cells at inter-rhombomere borders are critical for patterning of the hindbrain (Guthrie & Lumsden, 1991; Heyman, Faissner, & Lumsden, 1995; Xu et al., 1995). In chick, it has been demonstrated that border cells include a population of Sox2-expressing neural progenitor cells that give rise to neurons of both adjacent rhombomeres (Peretz et al., 2016). In zebrafish, these boundary cells express the chemorepellants Sema3fb and Sema3gb, which are critical for maintaining the positioning of Nrp2a-expressing neuronal populations within the rhombomere (Terriente, Gerety, Watanabe-Asaka, Gonzalez-Quevedo, & Wilkinson, 2012). Boundary cells may help to determine cell affinity properties that drive the proper segregation of more differentiated rhombomere cells. The delta ligand is expressed in cells neighboring the boundary cells and activates Notch signaling within the boundary cells. Hyperactivation of Notch signaling resulted in aberrant segregation of cells to boundaries, whereas mosaic loss of Notch signaling resulted in cells segregating away from boundaries. Notch signaling is also required to prevent premature neuronal differentiation of boundary cells, thereby coupling the regulation of differentiation with the affinity properties that define rhombomeric organization (Cheng et al., 2004).

## Cellular organization of NCCs

Neural crest cells arise at the border of the non-neural ectoderm and the neural plate, with induction of neural crest beginning at early gastrula stages and continuing through closure of the neural tube. Induction of NCCs at the neural plate border involves a host of signaling and tissue interactions, including BMP signaling as well as Wnt, FGF, and retinoic signaling (Simões-Costa & Bronner, 2015). Following induction, NCCs undergo EMT and depart from the neural tube. Directional migration of cranial NCCs to the branchial arches occurs in segmental migratory streams, followed by their entry into the branchial arches, termination of migration and differentiation. Each of these steps requires dynamic changes in cellular organization properties in order for NCCs to arrive in their appropriate destination and give rise to properly organized craniofacial elements.

## Delamination

Induction of NCCs at the neural plate border initiates a series of molecular and physical cellular changes through EMT to allow delamination and separation of NCCs from the neural tube and the adoption of a migratory phenotype. Changes in adhesion, cellular polarity and motility are tightly regulated in time and space. The changes in cadherin expression as NCCs undergo EMT are somewhat different between organisms, but have been well studied in the chick. Though initial studies posed a classical EMT view of cadherin “switching” in which E-cadherin expression is lost and N-cadherin expression is gained, more recent detailed temporal studies have demonstrated that the situation is much more complicated and nuanced (Dady & Duband, 2017; Nakagawa & Takeichi, 1995, 1998).

The early neural plate expresses E-cadherin and N-cadherin, whereas the non-neural ectoderm expresses E-cadherin but not N-cadherin (Dady & Duband, 2017). As NCC induction occurs, cadherin-6B is expressed within NC progenitor cells, which still express E-cadherin but do not express significant levels of N-cadherin. Cadherin-6B expression is initially dispersed, in a salt-and-pepper pattern, among other cells of the neural tube, but this expression resolves, presumably by partitioning from non-neural ectoderm expressing E-cadherin, but not cadherin-6B, and from N-cadherin-expressing neural plate cells (Dady & Duband, 2017). In NCC cells beginning to emigrate, E-cadherin is still expressed, though at somewhat reduced levels, and N-cadherin is still not expressed (Dady, Blavet, & Duband, 2012; Dady & Duband, 2017). The most striking change in cadherin expression through NCC delamination is in cadherin-6B, which is dramatically down regulated in migrating NCCs. As NCCs complete delamination, E-cadherin is finally lost, and the expression of cadherin 7 is dramatically up-regulated (Dady, Blavet, & Duband, 2012; Dady & Duband, 2017; Nakagawa & Takeichi, 1995, 1998). These patterns of expression suggest a tempting model in which combinatorial patterns of cadherin expression drive the segregation of the NCC from neural and non-neural ectoderm by differential affinity (Dady, Blavet, & Duband, 2012; Dady & Duband, 2017; Nakagawa & Takeichi, 1995, 1998). Unfortunately, it is not possible to discern dynamic changes in expression from cellular reorganization using static expression analysis, but future approaches utilizing live imaging or genetic lineage tracing of different cadherin-expressing populations will be of great value to answering this question. Nevertheless, it is clear that changes in cadherin expression through NCC EMT and delamination are critical, with cellular roles beyond regulation of differential affinity. Knockdown of cadherin-6B (Cad6B) in chick NCCs resulted in their premature delamination from the neural tube, while overexpression disrupted delamination with NCCs remaining clustered near the neural tube (Coles, Taneyhill, & Bronner-Fraser, 2007).

As might be expected, such dynamic regulation of cadherin-6B is complex and involves transcriptional and post-translational mechanisms. Tetraspanin18 (Tspan18) is critical to maintenance of cadherin-6B protein in premigratory NCCs (Fairchild & Gammill, 2013). When Tspan18 is lost this results in destabilization and early loss of cadherin-6B protein. Tspan18 is repressed by FoxD3, to alleviate stabilization of cadherin-6B during EMT enabling subsequent NCC migration (Fairchild & Gammill, 2013). In premigratory NCCs clathrin-mediated endocytosis and macropinocytosis also remove cadherin-6B from the cell surface (Padmanabhan & Taneyhill, 2015). Cadherin-6B down regulation is directly transcriptionally controlled both by direct suppression by the Snail2 transcription factor, and post-translationally by proteolytic cleavage by ADAM metalloproteases ADAM 10 and 19; depletion of these metalloproteases leads to the extended maintenance of cadherin-6B in the premigratory NCCs (Schiffmacher, Padmanabhan, Jhingory, & Taneyhill, 2014; Schiffmacher, Xie, & Taneyhill, 2016; Strobl-Mazzulla & Bronner, 2012; Taneyhill, Coles, & Bronner-Fraser, 2007). In addition to dismantling adherens junctions to promote delamination and migration, the cleavage of cadherin-6B results in a proteolytic product, CTF2, that functions as a transcriptional regulator to feedback and reinforce the EMT gene regulatory program (Schiffmacher et al., 2016). In addition to cadherin-6B, levels of N-cadherin must be regulated for chick NCC emigration as its overexpression prevents NCC delamination (Nakagawa & Takeichi, 1998; Shoval, Ludwig, & Kalcheim, 2007). During

NCC migration in *Xenopus*, E-cadherin expression levels are reduced, though it is still required for proper NCC migration (Huang, Kratzer, Wedlich, & Kashef, 2016). Meanwhile, in *Xenopus*, N-Cadherin expression increases upon the initiation of migration, promoting their collective migration (Huang et al., 2016; Scarpa et al., 2015). In *Xenopus* embryos, delamination of NCCs from the neural tube is therefore often referred to as partial EMT as NCCs initiate migration as a sheet rather than as individual mesenchymal cells (Sadaghiani & Thiébaud, 1987; Theveneau et al., 2010). Either overexpression or knockdown of N-cadherin blocks NCC migration, demonstrating the need for tight regulation of this cell adhesion molecule (Theveneau et al., 2010). *Xenopus* NCCs transition from collective migration to single cell migration between the neural tube and the branchial arches. A recent study sought to investigate the adhesive and mechanical changes associated with the dissociation of cells at early stages of *Xenopus* NCC migration using atomic force microscopy (AFM) (Blaue, Kashef, & Franz, 2018). These studies showed a uniform distribution of cell adhesion in NCC explants including semi-detached leader cells at the explant edge, suggesting that dissociation may not require weakening of cell-cell adhesions by changes in cadherin expression as previously hypothesized. Instead, NCC delamination requires a local decrease in tension mediated by increased expression of cadherin-11 to maximize cell-substrate contact and promote cell spreading and high substrate traction. Together these data provide a possible mechanism by which NCC cells transition from collective organization to a single-cell migratory phenotype (Blaue et al., 2018). While informative, all of these experiments were performed in *in vitro* explant culture; it will be necessary to test this role of cadherin-11 and cell-substrate traction in the dissociation of *Xenopus* NCCs in the embryo. Additionally, analyzing the mechanical changes associated with EMT and delamination of NCCs in other species will be informative to the mechanisms of NCC migration across species.

Along with changes in cadherin expression, concurrent changes in cell polarization and actomyosin contractility must also occur in the cell during NCC EMT. Interestingly, in zebrafish hindbrain, cadherin-6 has been shown to be specifically required for accumulation of F-actin in NCCs to promote their detachment, further demonstrating that in certain circumstances, cadherin expression may promote cell motility over cell aggregation (Clay & Halloran, 2014). Notably, cadherin-6 is not expressed at this stage in the midbrain, consistent with the requirement of down regulation of cadherin-6B to allow NCC delamination in the chick and emphasizing regional specific differences in regulation of NCC delamination (Coles et al., 2007). *In vivo* timelapse imaging in the zebrafish hindbrain was used to reveal a number of cell behaviors during EMT including cell rounding, membrane blebbing, and filopodial extension upon the onset of migration. Disruption of myosin or Rho-kinase (ROCK), both critical for actomyosin contractility, prevented blebbing and reduced NCC EMT and migration, demonstrating a critical role for regulation of actomyosin dynamics in NCC delamination and migration (Berndt, Clay, Langenberg, & Halloran, 2008). Further studies in zebrafish have demonstrated that Rho/ROCK activation is restricted to the apical region of NCCs by Arhgap1 and that this Rho/ROCK activation and localization is essential for detachment from the neuroepithelium (Clay & Halloran, 2013). These studies suggest that ROCK-mediated changes in actomyosin contractility drive

stereotypical cell behaviors including cell rounding and membrane blebbing that are critical for the initiation of NCC migration.

## Neural Crest Segregation

Rhombomeres compartmentalize cell lineages along the A-P axis of the hindbrain, resulting in segmentation of different NCC populations during emigration from the neural border (Minoux & Rijli, 2010). Though NCCs are not generated in a segmental pattern (Sechrist, Serbedzija, Scherson, Fraser, & Bronner-Fraser, 1993), their positionally segmented migration reflects rhombomeric boundary organization (Osumi-Yamashita, Ninomiya, Doi, & Eto, 1996). NCCs from rhombomeres r2, r4, and r6 migrate through the cranial mesenchyme in three sharp, highly stereotyped streams, avoiding the mesenchyme adjacent to r3 and r5 (Fig. 2B) (Lumsden, Sprawson, & Graham, 1991). Some NCCs from r3 and r5 undergo apoptosis, the rest migrate to join with NCCs generated in more rostral and caudal rhombomeres (Farlie et al., 1999; Graham, Heyman, & Lumsden, 1993; Kulesa & Fraser, 1998).

The receptor tyrosine kinase gene *ErbB4* is expressed in rhombomeres r3 and r5, initially within the neuroectoderm, and shifting to the pial surface at these rhombomere boundaries. Its loss non-autonomously allowed invasion of transplanted wild-type r4 NCCs destined for branchial arch 2 (BA2) into the mesenchyme adjacent to r3, leading ultimately to inappropriate r4-derived NCC contribution to BA1 (Gassmann et al., 1995; Golding, Trainor, Krumlauf, & Gassmann, 2000). In contrast, the neural crest-free boundary adjacent to r5 does not require *ErbB4* expression for its maintenance, and instead is regulated by unknown factors from the surface ectoderm overlying r5 (Golding et al., 2004). The *Xenopus* hindbrain is more compressed along the anterior-posterior axis and NCC-free zones are not observed. However, hindbrain origin position is maintained between streams, which may indicate that NCC-free zones are not a general requirement for segmentation of the migratory neural crest (Farlie et al., 1999).

Though rhombomere segmentation is necessary for normal initial NCC segmental migration, rhombomeres do not provide the only segmental cues as surgical removal of r3 resulted in invasion of NCCs into r3-adjacent mesenchyme, but maintenance of NCC segmental migration more ventrally (Golding, Dixon, & Gassmann, 2002; Golding et al., 2004). As in rhombomere boundary segregation, the Eph/ephrin signaling family has been implicated in maintaining segmented NCC streams (Fig. 2B). In *Xenopus*, the rhombomeric patterns of Eph/ephrin expression are extended into the migratory NCC streams such that EphA4 expression in r3 and r5 is maintained in r5-derived NCCs migrating toward the third arch, and EphB1 is expressed in NCCs migrating toward the third and fourth branchial arches (Fig. 2B) (Smith et al., 1997). Ephrin-B2, in contrast, is expressed in mesoderm along the migration pathway in a complementary pattern during NCC migration, consistent with the known roles of Eph/ephrin signaling in repulsive migration. Inhibition of EphA4 or EphB1 by overexpression of a dominant-negative mutant receptor resulted in expansion of the r5 NCC stream both rostrally and caudally from the outset of NCC emigration, with misguidance into second and fourth arch territories. Overexpression of ephrin-B2 to ectopically activate signaling resulted in the invasion of NCCs into ectopic sites (Smith et

al., 1997). The fact that EphA4 and ephrin-B2-expressing cells come into contact in the hindbrain and during early NCC migration, but are separated during migration into the arches, suggests that the NCC segmentation function of Eph/ephrin signaling occurs early; to what extent these functions may be related to even earlier disruption of rhombomere boundaries is not clear, but the fact that overexpression of ephrin-B2 can lead to a variety of redirections of the NCCs indicates that Eph/ephrin signaling is capable of redirecting NCCs relatively late in their migration. Though loss of function of ephrin-B2 in mice also results in disruption of NCC development and a hypoplastic second branchial arch, this phenotype is attributable to a role for ephrin-B2 within the vascular endothelium for NCC survival rather than migratory guidance (Davy & Soriano, 2007; Lewis, Hwa, Wang, Soriano, & Bush, 2015). It is possible that redundancy in function may explain the lack of an obvious guidance phenotype in ephrin-B2 loss of function models. Defects in migration of NCCs have been documented upon loss of the related ephrin-B1 in mice, as NCCs destined for BA3 and 4 inappropriately intermix upon complete loss of ephrin-B1 or its loss specifically from NCCs (Davy, Aubin, & Soriano, 2004). Several Eph receptors are also expressed in NCCs in mouse, though they are generally not as strikingly segmentally restricted to migratory NCC populations as in *Xenopus* (Adams et al., 2001; Agrawal, Wang, Kim, Lewis, & Bush, 2014; Gale et al., 1996).

Both embryological and genetic support exists for the role of Semaphorin/ Neuropilin/ Plexin signaling in cranial NCC segmentation from the earliest stages. *Sema3A* and *Sema3F* exhibit restricted expression of variable levels within r1, r3 and r5 (Fig. 2B). The *Npn1* and *Npn2* co-receptor genes are expressed in NCC streams in the periorbital region and streams derived from r2, r4 and r6; the Plexin-A1 receptor is expressed within NCCs migrating from r4 (Fig. 2B) (Eickholt, Mackenzie, Graham, Walsh, & Doherty, 1999; Gammill, Gonzalez, & Bronner-Fraser, 2007; Melendez-Herrera & Varela-Echavarria, 2006; Osborne, Begbie, Chilton, Schmidt, & Eickholt, 2005; Yu & Moens, 2005). In chick, implantation of *Sema3A*-soaked beads in the hindbrain prevented NCC emigration, whereas expression of a Neuropilin-Fc signaling competitor resulted in invasion of NCCs into areas normally inhibitory to their migration (Osborne et al., 2005). Likewise, loss of *Npn2* or *Sema3F* in mouse and zebrafish resulted in loss of sharp NCC boundaries, with bridges of cells crossing over between NCC streams 1 and 2 (Gammill et al., 2007; Yu & Moens, 2005). Whereas no skeletal defects were observed in mouse mutants lacking *Sema3F* or *Npn2*, consistent with the ability of NCCs to adopt the identity of their new position, the trigeminal ganglia was less condensed, and defects in the fasciculation of trigeminal nerve branches occur at later stages in *Npn2* null mice (Gammill et al., 2007; Giger et al., 2000). Interestingly, in the basal vertebrate, the lamprey, *Sema3F/Npn* signaling does not work to regulate segmental migratory guidance; instead, *Sema3F* functions in the positioning of NCC derivatives including pigment, cranial sensory neurons, and elements of the head and pharyngeal skeleton (York, Yuan, Lakiza, & McCauley, 2018). This suggests that during evolution, the roles for this pathway in the segmental organization of the head have changed, allowing rearrangement of the vertebrate head skeleton (York et al., 2018).

The transcriptional control of NCC guidance factors is beginning to be understood as well. In mutant embryos, lacking T-box transcription factor, *Tbx1*, migratory streams are maintained until entry of NCCs into the branchial arches, at which time r4-derived NCCs

inappropriately invade the first branchial arch, which may explain the cranial nerve fusions and skeletal anomalies that arise in these mutants (Moraes, Nóvoa, Jerome-Majewska, Papaioannou, & Mallo, 2005; Vitelli, Morishima, Taddei, Lindsay, & Baldini, 2002). *Tbx1*, which is expressed in the branchial arch mesoderm, endoderm, and ectoderm but not neuroectoderm or NCCs, is required for normal levels of *Fgf8* expression within the branchial arch ectoderm, providing one mechanism by which *Tbx1* may non-autonomously regulate NCC development (Chapman et al., 1996; Garg et al., 2001; Vitelli et al., 2002). However, though *Fgf8* is important for NCC survival and branchial arch formation, it does not appear to directly regulate NCC segmentation, as hypomorphic loss of *Fgf8* did not result in defects in the segmentation of NCC cells (Abu-Issa, Smyth, Smoak, Yamamura, & Meyers, 2002).

More recently, it has been demonstrated that in *Tbx1*<sup>-/-</sup> mutant mouse embryos, expression of the chemoattractant *Sdf1* is reduced in the pharyngeal endoderm, and expression of *Cxcr4*, its receptor, is reduced within NCCs, suggesting that *Tbx1* may regulate *Sdf1* to properly guide NCCs into the arches (Escot et al., 2016). Indeed, disruption of *Sdf1/Cxcr4* signaling results in NCC guidance defects in chick, *Xenopus* and zebrafish (Escot et al., 2016; Olesnicki Killian, Birkholz, & Artinger, 2009; Theveneau et al., 2010). Rather than acting as a repulsive cue to maintain NCC segmentation, *Sdf1* promotes directional polarization of neural crest cells expressing *Cxcr4* by directionally stabilizing protrusions following NCC contacts (Theveneau et al., 2010). In *Xenopus* and zebrafish, *Sdf1* is expressed in the pre-placodal region at the border of the neural plate before NCC migration begins and is later restricted to discrete domains corresponding to individual placodes. The expression of *Sdf1* attracts NCCs, while in turn the physical NC-placode contact directionally displaces the placode, which remains segregated from the NCCs in a “chase and run” mechanism (Theveneau et al., 2013).

## NCC Migration

The directed segmental migration of NCCs to the PAs involves multiple signaling pathways that coordinate complex cell behaviors. A well-established mechanism for how directed migration occurs is commonly referred to as contact inhibition of locomotion (CIL), which is also a mechanism for boundary formation and maintenance (Fig. 3B, C). CIL encompasses a number of constituent cell behaviors in which two cells come into contact and either cease movement or undergo active directional migration away from each other (Fig. 3B,C). The specific cellular details of CIL events can vary significantly, leading to the description of a variety of CIL subtypes (Martz & Steinberg, 1973; Stramer & Mayor, 2017). Whereas type I CIL describes the situation in which the leading edge of a cell undergoes contraction upon contact with another cell, type II CIL is essentially a case of differential adhesion, wherein another cell's surface is less adhesive than the substrate, causing the cell to prefer not to migrate over the other cell (reviewed in Stramer & Mayor, 2017). It is notable, however, that neither CIL type specifies what happens following the contact, though type I CIL has been associated with active movement away from a collision partner and type II CIL has been considered as a passive response that stops cell movement (Fig. 3B) (Stramer & Mayor, 2017).

CIL behavior was first described in fibroblasts (Abercrombie & Heaysman, 1953) and has since been observed in other cell types, including *Xenopus* NCCs in culture and *in vivo* (Carmona-Fontaine et al., 2008). Time-lapse imaging revealed *Xenopus* NCCs making contact, collapsing protrusions and changing the direction of their migration, while NCCs encountering another cell type did not demonstrate these behaviors and invaded the neighboring tissue (Carmona-Fontaine et al., 2008). The cumulative effect of CIL interactions within a NCC stream is the coordinated directional polarization and migration of the cells within that stream. Cell contact and intercellular communication is required during CIL prior to detachment, repolarization and movement away (Fig. 3B, C). Both N-cadherin and cadherin-11 are essential for proper CIL behavior in *Xenopus* NCCs, with disruption of either cadherin leading to loss of CIL and non-directional migration (Becker, Mayor, & Kashef, 2013; Theveneau et al., 2010). At cell-cell contacts, N-cadherin signaling function inhibits Rac1 activity and thus inhibits protrusions while promoting Rac1 activation and protrusions at the cellular free edge (Theveneau et al., 2010). Recently, it was shown that N-cadherin expression is dependent on PDGFR $\alpha$ /PDGF-A signaling (Bahm et al., 2017). The PDGFR $\alpha$  receptor tyrosine kinase and its ligand PDGFA are co-expressed in CNCCs, and their inhibition prevents N-cadherin expression, thus resulting in a loss of CIL and inhibiting NCC migration (Bahm et al., 2017). This pathway therefore achieves cell-autonomous regulation of CIL by upregulating N-cadherin during EMT. A role for PDGF signaling in NCC migration has been demonstrated in zebrafish, mice and *Xenopus*, suggesting this may be a conserved mechanism for driving the directional migration of NCCs (Bahm et al., 2017; Eberhart et al., 2008; He & Soriano, 2013; Tallquist & Soriano, 2003).

Unlike N-cadherin, cadherin-11 localizes to cellular protrusions such as lamellipodia and filopodia, and is necessary for their protrusion formation and normal NCC migration (Kashef et al., 2009). While the cytoplasmic tail of cadherin-11 has been shown to drive this protrusive phenotype, specifically reducing cadherin-11s adhesive function results in loss of CIL behavior and increased invasiveness (Becker et al., 2013; Kashef et al., 2009). As in NCC delamination, post-translational regulation of cadherin expression and function is critical for regulation of CIL. Cadherin-11 is regulated through cleavage by ADAM13, and this cleavage, which creates the extracellular fragment EC1–3, is essential for NCC migration (Abbruzzese, Becker, Kashef, & Alfandari, 2016; Cousin, Abbruzzese, McCusker, & Alfandari, 2012). In cells expressing a non-cleavable variant of cadherin-11, migration is inhibited, and migratory defects can be rescued by expression of the EC1–3 cleavage product (Abbruzzese et al., 2016). It was recently shown that the EC1–3 cleavage product stimulates phosphorylation of AKT through interactions with ErbB2, which is necessary for NCC migration (Mathavan, Khedgikar, Bartolo, & Alfandari, 2017). These studies indicate that cadherin-11 cleavage products have a signaling function in regulating NCC migration, further demonstrating the diverse and complex ways in which cadherins regulate NCC migration.

The repolarization of cells after contact is a critical component of NCC directional migration. In addition to N-cadherin signaling inhibiting Rac, an increase in RhoA activity at the site of cell-cell contact, regulated by the planar cell polarity (PCP) pathway is required for CIL (Matthews et al., 2008). RhoA and the proteoglycan Syn4, a proposed regulator of

cell migration, inhibit Rac at the site of contact (Carmona-Fontaine et al., 2008; Matthews et al., 2008; Theveneau et al., 2010). This activation of RhoA at the site of contact, along with strong inhibition of Rac, ultimately promotes the formation of directional protrusions away from the site of contact. Further, in both *Xenopus* and chick, this pathway was shown to act through the actin binding protein calponin2 (Cnn2), which localizes to the leading edge of NCCs (Ulmer et al., 2013). Cnn2 knockdown results in random protrusion formation and migratory defects, suggesting an important role for Cnn2 in polarizing the actin cytoskeleton, promoting protrusion formation, and the formation of directional protrusions for directional migration (Ulmer et al., 2013). Knockdown of Cnn2 rescued migratory defects observed as a result of loss of Wnt signaling and ROCK, suggesting Cnn2 is acting downstream of these pathways in NCC migration (Ulmer et al., 2013). Along with inhibition of Rac1 at the contact edge, an increase in Rac1 activity is required away from the contact to drive cellular repolarization and lamellipodia formation (Scarpa et al., 2015). In cells expressing E-cadherin, which do not separate upon contact, stimulation of protrusion formation through Rac1 was sufficient to induce the separation of these cells, suggesting that this repolarization and protrusion formation at the new leading edge is sufficient to tear the adhesions at the edge of contact and lead to separation of these two cells after collision (Scarpa et al., 2015).

It has been suggested that CIL behavior alone would result in the spreading of migrating neural crest cells rather than maintenance of migratory streams. However, a mechanism of mutual cell-cell coattraction could counterbalance the tendency of cells to disperse through mechanisms such as CIL. In *Xenopus*, it has been shown that NCCs are attracted to one another through the complement fragment C3a and its receptor C3aR (Carmona-Fontaine et al., 2011). Loss of coattraction through antibodies blocking C3a/C3aR signaling disrupted coordinated movements of the NCCs. This disruption of movement, however, occurred in a variable fashion ranging from slight disruption of migratory streams to complete disorganization and lack of migration. These findings led the authors to propose a migratory mechanism in which coattraction and CIL form a balance to allow cells to self-organize and migrate (Carmona-Fontaine et al., 2011). Mathematical modeling of these two parameters of coattraction and CIL demonstrated how this balance could result in maintenance of the directionality of migration and recapitulated many properties of NCC migration *in vitro* and *in vivo* (Woods et al., 2014).

While CIL behavior and coattraction occur in the migration of NCCs in both amphibians and zebrafish (Carmona-Fontaine et al., 2011), there is some question as to whether this CIL mechanism occurs in all species or even if collective migration occurs in NCCs of all species. In chick, detailed imaging analysis has shown that chick NCCs maintain cell-cell contacts through dynamic long- and short- range filopodial protrusions while migrating (Teddy & Kulesa, 2004). Cell-cell contact through these protrusions was shown to result in a cell changing direction to favor the neighboring cell's path suggesting that protrusions may mediate cell communication to refine directionality within the migratory stream (Teddy & Kulesa, 2004). Further, it was noted that cell morphology and protrusion dynamics differ between regions within the neural crest migratory streams, with cells at the leading edge being non-polar, containing many protrusions, and cells away from the leading edge displaying a bipolar morphology (Teddy & Kulesa, 2004). A recent study from Genuth et al.



also using detailed live imaging of NCCs *in vivo* in the avian embryo to analyze protrusion dynamics made somewhat different conclusions, though differences in stages of analysis, and methods of imaging and quantification exist between the two studies (Genuth, Allen, Mikawa, & Weiner, 2018; Teddy & Kulesa, 2004). Notably, Genuth et al. did not observe differences in protrusion dynamics dependent on cell positioning within the migratory stream. Further, the authors of this study showed that chick NCCs have a weak spatial bias in the generation of filopodial protrusions followed by a strong spatial bias in the generation of large protrusions in the direction of movement (Genuth et al., 2018). These findings differ from those in *Xenopus*, in which NCCs undergo co-attraction and CIL, with cells migrating as a stream with only edge cells extending protrusions (Carmona-Fontaine et al., 2008). Additionally, in contrast to the CIL model of migration where cell-cell contact results in protrusion collapse, Genuth et al., observed that in the majority of migratory NCCs, protrusions continued to be extended after contact with another cell, and these cells maintained a forward trajectory following cell-cell contact. Though a thorough test of the ability of chick NCCs to undergo CIL, would require examination of cell behaviors in a lower density context, these findings nevertheless suggest a different mechanism from CIL in which chick NCCs migrate through a search and polarity refinement mechanism. Additional mechanistic studies will be necessary to determine the role of protrusion based cell-cell contacts in NCC migration as well as the role of these protrusions in sensing the local environment, and how these inputs are coordinated to result in directional collective migration.

How this migration occurs in mouse has yet to be determined, but differences between *Xenopus* and mouse NCC migration mechanism have already been noted. Mouse NCC migration was shown to occur independently of PCP signaling, which is essential in both *Xenopus* and zebrafish (Pryor et al., 2014). Using *Vangl2<sup>Lp/Lp</sup>* mice, which lack PCP signaling, the authors showed that despite neural tube closure defects and lack of PCP signaling, normal NC specification, migration, and derivative formation occurred in these embryos, suggesting that this signaling pathway is dispensable for segmental NCC migration in mice (Pryor et al., 2014). The pathways critical for polarization in NCC migration in mice are not clear, but these discrepancies between species suggest that control of NCCs migration may be achieved by multiple mechanisms *in vivo*.

As a mechanism of developmental boundary formation, CIL behavior has parallels to cell segregation mechanisms but also has important differences. First, the cell-cell repulsion model that has been proposed to drive segregation could be considered a general subtype of CIL (Fig. 3A, B). Indeed, it has been demonstrated that Eph/ephrin signaling, a potent driver of cell segregation by cellular repulsion (Poliakov et al., 2008), does indeed also activate CIL in some cell types (Astin et al., 2010; Villar-Cerviño et al., 2013). While both CIL and repulsive cell sorting result in the migration of two cells away from one another following contact, directional Rac1 repolarization away from the site of contact has been demonstrated for CIL but has not been specifically shown for repulsive migration related to cell segregation (Fig. 3A,B). Second, whereas cadherins drive cell segregation by differential affinity mechanisms that promote homotypic adhesion, homotypic cadherin interactions are actually required for repulsive interactions in CIL (Fig. 3B). Indeed, most examples of CIL in the developing embryo involve homotypic repulsion, while cell segregation by repulsion

is by definition between heterotypic cellular contacts. Finally, it has been demonstrated that actomyosin contractility is required for Eph/ephrin mediated cellular collapse (Prosperi et al., 2015), whereas upon treatment with blebbistatin to inhibit myosin contractility, NCCs were still able to switch polarity, detach and migrate, suggesting that CIL behavior is not dependent upon myosin contractility (Kadir, Astin, Tahtamouni, Martin, & Nobes, 2011). Instead, RhoA activation is necessary for cellular repolarization by inhibition of Rac1 in CIL; though it has not yet been determined whether local regulation of Rac1 activity is required for cell segregation, its pharmacological inhibition did not disrupt Eph/ephrin-mediated cell segregation, suggesting that Rac1-mediated repolarization is most likely not required (O'Neill et al., 2016). Therefore, whereas CIL, as most commonly described, is a potent regulator of cellular organization, it is distinct from other modes of cellular segregation and boundary formation, though they share some cell biological characteristics.

It is important to note that segmentation of the PAs and intervening pharyngeal pouches, out-pocketings of the foregut endoderm that help to organize development of the head and neck, does not solely rely on maintenance of distinct NCC-migratory streams, but also heavily involves the endoderm. In *Tbx1*<sup>-/-</sup> mice and mice lacking *Tbx1* specifically from the endoderm, pharyngeal pouches fail to evaginate from the foregut endoderm. Loss of *Fgf3* and *Fgf8* from the pharyngeal endoderm only partially disrupted pouch morphogenesis indicating that *Fgf* signaling is not required for pouch formation (Jackson, Kasah, Mansour, Morrow, & Basson, 2014). However, *Fgf8* is required, together with *Fgf3*, for segmentation of the pharyngeal endoderm into pouches in zebrafish (Crump, Maves, Lawson, Weinstein, & Kimmel, 2004). Notably, initial rhombomere organization again plays an important role, as *Fgf8* and *Fgf3* are segmentally restricted within the midbrain/hindbrain boundary and r4, and expression from the neural tube as well as the mesoderm was required for normal early pharyngeal pouch segmentation (Crump et al., 2004; Maves, Jackman, & Kimmel, 2002). Eph/ephrin signaling also has a role in pharyngeal morphogenesis beyond guidance of NCCs. In zebrafish, Eph/ephrin expression regulates morphogenesis of the pharyngeal pouches. Signaling between *Efnb2a/Efnb3b* and EphB4a within the pouch endoderm is required to increase intercellular adhesion to regulate segmental pouch outgrowth (Choe & Crump, 2015). Finally, Wnt signaling is an important pathway for endoderm segmentation and pouch formation (Choe et al., 2013). *Wnt11r*, expressed in discrete domains of the head mesoderm, along with Rac1 are important to initial outgrowth of the pouch forming cells (Choe et al., 2013). Later, *Wnt4a*, displaying segmental expression in the head ectoderm, and Cdc42 signaling are required to organize the developing pouch (Choe et al., 2013). Requirement for both *Wnt11r* and *Wnt4a* in pharyngeal pouch morphogenesis suggests roles for both the mesoderm and ectoderm in segmentation of the pharyngeal pouches.

## Boundaries in skull vault development

Later in craniofacial development, tissue boundaries are critical for establishing normal skeletal structure. The skull vault develops from neural crest and paraxial mesoderm-derived cells that do not mix during development. Instead, they maintain a boundary that first appears in mice at E9.5 and remains a distinct interface through the neonatal stage (Fig. 2C) (Chai et al., 2000; Jiang, Iseki, Maxson, Sucov, & Morriss-Kay, 2002). The transcription factor Twist1 is a key regulator of mesoderm formation and maintenance of NCC/mesoderm

boundaries. In mice, homozygous loss of *Twist1* results in NCC invasion into the paraxial mesoderm, and loss of *Twist1* specifically within the mesoderm led to an invasion of mesenchyme into the NCC-derived ganglia (Bildsoe et al., 2013; Soo et al., 2002). Though the cellular mechanisms by which NCC/mesoderm intermixing is prevented are unknown, it is notable that in chimera experiments in mice, *Twist1*<sup>-/-</sup> head mesenchyme cells strikingly segregate from wild-type cells (Chen & Behringer, 1995). Interestingly, in epithelial cell lines, Twist-1 promotes EMT through repressing E-cadherin resulting in a loss of E-cadherin-mediated cell-cell adhesion, suggesting that Twist1 may regulate adhesion differences to drive cell segregation (Yang et al., 2004). Further, Twist1 loss in the coronal suture results in reduced expression of ephrin-A2, ephrin-A4, and EphA4 suggesting another possible mode by which Twist might regulate segregation (Ting et al., 2009). It will be extremely interesting to determine the expression profiles of drivers of cell segregation such as Eph/ephrins and cadherins in *Twist1*<sup>-/-</sup> head mesenchyme cells.

In mammals, the neural crest/mesoderm boundary will ultimately coincide with the coronal suture, with NCC-derived cells forming the frontal bones and mesoderm-derived cells forming the parietal bones and coronal suture mesenchyme (Fig. 2C) (Jiang et al., 2002; Merrill et al., 2006; Yoshida, Vivatbutsi, Morriss-Kay, Saga, & Iseki, 2008). Calvarial sutures are fibrous joints that allow passage through the birth canal and accommodate the growth of the underlying brain while preventing the premature fusion of the calvarial bones of the skull (Ishii, Sun, Ting, & Maxson, 2015). The suture serves as a growth center to regulate the proliferation and differentiation of osteoprogenitors in the appositional growth of the calvaria during development and houses the mesenchymal stem cells that are the main progenitor population for craniofacial bones during postnatal growth (Zhao et al., 2015). An overabundance of NCCs, such as in mouse embryos lacking the ciliary protein Fuz, drives an expansion of the NCC-derived frontal bone at the expense of the mesoderm-derived parietal bone, but not an intermixing between these populations (Tabler, Rice, Liu, & Wallingford, 2016). In contrast, mice with mutations in engrailed 1, a protein that plays a role in lineage boundaries in multiple contexts (Araki & Nakamura, 1999; Dahmann & Basler, 2000) show premature migration of neural crest-derived cells into the coronal suture territory, resulting in a shifted mesoderm/NCC boundary (Deckelbaum et al., 2012). Thus, in this context, engrailed 1 appears to be an important regulator of cell movement and therefore boundary formation at this interface.

The mesenchyme precursors that generate the coronal suture originate from the cephalic paraxial mesoderm cells that migrate to establish a lineage boundary with the neural crest derived mesenchyme (Deckelbaum et al., 2012). As at earlier stages, Twist1 is a key regulator of suture boundaries; *Twist1*<sup>+/-</sup> mice exhibit coronal synostosis (a premature fusion of the frontal and parietal bones), with inappropriate invasion of NCC-derived mesenchymal cells into the mesoderm-derived coronal suture mesenchyme (Carver, Oram, & Gridley, 2002; Merrill et al., 2006). This suture boundary is apparently distinct from the earlier NCC/mesoderm boundary, as NCC invasion into mesoderm-derived tissues in *Twist1*<sup>+/-</sup> embryos was not observed prior to the establishment of the suture at E14.5 (Merrill et al., 2006). Other studies have proposed that the suture boundary may actually be unidirectional in nature, preventing NCC mixing into the suture and parietal bone, while allowing a small number of Mesp1-cre lineage mesoderm cells to contribute to the frontal

bone (Deckelbaum et al., 2012). Nevertheless, an important function of Twist1 at this suture boundary is to prevent aberrant cell intermixing, which is achieved in part by the regulation of Eph/ephrin-mediated cell segregation; expression of ephrin-A2, ephrin-A4 and EphA4 were reduced in *Twist1*<sup>+/-</sup> sutures, and loss of signaling through EphA4 resulted in partial suture fusion (Merrill et al., 2006; Ting et al., 2009). Though little is known about the cell behaviors involved in Eph/ephrin segregation at the suture boundary, it is notable that Eph/ephrin signaling can regulate boundary formation in contexts as distinct as the suture mesenchyme and hindbrain neuroepithelium, underlining the fact that the Eph/ephrin signaling pathway is a powerful regulator of cell segregation independent of cell type and developmental context. Eph/ephrin signaling also impacts calvarial bone formation by regulation of gap junction communication, providing a potential mechanism by which suture boundary formation and regulation of bone formation might be coupled (Davy, Bush, & Soriano, 2006).

Disruption of coronal suture boundaries was also observed in mice lacking the Notch ligand Jagged1 from the suture mesenchyme, which exhibit invasion of mesoderm-derived cells into the frontal bone (Yen, Ting, & Maxson, 2010). The cellular mechanisms by which Jagged1 signaling regulates suture boundary formation are not yet known and it is not clear whether disruption of Jagged1 signaling leads to loss of boundaries by aberrant cell segregation or a change in cell fate specification. Twist1 also regulates Jagged1 expression in the suture, and compound loss of Twist1 and Jagged1 resulted in a more severe craniosynostosis phenotype. *Twist1*<sup>+/-</sup> adult mice also exhibit a reduction in Glil-expressing MSCs in their sutures, consistent with the long-term importance of establishing developmental boundaries (Zhao et al., 2015). Together, these studies put *Twist1* at the top of a regulatory hierarchy for the establishment and maintenance of normal coronal suture boundaries. Though the concurrence of the coronal suture with the NCC/mesoderm boundary in mice provides powerful Cre-recombinase genetic tools for observing and manipulating the coronal suture boundary, this coincidence may not be generally significant. In fact, there are species-specific differences in the location of this boundary; in chick, *Xenopus* and zebrafish, the neural crest/mesoderm boundary occurs within the frontal bone, and the coronal suture occurs between bones of mesodermal origin (Fig. 2C) (Kague et al., 2012; Matsuoka et al., 2005; Piekarski, Gross, & Hanken, 2014). Nevertheless, loss of Twist1 and its partner Tcf12 in zebrafish results specifically in coronal synostosis by a directional acceleration of bone production and exhaustion of coronal suture progenitor cells (Teng et al., 2018). These findings indicate that what is unique about the coronal suture is not related to embryonic origin, but rather that boundary maintenance can be achieved by exquisite control of directional growth dynamics. It will be exciting to determine whether other sutures also exhibit boundary characteristics, such as restriction of mesenchymal intermixing.

## Cell segregation in craniofacial dysmorphology

Generally, it is difficult to determine to what extent human craniofacial conditions explicitly result from disruption of developmental boundaries, though a few examples exist. As discussed above, disruption of suture boundaries is likely to contribute to coronal synostosis upon heterozygous loss of function of *Twist1* in Saethre-Chotzen syndrome (Howard et al., 1997). Similarly, mutation of *EFNA4* has been identified in humans with coronal synostosis

(Merrill et al., 2006). Craniofrontonasal syndrome (CFNS) is caused by mutations in *EFNB1*, a gene found on the X-chromosome that encodes the EPHRIN-B1 signaling protein (Twigg et al., 2004; Wieland et al., 2004). As *EFNB1* is an X-linked gene, heterozygous female CFNS patients harbor cellular mosaicism for *EFNB1* mutation due to random X-inactivation, and thus mosaic expression of EPHRIN-B1. CFNS results in coronal craniosynostosis, hypertelorism, frontonasal dysplasia and cleft lip and palate, affecting females heterozygous for *EFNB1* mutations more severely than hemizygous males with no functional *EFNB1*, suggesting that mosaicism underlies disease severity. Indeed, males with somatic mosaic *EFNB1* mutations, resulting in mosaic EPHRIN-B1 expression, also exhibit severe phenotypes similar to heterozygous females, supporting that mosaicism for EPHRIN-B1 underlies the disease phenotype (Twigg et al., 2013). *Efnb1*<sup>+/-</sup> mice exhibit many of the same craniofacial phenotypes as CFNS patients, and mosaicism for ephrin-B1 expression results in aberrant segregation of cells in the neuroepithelium and the appearance of ectopic ephrin-B1 boundaries in NCC-derived mesenchyme of the craniofacial region (Fig. 2D) (Bush & Soriano, 2010; Compagni, Logan, Klein, & Adams, 2003; Davy et al., 2006). Cell segregation also occurs in patient hiPSC-derived neuroepithelial cells, supporting the relevance of aberrant segregation in human CFNS (Niethamer et al., 2017). The CFNS disease model has also been instructive in studying the molecular and cellular mechanisms by which Eph/ephrin-mediated segregation and boundary formation may occur more generally *in vivo*. For example, whereas bidirectional signaling has previously been associated with cell segregation, mouse genetics approaches demonstrated that unidirectional forward signaling is necessary and sufficient for cell segregation in this context (O'Neill et al., 2016). Cell segregation in *Efnb1*<sup>+/-</sup> embryos required ROCK function, but not the function of Cdc42 or Rac1, indicating that though actomyosin contractility is required for segregation, repolarization of cells by Rac1 or Cdc42 is not required. These data support a model in which unidirectional signaling influences cortical actomyosin contractility to drive segregation (O'Neill et al., 2016). How aberrant cell segregation and ectopic boundaries ultimately disrupt craniofacial morphogenesis remains to be determined.

## Conclusion

The cellular behaviors underlying boundary formation in craniofacial morphogenesis are complex and only beginning to be uncovered. While rhombomere boundaries and neural crest migratory streams have been well-studied, much remains to be learned regarding the mechanisms of regional identity plasticity as well as the molecular and physical mechanisms driving cell segregation and how these are coupled to regulate boundary formation.

Rhombomere boundaries serve as critical organizational centers, segregating the neural ectoderm into segments and establish the initial patterning for NCC migration and pharyngeal morphogenesis. The migration of NCCs in distinct migratory streams to populate specific regions of the developing head and face are critical for proper morphogenesis. These processes begin at the earliest stages of craniofacial development, and though incompletely understood, we have a considerable amount of data on the cell behaviors underlying cell segregation and boundary formation at the early stages of craniofacial development. In contrast, we have very little information on post-migratory cellular organization of the

craniofacial mesenchyme, though studies of craniofacial dysmorphology emphasize the continual importance of proper boundary formation and tissue flow. Whether and how cell segregation acts throughout later stages of craniofacial morphogenesis to enable proper cellular organization therefore remains an open question. Many commonalities exist between the mechanisms establishing different boundaries throughout craniofacial development, and each utilize an overlapping toolkit of cellular mechanisms that includes cell-cell adhesion and actomyosin cytoskeletal dynamics to regulate cell migration, cell polarization and interfacial tension to achieve craniofacial organization. Many cell behaviors discussed here have been studied *ex vivo* or in cell culture contexts; for many such cell behaviors, such as how actomyosin-mediated cortical tension influences the strength of cell contacts in NCC EMT, remains unknown. Further, it is important to consider that each of these mechanisms has broad pleiotropic roles; for example, cadherin regulation of cell behavior goes far beyond cell-adhesion function and we are just beginning to uncover the detailed molecular mechanisms regulating, and regulated by cadherins in these cellular organization processes. It is unlikely that there is a universal mechanism governing self-organization in different cell types; rather, multiple mechanisms likely influence the physical properties of cells to achieve different organization according to a few basic principles, including those described above. Indeed, the examples discussed here are likely just a few of the boundaries that contribute to craniofacial development; for example, relatively little is understood about how NCC-derived mesenchymal populations generate boundaries for the formation of distinct skeletal elements. A detailed mechanistic understanding of the organizational principles that underlie craniofacial morphogenesis is critical to understanding how this complex process occurs.

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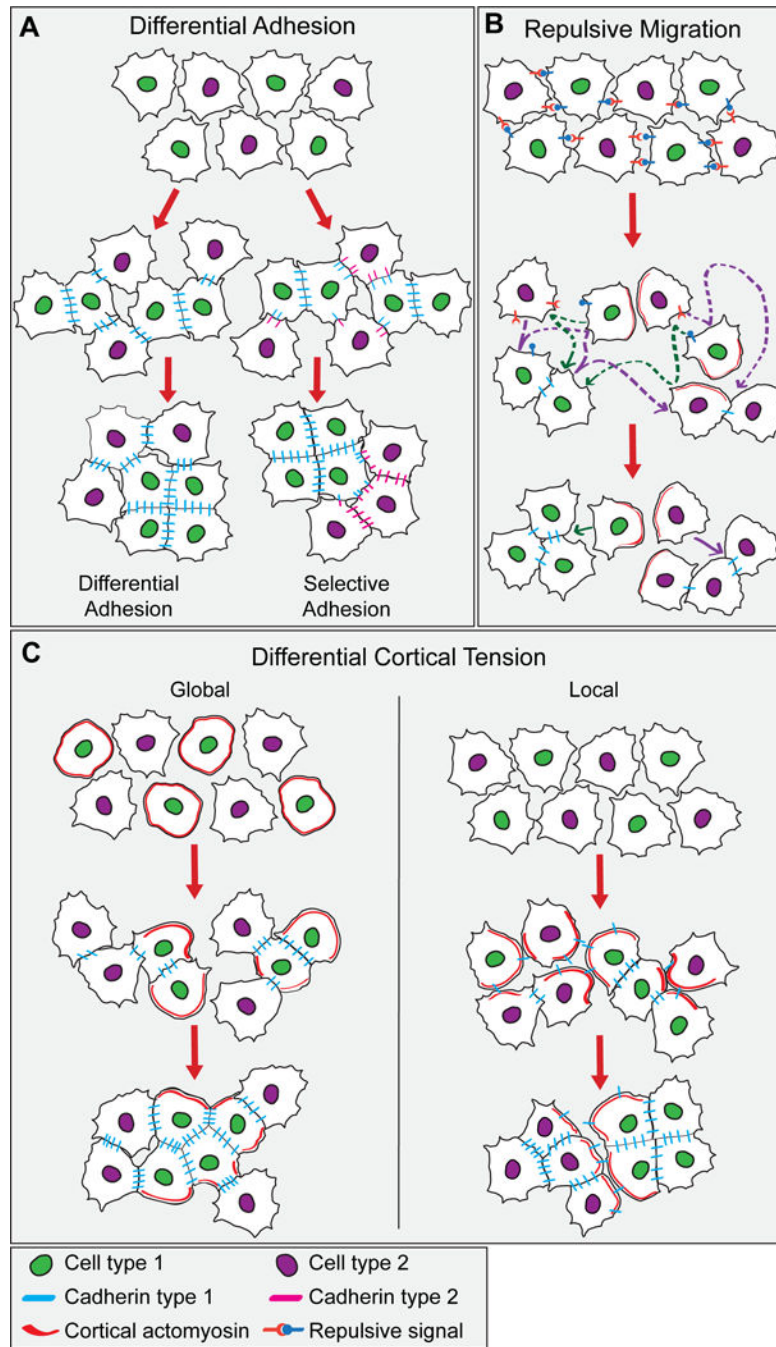
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**Figure 1. Cellular mechanisms underlying cell segregation and boundary formation based on differences in cell-cell adhesion or cortical actomyosin contractility.**

(A) Differential cell adhesion can result in cell segregation either through differential adhesion resulting from different levels of cadherin expression, or selective adhesion, resulting from the types of cadherins expressed. Cells with greater adhesion will aggregate and be surrounded by the less adhesive cell population. (B) Cell segregation can also be achieved through cell-cell repulsion in which a local repulsive cue triggers collapse of cellular processes and repulsive migration. Over reiterative repulsive interactions cell



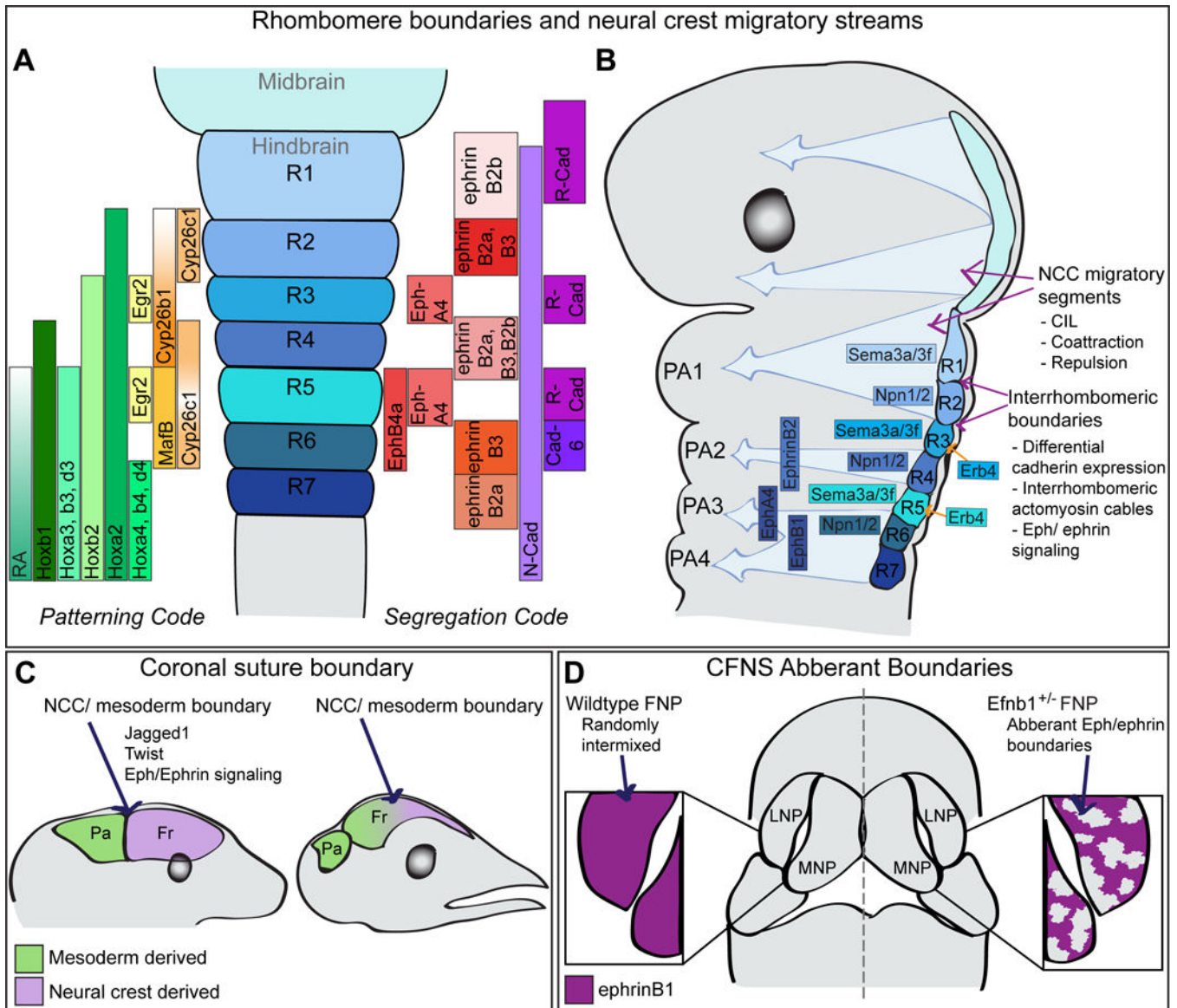
segregation is achieved. (C) Differences in cortical actomyosin contractility can lead to cell segregation. Cortical contractility can be localized to a specific interface, preventing heterotypic cell pairs from making stable cell contacts, thus only forming stable contacts with like cells, giving rise to two separate populations. Contractility can also be globally high in one cell type, resulting in those cells aggregating and being surrounded by the less contractile cell type to minimize these high-tension interactions.

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**Figure 2. Boundaries relevant to craniofacial morphogenesis in the embryo.**

(A) Rhombomeres segment the neural ectoderm, acting as organizing centers along the rostrocaudal axis. Two major mechanisms contribute to rhombomere organization and boundary formation; 1) The patterning code, involving changes in gene expression to match positional identity; 2) The segregation code, giving rise to the spatial segregation of cells with distinct identities. (B) Proper migration of NCCs is required for craniofacial morphogenesis with the NCCs migrating from the rhombomeres into the branchial arches. Various repulsive cues such as Ephs/ephrins and semaphorins are required for stream maintenance. (C) In mice the NCC/ mesoderm boundary occurs at the coronal suture between the frontal bone (Fr) and parietal bone (Pa). Conversely, in chick the NCC/ mesoderm boundary falls within the frontal bone with the coronal suture occurs between two mesoderm derived tissues. (D) Aberrant cell segregation occurs in *Efnb1*<sup>+/-</sup> embryos. Ephrin-B1 expression appears uniform in the WT frontonasal process (FNP), while patches

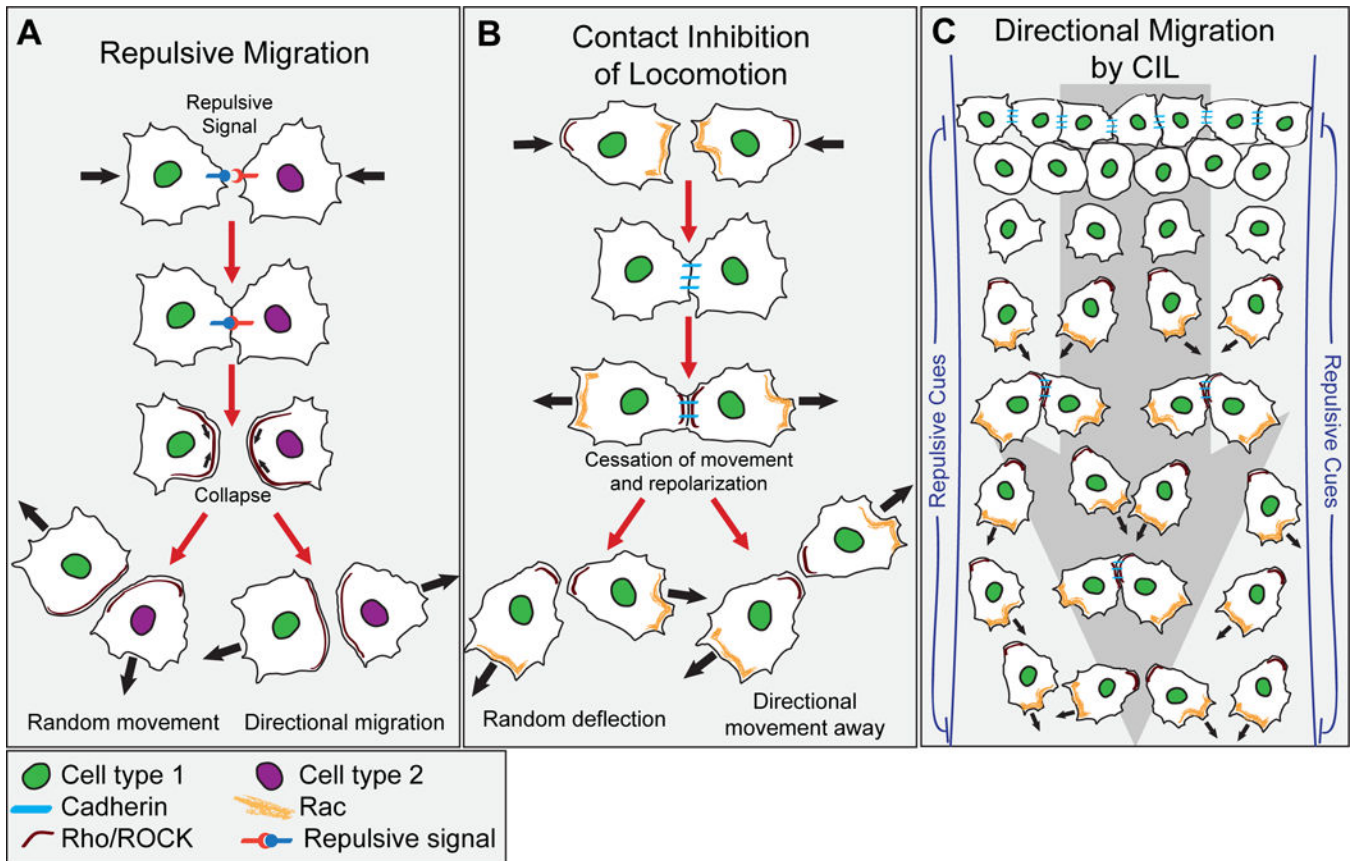
of Ephrin-B1 expression and non-expression occur due to segregation in the FNP of *EfnB1*<sup>+/-</sup> embryos. Lateral nasal process (LNP), medial nasal process (MNP).

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**Figure 3. Migratory guidance mechanisms resulting in segregation or maintenance or segregated cell populations.**

(A) Migratory guidance and cell segregation can be achieved through a repulsive migratory mechanism by which heterotypic cell contacts, providing a repulsive signal, triggers cells to collapse, resulting in cells moving apart in either a directional or random fashion. (B) Contact inhibition of locomotion (CIL) is an underlying mechanism of migratory guidance and cell segregation. CIL is characterized by two cells coming into contact with one another and either ceasing movement or undergoing directional migration away with repeated interactions resulting in directional migration as has been seen in NCCs (C). NCCs delaminate from the neural tube and undergo directional migration by CIL. Repulsive cues are also required for the maintenance of migratory streams.