Hindbrain boundaries: addressing the crossroad between tissue segmentation and cell fate regulation

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ON BOUNDARIES

La frontera és la cristal·lització de la violència que reprodueix les relacions socials. […] La veritat d'una frontera està en aquells que no l'han poguda passar.

Marina Garcés.

As a brand of vital materialism, posthuman theory contests the arrogance of anthropocentrism and the 'exceptionalism' of the Human as a transcendental category. It strikes instead an alliance with the productive and immanent force of zoe, or life in its nonhuman aspects. This requires a mutation of our shared understanding of what it means to think at all, let alone think critically.

Rosi Braidotti.

The machine is not an *it* to be animated, worshipped, and dominated. The machine is us, our processes, an aspect of our embodiment. We can be responsible for machines; *they* do not dominate or threaten us. We are responsible for boundaries; we are they.

Donna Haraway.

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ABSTRACT

The hindbrain boundary cell population (BCP) is specified at the interface between adjacent compartments during embryonic development of the posterior brain. Hindbrain BCP is a non-neurogenic population that acts as both a signaling center and an elastic mesh that prevents cell intermingling between adjacent compartments. Remarkably, boundary cells display mechanical characteristics that emphasize the impact of tissue segmentation on boundary architecture: they display specific cell morphology and contain actomyosin cable-like structures that provide the boundaries with the tension necessary for carrying out their physical barrier role.

Considering the mechanical microenvironment in the BCP and its identity specificities, we propose YAP/TAZ-TEAD activity as the molecular scaffold that underpins the crossroad between hindbrain segmentation and proliferative capacity modulation. In this work we show that mechanical stimuli in the BCP trigger YAP/TAZ-TEAD activity. In turn, this activity is responsible for transiently modulating the proliferative capacity of boundary cells, which eventually differentiate into neurons.

RESUM

La població cel·lular de les fronteres del romboencèfal (PCF) s'especifica a la interfície entre compartiments adjacents durant el desenvolupament embrionari del cervell posterior. La PCF del romboencèfal és una població no neurogènica que actua com a centre senyalitzador i com a barrera elàstica que evita la barreja de cèl·lules entre compartiments adjacents. Cal destacar que les cèl·lules de les fronteres presenten característiques mecàniques que fan palès l'impacte de la segmentació del teixit sobre l'arquitectura de les fronteres: presenten una morfologia cel·lular específica i contenen estructures d'actomiosina de tipus cable que proporcionen a les fronteres la tensió necessària per portar a terme la funció de barrera física.

Considerant el microambient mecànic a la PCF i les seves especificitats en termes d'identitat, proposem l'activitat YAP/TAZ-TEAD com la bastida molecular present a la intersecció entre la segmentació del romboencèfal i la modulació de la capacitat proliferativa. En aquesta investigació demostrem que els estímuls mecànics presents a la PCF desencadenen l'activitat YAP/TAZ-TEAD. Al seu torn, aquesta activitat és transitòriament responsable de la modulació de la capacitat proliferativa de les cèl·lules de les fronteres, les quals acabaran diferenciant-se en neurones.

PREFACE

The architectural and functional complexity of the nervous system is without a shadow of a doubt of crucial importance for understanding biodiversity. The nervous system harbors the toolkit for biological roles encompassing from the regulation of basic physiological functions such as motor coordination or breathing to the scaffolding of sentience and self-awareness.

The generation of cell diversity is one of the keystones in developmental biology. This intellectual aspiration is inevitably bound to questions such as how cell fates are regulated, how cell behavior and cell fate are intertwined and how the spatiotemporal dynamics of morphogenesis conditions but also relies on the orchestration and regulation of progenitors and differentiated cells.

Developmental neurobiology aims at unveiling the nuts and bolts of the ontogeny of the nervous system. Moreover, this discipline, at its intersection with evolutionary biology, allows addressing how the genetic programs for the generation of neurophysiological and neuroanatomical plans are distributed in the phylogeny. Interestingly, the posterior brain, also known as rhombencephalon or hindbrain, is the most conserved brain region along evolution, with tissue segmentation as probably the most determinant event in the developmental story of this territory.

Splitting up the posterior brain into compartments during embryonic development highlights the relevance of the challenge of gradually increasing the refinement of gene expression patterns, which goes hand by hand with the establishment of cell lineage restriction boundaries. Importantly, and possibly due to its strategic location within

tissues, compartment boundaries display several roles involved in the making of tissue and organ architecture.

My thesis work has combined classical developmental biology tools and recently developed methods on *in vivo* imaging and genome edition to depict an integrative view of the progenitor biology of hindbrain interhombomeric boundary cells. Thus, from the spatiotemporal characterization to the molecular regulation of boundary cell behavior and cell fate we provide important insights on the principles of boundary progenitor homeostasis. Importantly, this work addresses the interconnection between the tissue microenvironment and the downstream biochemical responses and cell behaviors. Furthermore, this thesis also contains novel results regarding hindbrain boundary cell lineage that set a framework for future mechanistic interrogations.

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CHAPTER 1: INTRODUCTION.

FROM MORPHOGENESIS TO PROGENITOR DYNAMICS.

1.1. TISSUE SEGMENTATION AND COMPARTMENT BOUNDARIES.

Embryonic development is a crucial period in the life of a multicellular organism, during which limited sets of progenitors are responsible for producing all the cellular variability in the adult body. In this sense, complexity displays an increasing progression during ontogeny. At its dawn, vertebrates and higher invertebrates are a single cell that, by way of a highly dynamic developmental process, will result into an entirely functional multicellular organism. Tens of thousands to trillions of cells comprise the resulting organism, and these cellular building blocks are arranged in specialized tissues and organs able to perform highly elaborate tasks. Thus, the timely delimitation of specific territories and their arrangement in particular shapes become defining features governing embryo development (Meinhardt, 2009; Lander, 2011).

Morphogenesis, in short, is the generation of form. In the context of developmental biology, it refers to the generation of shape and to the organization of tissues in animal and plant embryos. One of the fundamental concepts in morphogenesis is tissue separation, which leads to the physical segregation of two cell embryonic populations (Tepass, Godt and Winklbauer, 2002; Dahmann, Oates and Brand, 2011; Batlle and Wilkinson, 2012; Fagotto, 2014). Depending on the ability of these cells to interact with their adjacent neighbors, namely, whether the cells at the interface between two distinct cell populations can mix, tissue separation can result in the segmentation of immiscible compartments. In this sense, segmentation can scaffold the regional organization both along the embryonic axes and within tissues by physically segregating two cell populations (Alexander, Nolte and Krumlauf, 2009). Indeed, the generation and stabilization of boundaries between neighboring groups of cells is pivotal for embryonic development since cell populations with different functions and/or fates must be physically separated (see Fig. 1 for two key examples; Dahmann, Oates and Brand, 2011).

The discovery of tissue compartments rests on the legacy of *Drosophila* imaginal disc studies. Clonal analysis experiments in the 1970s showed that genetically induced clones do not cross between the anterior and the posterior portions of the wing disc, indicating the existence of a stable boundary responsible for keeping segregated cell lineages (Fig. 1A; Garcia-Bellido, Ripoll and Morata, 1973; Lawrence, 1973; Morata and Lawrence, 1975). The concept of tissue segmentation gained further relevance in embryogenesis when in the 1990s it was described in other systems such as the developing chicken hindbrain and the mammalian brain (Fraser, Keynes and Lumsden,

1990; Ingham and Martinez Arias, 1992; Levitt, Barbe and Eagleson, 1997; Dahmann and Basler, 1999).

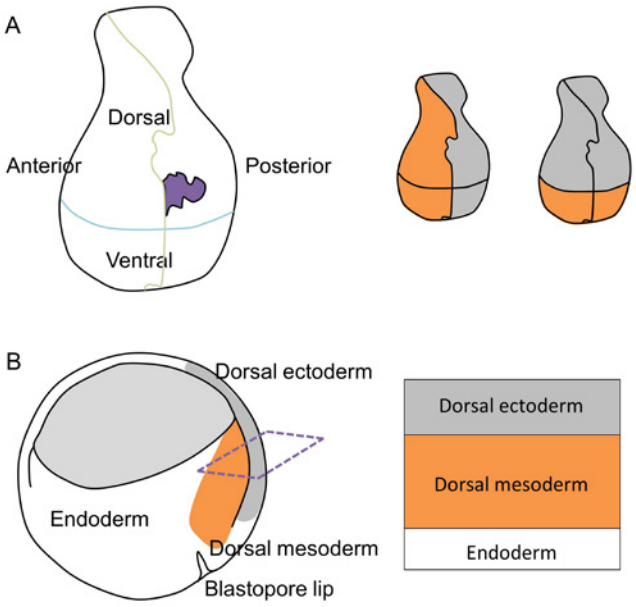

Figure 1. Tissue boundaries. (A) The wing imaginal disc is sharply partitioned into anteroposterior and dorsoventral compartments. The corresponding boundaries were discovered by clonal analysis (purple area) and are preserved during the massive growth of these structures during development. (B) In frog (and in fish) embryos, the first visible boundary is formed between the involution mesoderm and the overlying ectoderm. Transverse section at the positioned mark is shown. Adapted from Fagotto, 2014.

Importantly, patterning, or the setting up of positional information along time, results in the establishment of gene expression borders, which precedes boundary formation in the sense of cell movement restriction and morphological differentiation. Thus, a tight link exists between pattern formation and embryonic physical regionalization (Dahmann, Oates and Brand, 2011; Xu and Wilkinson, 2013; Fagotto, 2014). Indeed, in vertebrate embryos, boundaries are initially ragged delimitations of gene expression due to the interpretation of noisy positional cues, which end up refining into actual sharp boundaries. However, gene expression boundaries do not necessarily require physical separation, and these two concepts should not be considered equivalent (Fagotto, 2014).

Be that as it may, once compartment boundaries are formed, their integrity faces two major challenges: cell intercalation from dividing cells and tissue deformation as a result of morphogenesis. In this regard, two basic types of boundaries can be defined depending on how cells face integrity perturbation: non-lineage and lineage-based boundaries. Remarkably, cell behavior upon boundary disturbance leans on how cell

fate is determined in the cell populations involved. At non-lineage boundaries, fate determination is based on an instructive continuous singling input. Thus, when cells at non-lineage boundaries cross gene expression borders they will face a new upstream input and consequently switch their fate. As a result, no physical restriction exists between the adjacent cell populations and a clone could contribute to both sides of the boundary. Examples of non-lineage boundaries are the boundary between the wing pouch and the notum in the *Drosophila* wing disc (Zecca and Struhl, 2002), the somite boundaries (Tepass, Godt and Winklbauer, 2002; Dahmann, Oates and Brand, 2011), and the boundary between the vertebrate foregut and hindgut (Tremblay and Zaret, 2005). Contrarily, at lineage-based boundaries, fate determination on either side of the boundary is genetically inherited and does not require a continuous signaling input. In this case, boundary integrity between the two growing populations is maintained through a sorting mechanism that restricts cell intermingling to ensure tissue coherence. In vertebrates, examples of cell lineage boundaries are the midbrain-hindbrain boundary (Langenberg *et al.*, 2006) and hindbrain boundaries (Calzolari, Terriente and Pujades, 2014).

Therefore, lineage-based boundaries need to display full logistics for segregation maintenance comprising strategies such as cell signaling and physical mechanisms. In terms of cellular components, this involves the deployment of cell-cell adhesion complexes and the actin cytoskeleton. As to the former, cell-cell adhesion/repulsion by Eph/ephrin signaling has proven fundamental on tissue separation. Eph proteins are receptors with tyrosine-kinase activity that bind to their ephrin ligands and mediate contact-dependent cell interactions (Box 2; Taylor, Campbell and Nobes, 2017). Thus, the complementary expression of Eph receptors and ephrin ligands in tissues during embryogenesis leads to their activation only in compartment interfaces. Notable examples of Eph/ephrin signaling in tissue separation are found in germ-layer segregation (Rohani *et al.*, 2014) and hindbrain boundaries (see 1.4.2. Hindbrain morphogenesis and segmentation; Mellitzer, Xu and Wilkinson, 1999; Xu *et al.*, 1999). Concerning the dorsal ectoderm-mesoderm boundary of the *Xenopus* embryo, it has been described that ectodermal ephrins react with mesodermal Eph receptors and, conversely, ectodermal Eph receptors interact with mesodermal ephrins. As consequence, complementary Eph/ephrin pairs undergo selectively functional interactions in such a way that Eph/ephrin signaling activates Rho GTPases at the boundary resulting in actomyosin accumulation and contact repulsion (Rohani *et al.*, 2011, 2014; Fagotto *et al.*, 2013). On the other hand, physical mechanisms responsible for tissue separation can be also recruited. In this sense, barrier-like elements, such as

actomyosin structures, can generate differential mechanical tension leading to cell mixing avoidance. Interestingly, this seems to be a conserved mechanism in different systems expanding from *Drosophila* to vertebrates (Major and Irvine, 2005, 2006; Landsberg *et al.*, 2009; Monier *et al.*, 2010; Becam *et al.*, 2011; Rohani *et al.*, 2011, 2014; Curt, de Navas and Sánchez-Herrero, 2013; Fagotto *et al.*, 2013; Calzolari, Terriente and Pujades, 2014). For example, local regulation of actomyosin contractibility induces cell bond tension at compartment boundaries in *Drosophila* tissues (Landsberg *et al.*, 2009) and plays a role in cell sorting (Major and Irvine, 2005, 2006; Monier *et al.*, 2010). Thus, for instance, the formation of a long filamentous Actin (F-Actin) cable occurs at the adherens junction of boundary cells during the establishment of the dorsoventral (DV) boundary in the *Drosophila* wing disc (Major and Irvine, 2005, 2006). DV compartmentalization in the wing depends on contractile forces mediated by myosin. Indeed, Myosin II heavy chain mutations can specifically impair DV compartmentalization (Major and Irvine, 2005, 2006).

Notwithstanding, some boundaries appear to be transient, meaning that they end up disappearing and not being maintained in the adult structure. It is also highly relevant to note that compartment boundaries are not limited to the separation of future tissues and organs, but boundaries can play an important role in tissue patterning acting as signaling organizers (Kiecker and Lumsden, 2005). For example, in the developing *Drosophila* wing disc, the anteroposterior (AP) compartment boundary is the source of a Decapentapelagic (Dpp) gradient that scaffolds the specification of cell identities in a concentration-dependent manner (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). In this sense, graded activation of MAD, the Dpp transducer, and the inverse gradient of Brinker, a transcriptional repressor negatively regulated by Dpp, contribute to the transcriptional regulation of Dpp target genes in discrete domains (Affolter and Basler, 2007; Restrepo, Zartman and Basler, 2014) and, eventually, the location of the patterning elements of the adult wing (de Celis, Barrio and Kafatos, 1996).

During vertebrate brain development, compartment boundaries and organizers also serve important functions. In the developing Central Nervous System (CNS), gene expression borders establish a Cartesian-like coordinate system of positional information along the AP and DV axes responsible for prefiguring and positioning several compartment boundaries such as the *Zona Limitans Intrathalamica* (ZLI; Bulfone *et al.*, 1993), the Mid-Hindbrain Boundary (MHB; Alvarado-Mallart, Martinez and Lance-Jones, 1990) and the hindbrain boundaries (Moens and Prince, 2002; Tumpel, Wiedemann and Krumlauf, 2009). The MHB, also known as the isthmus, is the boundary between the midbrain and the hindbrain and has served as a model of local

signaling center for developmental biologists for decades. The MHB is essential for patterning cell fates anteriorly in the midbrain and posteriorly in the cerebellum (Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Dworkin and Jane, 2013) and emerges at the interface between an anterior *otx2* positive domain and a posterior *gbx2* positive domain, two gene expression territories present in the early neural plate. Importantly, Otx2 and Gbx2 are mutually repressive transcription factors that create a lineage restriction boundary at their expression juncture (Gibbs *et al.*, 2017). Indeed, expression of *wnt1* anteriorly and *fgf8* posteriorly to the presumptive MHB reinforce *otx2*/*gbx2* interface (Rhinn and Brand, 2001; Buckles *et al.*, 2004). Moreover, the establishment of the *Wnt*/*Fgf* signaling interface is crucial to the development of the mid-hindbrain, since bot $Wnt1^{-/-}$ and $Fgf8^{-/-}$ mice fail to develop the entire mid-hindbrain region (McMahon and Bradley, 1990; Chi, 2003).

Mounting evidences highlight the importance of contractility in tissue segmentation and boundary formation (Landsberg *et al.*, 2009; Monier *et al.*, 2010; Rohani *et al.*, 2011; Fagotto *et al.*, 2013; Calzolari, Terriente and Pujades, 2014). Despite the fact that we can already catch sight of the common principles of boundary formation (Dahmann, Oates and Brand, 2011; Fagotto, 2014) and of how physical stimuli are transformed into biological processes (Taber, 2014; Budday, Steinmann and Kuhl, 2015; Martino *et al.*, 2018), many research avenues remain to be explored. Consider, for example, the simplicity of the neuroepithelial tube, which ends up giving rise to an adult brain with strikingly complex architecture. Neurodevelopment is a complex and dynamic process that involves a precisely orchestrated sequence of genetic, environmental, biochemical, and physical events.

Understanding the mechanics of segmentation is central to determining the link between biophysical creation of form and structure and the mechanotransduction consequences at molecular and cellular scales. One overarching question has to do with the interplay between actomyosin assembly, tissue tension, cell behavior and cell fate decisions. In other words, how tissue segmentation and morphogenesis impacts on cell position, and therefore on cell identity, cell survival and proliferative capacity? In addition, tissue segmentation generates compartment boundaries that also act as signaling centers. Thus, compartment boundaries can play a role in dictating spatiotemporal coordinates for cell specification in adjacent territories. In this regard, formation and maintenance of boundaries are key events in the final architectural output of a tissue. The depiction of the spatial and temporal profile of boundary-dependent pattern formation and the eventual consequences in terms of cell population specification and distribution awaits further insight. Finally, a primary ambition in the

field seeks the integration between local behaviors and global properties. In line with this, the questions as to how the dynamism of morphogenetic events is in register with the developmental history of cell lineages and how these processes reciprocally affect each other posit a thrilling quest for developmental biologists.

1.2. NEURAL PROGENITORS AND NEUROGENESIS.

A single fertilized egg develops into a whole new individual with all the functions, the organs, the tissues and the many specialized cell types. The brain has often been referred as the most complex known structure. During embryonic development, the CNS derives from neuroepithelial cells that divide to generate all the mature neuronal and glial types, each of which has to emerge at the propitious spatiotemporal crossroad and in the correct proportions for appropriate development and physiological function. The process by which multipotent neuroepithelial cells give rise to neurons is termed neurogenesis.

As to neurogenesis, neural progenitors initially divide symmetrically to expand their pool and, once committed, they switch to asymmetric or symmetric differentiative divisions resulting in the generation of post-mitotic neurons. This implies that too little cell cycle exit will result in not enough differentiated cells being produced, whereas too much cell cycle exit will deplete the progenitor pool. On this account, the type and number of cell divisions of neural progenitors determine the number of neurons generated during embryonic development.

The process by which a particular type of neuron is generated from an initial multipotent progenitor can be subdivided into a series of sequential steps (Guillemot, 2007). First of all, a process of spatial patterning of the neural primordium assigns progenitor cells with unique positional identities (Guillemot, 2007). During early development, the vertebrate neural tube is subdivided into prospective different areas by gradient of morphogens such as Fgfs, Wnts, Shh, and BMPs. The signaling factor Sonic hedgehog (Shh), for example, is secreted from the notochord and the floor plate to establish a ventral-to-dorsal gradient of Shh activity that instructs subsequent patterns of neurogenesis (Ericson *et al.*, 1992; Roelink *et al.*, 1994; Chiang *et al.*, 1996; Teleman, Strigini and Cohen, 2001; Le Dréau and Martí, 2012). By contrast, BMP family and Wnt are secreted from the roof plate and regulate the patterning from dorsal-to-ventral cell types (Liem, Tremml and Jessell, 1997; Le Dréau and Martí, 2012). 13 determinants of DV identities, including members of the homeodomain and the bHLH families of transcription factors are expressed in restricted DV domains

according to the activity of the aforementioned patterning signals (Alaynick, Jessell and Pfaff, 2011; Le Dréau and Martí, 2012). The homeodomain factors of the Pax, Nkx and Irx families and the basic helix-loop-helix (bHLH) protein Olig2 provide positional identity along the DV axis, whereas Otx, Gbx, En and Hox families, among others, provide positional identity along the AP axis. In this sense, spatial patterning and fate specification are coupled, since soon after neural induction, neural cells acquire specific characteristics and fates depending on the domain they populate along the AP and DV axes of the neural tube (Guillemot, 2007; Le Dréau and Martí, 2012).

A paradigm for pattern formation could be, for example, the bHLH protein Olig2, which is required for the generation of the so-called pMN progenitor domain where motoneurons and oligodendrocytes are produced sequentially in time. Importantly, the restriction of developmental programmes to specific progenitor populations is assured by cross-repression of many fate determinants (Muhr *et al.*, 2001; Lee *et al.*, 2004). In agreement with this, Nkx2.2 and Olig2 establish a cross-repressive interaction that leads to the generation of distinct interneuron and motoneuron domains in the ventral spinal cord (Briscoe *et al.*, 2000; Novitch, Chen and Jessell, 2001).

Once pattern formation is accomplished, the neural tube is a structure organized according to three-dimensional coordinates that establish spatially defined domains where each territory displays the expression of different proneural bHLH and homeodomain genes. Proneural genes code for transcription factors that are both necessary and sufficient to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to differentiation (Bertrand, Castro and Guillemot, 2002). The main mouse proneural genes are *Mash1* (*Ascl1*), *Neurogenin* (*Neurog*) *1-3* and *Math1* (*Atoh1*) (Guillemot, 2007). Despite the fact that different proneural genes are expressed in different domains, they deploy a common mode of action that implies neuronal commitment, leading to cell cycle exit, differentiation and Notch signaling activation in adjacent progenitors (Box 1). Indeed, cell-cell contact Notch signaling pathway plays a major role in neuronal commitment (Box 1). Proneural transcription factors upregulate Delta ligand that is recognized by the Notch receptor of the adjacent cells, which induces the downregulation of proneural gene expression in the neighboring cells. As a result, one of the cells will display slightly higher level of proneural gene activity leading to the repression of proneural expression in the adjacent cell and to further increase proneural expression in the same cell, the future neural progenitor (Box 1). On the other hand, Delta ligand triggers Notch activity in adjacent cells, which upregulates *Hes/Her* transcriptional repressors that inhibit proneural gene expression and therefore neurogenesis (Box 1). Thus,

Notch-mediated lateral inhibition through cell-cell signaling is one of the mechanisms responsible for keeping the balance between progenitor maintenance and differentiation.

Box 1: Notch signaling pathway.

In contrast to signaling pathways such as Wnt, Shh and BMP/TGF-β, Notch signaling occurs via cell-cell communication. The Notch pathway is involved in binary fate decisions, including cell type specification in the vulva of *Caenorhabditis elegans*, the lateral inhibition of neurogenesis, vertebrate mesoderm segmentation, and the formation of compartment boundaries in *Drosophila* imaginal discs and the chick diencephalon (reviewed by Artavanis-Tsakonas, 1999; Irvine, 1999; Kimble and Simpson, 1997; Pourquie, 2003; Tepass et al., 2002).

The transmembrane Notch receptor is activated upon binding to a juxtaposed transmembrane Delta or Serrate/Jagged ligand. As consequence, the receptor undergoes proteolytic cleavage first by an ADAM metalloprotease and then by the γ-secretase complex. This cleavage results in the release of a cytoplasmic fragment, the COOH-terminal portion of the Notch receptor, the so-called Notch Intracellular Domain (NICD). The NICD translocates to the nucleus where it acts as a cofactor interacting with CSL (Suppressor of Hairless, Sh(H) in *Drosophila*) and mastermind/MAML to regulate transcription of target genes. The formation of the complex CSL-NICD-MAML transforms CSL into an activator of gene expression, whereas in the absence of NICD, CSL forms a transcriptional repressor complex. Importantly, among the transcriptional targets are members of the *Hes* (Hairy-Enhancer of Split) and *Hey/Hrt* (Hes related type) genes, which code for bHLH proteins that repress proneural gene expression (Artavanis-Tsakonas, Rand and Lake, 1999; Bray, 2006; Fortini, 2009; Siebel and Lendahl, 2017).

Notch signaling can act in two different modes: lateral inhibition and lateral induction (Fig. 2). Lateral inhibition derives in a binary cell fate decision by which adjacent cells will be driven to differ from one another, resulting in salt-and-pepper patterns of gene expression. Mechanistically, a ligand-producing cell signals the adjacent cell resulting in the downregulation of ligand expression. As consequence, cells from an initially equipotent field either activate or silence Notch. Thus, high Notch and low Delta maintain the progenitor state by inducing *Hes*/*Hey* genes, whereas low Notch and high Delta allow for the expression of proneural genes and eventually differentiation. In contrast, lateral induction promotes coherent signal activation and coordinated cell behavior. In this case, a positive-feedback loop in which a *Serrate*-expressing cell stimulates adjacent cells to upregulate *Serrate* expression and Notch activation (Neves *et al.*, 2013).

The interactions between Notch and either of its ligands can be modulated by the Fringe family of glycosyltransferases (Lunatic Fringe, Manic Fringe and Radical Fringe) located in the Golgi apparatus (Neves *et al.*, 2013). Fringe proteins potentiate Notch signaling induced by Delta while inhibiting signaling induced by Serrate/Jagged1 (Brückner *et al.*, 2000; Hicks *et al.*, 2000; Shimizu *et al.*, 2001; Lei, 2003; Okajima, Xu and Irvine, 2003; Yang, 2004; Neves *et al.*, 2013). Importantly, through *cis*-inhibition,

Notch ligands can also inhibit signaling by co-expressed Notch in a cell-autonomous fashion (Glittenberg *et al.*, 2006).

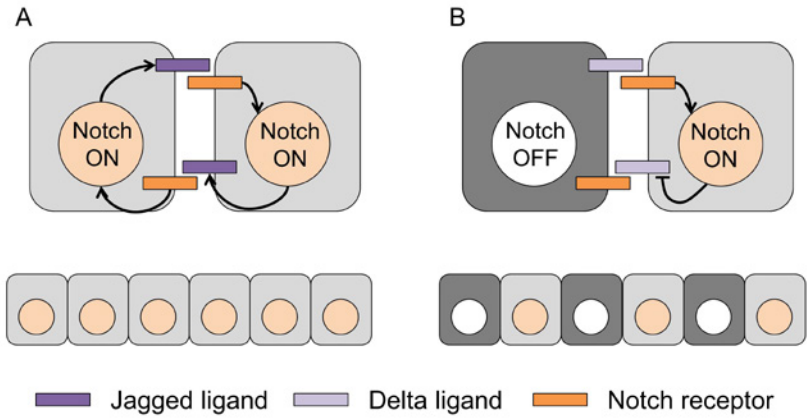

Figure 2. Notch has two modes of action. (A) Lateral induction is characterized by a positive feedback loop between Notch and the Notch ligand Jag1. All cells in the population express both Jag1 and show Notch activity. As consequence, they adopt the same fate. (B) Lateral inhibition is described as a negative feedback loop by which Delta1 induces Notch activity in the neighboring cell, and this causes the suppression of the expression of *Delta1*. The result is that the ligand delivering cell shuts down Notch activity and becomes fated to differentiate, while the surrounding cells repress *Delta1* expression, maintain high levels of Notch activity and are kept as progenitors. Adapted from Neves *et al.*, 2013.

Afterwards, the proneural proteins of the Delta-expressing cell will trigger the neurogenic programme in neural progenitors by way of inducing the expression of neuronal differentiation bHLH factors in post-mitotic cells that contribute to the neuronal differentiation programme. The main neuronal differentiation genes are *NeuroM* (*NeuroD4*) and *NeuroD* (*NeuroD1*) (Guillemot, 2007). Thus, after neuronal commitment, progenitors are specified to become neurons of a particular type. Neural progenitors then undergo cell cycle exit and migrate towards differentiated areas of the neural tube to initiate a programme of terminal differentiation (Guillemot, 2007). This implies that neural progenitors and differentiated cells are differently distributed in the neural tube.

Neural progenitors display apico-basal polarity, with cell processes that span the neuroepithelium, with the apical membrane exposed to the ventricle and their basal side contacting the basal membrane. Newborn neurogenic daughter cells need to withdraw or abscise their apical endfoot in order to migrate basally and differentiate. Proneural genes expressed in the post-mitotic daughter cell induce downregulation of cadherins and other factors to mediate delamination from the ventricular surface (Rousso *et al.*, 2012; Itoh *et al.*, 2013; Das and Storey, 2014). As differentiating cells lose their contact with the ventricle and migrate basally, generate a new domain called the mantle zone that progressively thickens as more differentiated cells are added to it.

Noteworthy, an important mechanism for the generation of neuronal diversity is the temporal control of neuronal fate specification, namely, the regulation of the sequential production of distinct types of neurons at different temporal windows from the same progenitors. For example, in the zebrafish hindbrain, neurons are spatially organized in an age-related consistent pattern in which the oldest neurons are ventrally positioned and the youngest ones populate the dorsal regions (see 1.4.3. Hindbrain neurogenesis; Kinkhabwala *et al.*, 2011). Indeed, it has long been known that a correlation exists between the spatial location of neurons in different layers of the cerebral cortex or the retina and the time of their birth. For instance, neurons located in the deepest layer of the cortex are generated first, and neurons positioned on top of them are generated at progressively later times during cortical development (McConnell, 1995).

During embryonic development, neural progenitors proliferate, a process that counterbalances terminal differentiation (Das and Storey, 2014). However, how differentiation rate is regulated both in time and space is not well understood. Cell cycle length and cell division mode regulation, Notch signaling and proneural genes activated downstream of domain identity regulators could play a role (Kageyama and Nakanishi, 1997; Novitch, Chen and Jessell, 2001; Calegari, 2003; Lee *et al.*, 2005; Marklund *et al.*, 2010; Martynoga *et al.*, 2012; Saade *et al.*, 2013). Indeed, different modes of cell division are tightly regulated to balance growth and differentiation during organ development and homeostasis. In line with this, Shh signaling, for example, not only is involved in DV pattern formation (Briscoe, 2009), but also primes proliferation and survival of neural progenitors in the developing nervous system (Cayuso, 2006; Saade *et al.*, 2013). Indeed, in chick spinal cord Shh signaling promotes progenitor expansion by symmetric proliferative and asymmetric self-renewing divisions at the expense of neuronal differentiation, which results in the temporal control of motoneuron formation (Saade *et al.*, 2013). Accordingly and taking into account that the notochord and the floor plate are the sources of the Shh gradient, the temporality of neuron production progresses from ventral to dorsal (Saade *et al.*, 2013; Kicheva *et al.*, 2014).

Signaling factors such as the aforementioned Shh gradient can regulate neuronal production. However, other mechanisms can play a role in the balance proliferation/differentiation. For example, members of the SoxB1 (Sox1, Sox2, Sox3) family of proteins act as inhibitory factors responsible for progenitor maintenance (Bylund *et al.*, 2003; Wegner, 2011). Remarkably, neurogenesis regulation also occurs in a large-scale level by the spatial organization in the neural epithelium of neurogenic and non-neurogenic regions (Bally-Cuif and Hammerschmidt, 2003; Stigloher *et al.*, 2008). A remarkable example of this is found in the Mid-Hindbrain Boundary, where

Notch-independent expression of the bHLH transcription factor Her5 links patterning to regional inhibition of neurogenesis (Geling *et al.*, 2003).

Thus, proliferation and differentiation rates in the developing nervous system can be regulated via growth factors and intrinsic mechanisms. However, living cells are constantly exposed to mechanical stimuli arising from the surrounding microenvironment, and, indeed, it has been recently shown that *in vitro* cell proliferation can respond significantly to externally applied forces (Aragona *et al.*, 2013; Streichan *et al.*, 2014; Benham-Pyle, Pruitt and Nelson, 2015). Moreover, stem cell differentiation in culture depends on the mechanical properties of the microenvironment (Engler *et al.*, 2006; Leipzig and Shoichet, 2009; Gilbert *et al.*, 2010; Seidlits *et al.*, 2010; Arulmoli *et al.*, 2015; Pan *et al.*, 2016). Nonetheless, since the architecture of the neural tube is much more complex and dynamic than 2D-cell monolayers, the extent to which these observations can be extrapolated to nervous system development is largely unknown. Notwithstanding, a recent study shows that cell crowding at the apical surface of the zebrafish spinal cord can displace nuclei basally and that these progenitors far from the apical surface differentiate (Hiscock *et al.*, 2018). Thus, this study posits that neurogenesis dynamics within the zebrafish neural tube can be regulated by the mechanical properties of the tissue, implying that it does not consist in an entirely deterministic, nor cell-autonomously programmed process (Hiscock *et al.*, 2018).

On the whole, the understanding of proliferation rate and cell division mode regulation is one of the mainstays of developmental neurobiology. In addition, despite the fact that we currently dispose of a better understanding of the mechanisms controlling neuronal specification and identity in several CNS regions, it remains unclear how non-neurogenic populations are strategically maintained and what triggers non-neurogenic-to-neurogenic switch at specific spatiotemporal coordinates. Last but not least, an ultimate integrative approach will have to address the connection between the balance proliferation/differentiation and the geometrical and physical aspects of tissue architecture.

1.3. CELL LINEAGES AND EMBRYOGENESIS.

Tissue segmentation results in the subdivision of the embryo into spatially segregated compartments confined by boundaries. Both compartments and boundaries are built up by cells and, noteworthy, understanding the complex dynamics of the formation of embryonic compartments, tissues, organs and even entire organisms as a function of the substratal cell behavior is a central goal of developmental biology (Keller *et al.*,

2008; Khairy and Keller, 2011; Amat and Keller, 2013). Cell lineages, namely the developmental history of positions, movements and divisions of cells, become of crucial importance when it comes to depicting a systematic characterization of functional relationships during embryogenesis and to provide key insights into the quantitative rules underlying developmental building plans (Amat and Keller, 2013).

The developmental pathways that convey the information responsible for the commitment of cells to specific fates are framed in a scenario where typology and topography converge (Stent, 1998). Thus, cell position, cell identity, cell behavior and tissue morphology are irredeemably related in the developmental plan of tissues and the comparison of individuals between species might reveal conserved and emerging morphogenetic rules of embryogenesis (Keller *et al.*, 2008).

Cell lineages, thus, are in register with the morphogenetic program of tissues and organs (Keller *et al.*, 2008; McMahon *et al.*, 2008; Fernandez *et al.*, 2010; Bosveld and Nodal, 2012), scaffold the link between developmental history and cell function (Murray *et al.*, 2008) and reconstruct the framework in which cell fate decisions are directed by differential gene expression (Liu *et al.*, 2009; Held *et al.*, 2010). Importantly, cross-correlation of cell lineage reconstructions and parallel developmental mechanisms such as gene expression data or the mechanical microenvironment may allow quantitative mapping of the genetic programs for developmental building plans and the integration of physical forces acting during morphogenesis across temporal and spatial scales (Oates *et al.*, 2009; Khairy and Keller, 2011).

In this sense, clonal history has traditionally been investigated by microscopically tracking cells during development (Sulston and Horvitz, 1977; Behjati *et al.*, 2014), monitoring the heritable expression of genetically encoded fluorescent proteins (Livet *et al.*, 2007). However, more recently, next-generation sequencing technologies have allowed the field of cell lineage reconstruction to exploit somatic mutations (Behjati *et al.*, 2014), microsatellite instability (Reizel *et al.*, 2011), transposon tagging (Sun *et al.*, 2014), viral barcoding (Naik *et al.*, 2013), CRISPR-Cas9 genome editing (Frieda *et al.*, 2016; Guernet *et al.*, 2016; McKenna *et al.*, 2016; Kalhor, Mali and Church, 2017; Schmidt *et al.*, 2017), Cre-loxP recombination (Tanay and Regev, 2017) and single-cell transcriptomics (Alemany *et al.*, 2018) providing powerful platforms for unbiased cell-type classification.

Thus, a myriad of cell lineage reconstruction techniques are today made available to address the ultimate link between the genealogy of embryonic progenitors and adult cell fate. In this sense, unveiling the clonal dynamics of embryonic development and

cell identity at single-cell resolution is both a major challenge and one of the overarching goals in developmental biology. On the whole, cell lineage-based reconstruction of embryonic development offers a great opportunity to address system-level questions and to inquire for recurrent motifs in the spatiotemporal patterns of cell behavior scaffolding specific dynamic processes (Khairy and Keller, 2011).

1.4. THE HINDBRAIN AS A MODEL OF STUDY.

The morphogenetic mechanism of tissue segmentation is an ancient developmental strategy leading to the spatiotemporal organization of cell populations. It is worth to note that with the physical segregation of compartments, a new cellular niche emerges at the interface of adjacent territories, the so-called compartment boundaries. Currently, we can finally catch sight of the biological functions that boundary cells carry out during embryonic development. They play a major role in restricting cell intermingling between adjacent cell populations and they are involved in tissue patterning working as signaling centers. However, we still miss a comprehensive picture of how the molecular and behavioral identity of these cells is regulated. Furthermore, cell lineage relationships within boundary tissues are a black box waiting to be addressed: how boundary cell position, proliferative behavior and cell identity are intertwined? Boundary cells become even more enticing when one considers the mechanics of tissue segmentation, which implies the generation of specific microenvironments at boundary populations in terms of cell morphology, actomyosin arrangements and tissue forces. It is tempting, thus, to consider that mechanobiological underpins might be scaffolding the behavioral and fate specificities of boundary cells.

In order to address the aforementioned research avenues, we resorted to the zebrafish posterior brain as a model of study, which undergoes transient segmentation during embryonic development generating seven compartments with nothing less than six boundary cell populations displaying all the characteristics of compartment boundaries.

1.4.1. Anatomy, functions and developmental building plan.

Three primary vesicles and a narrow tube constitute the embryonic CNS primordium, which from anterior to posterior, they correspond to the forebrain (prosencephalon), the midbrain (mesencephalon), the hindbrain (rhombencephalon) and the spinal cord. In the adult vertebrate, the hindbrain will end up giving rise to the medulla, the pons and the cerebellum (Fig. 3A). Functionally, these structures are depicted as a complex coordinator center responsible for automated body systems such as breathing rhythms, circulation, sleep patterns and motor coordination (Alexander, Nolte and Krumlauf,

2009). Eight of the twelve cranial nerve emanate for the hindbrain to receive input from cranial sensory organs and provide motor output to head muscles, acting as a relay center for the sensations of taste, touch, hearing, and balance, while controlling chewing, eye movement, and facial expressions. The hindbrain contains a network of reticulospinal neurons (RSNs) responsible for integrating sensory input with motor impulses from the cortex and is involved in many aspects of motor coordination, such as locomotion and posture. The hindbrain also contains circuits with rhythmic pacemaker-like activities providing timing signal for breathing, swallowing and vocalization.

In vertebrates, the hindbrain develops as a series of segmental compartments. Thus, the embryonic hindbrain is transiently subdivided in the AP axis into seven segments, the so-called rhombomeres (r1 – r7). Rhombomere 1 will constitute the metencephalon, including the cerebellum and pons, whereas the remaining rhombomeres will constitute the myelencephalon. Each rhombomere is i) a unit of gene expression, meaning that they display a unique molecular signature that scaffolds the formation of neuronal architecture and cranial tissue (Lumsden and Keynes, 1989), and ii) a cell lineage-restricted compartment, since interhombomeric cell movement is restricted (Fraser, Keynes and Lumsden, 1990; Jimenez-Guri *et al.*, 2010). On that account, through segmentation, iterative units that independently respond to axial patterning signals create regional identity (Parker, Bronner and Krumlauf, 2016). In fact, rhombomeres exhibit a reiterative and compartment-restricted arrangement of proliferation, neurogenesis, axonal projections and neural crest migration (Fig. 3B; Hanneman *et al.*, 1988; Lumsden and Keynes, 1989; Trevarrow, Marks and Kimmel, 1990; Clarke and Lumsden, 1993; Eickholt *et al.*, 2001).

Remarkable examples of how segmentation is involved in the establishment of neuronal architecture are the RSNs and brachiomotor neurons. As to the former, each rhombomere contains a similar set of RSNs, yet they are specialized in morphology and function in each segment. Regarding brachiomotor neurons, they are generated in specific pair of adjacent rhombomeres and their axonal projections exit the hindbrain as cranial nerves from even-numbered rhombomeres to innervate individual branchial arches (Lumsden and Keynes, 1989).

Hox genes play a common role in instructing positional identities along the AP axis, indeed, they are crucial players in the establishment of the hindbrain AP segmental identity. *Hox* genes encode a family of highly conserved transcription factors found in nearly all animal genomes. Importantly, the coupling of a *Hox* gene regulatory network

to hindbrain segmentation is an ancient trait originating at the base of vertebrates (see 1.4.4. Evolutionary perspective; Parker, Bronner and Krumlauf, 2016).

Hox genes can be classified into 13 paralogue groups (PG) and display nested expression and spatial and temporal colinearity, meaning that their linear arrangement within the chromosome conditions when and where they will be expressed (Duboule and Dollé, 1989; Graham *et al.*, 1989; Kmita and Duboule, 2003). In the vertebrate hindbrain, *Hox* genes from the PG1-4 display nested and ordered segment-specific patterns of expression along the AP axis implying that they inform neuroepithelial cells their coordinates within this axis (Fig. 3B; Lumsden and Krumlauf, 1996; Alexander, Nolte and Krumlauf, 2009; Tumpel, Wiedemann and Krumlauf, 2009; Parker, Bronner and Krumlauf, 2016).

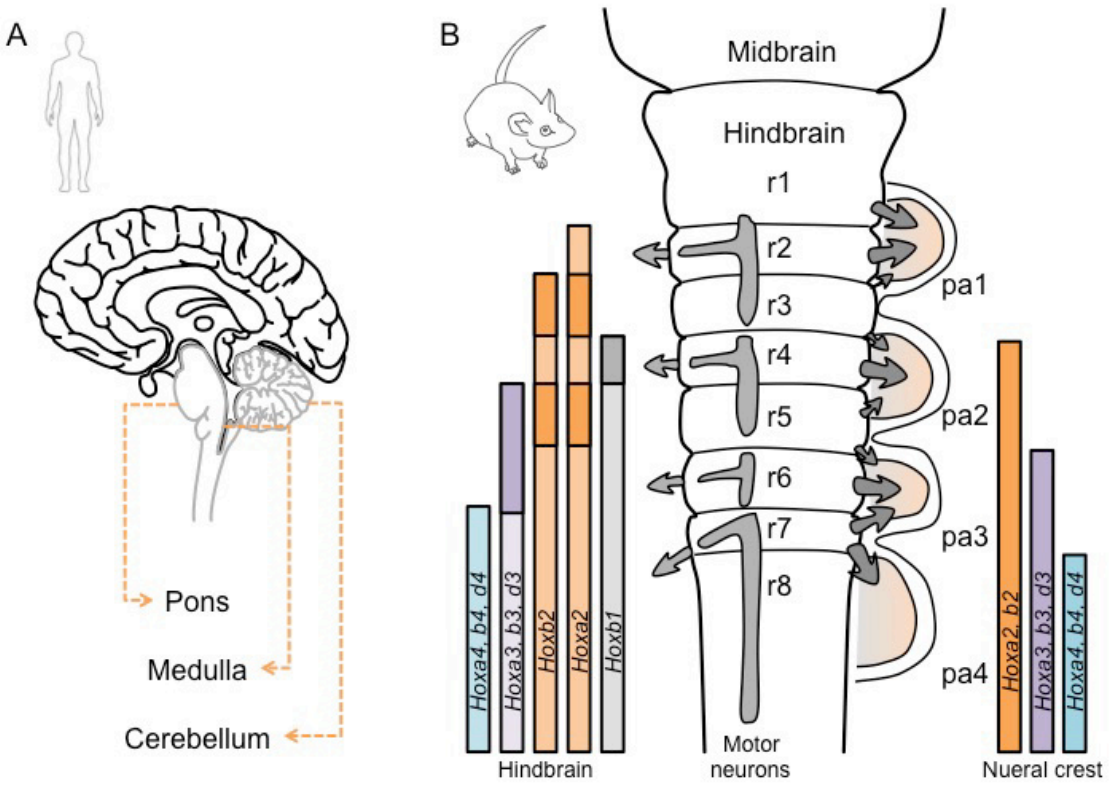

Figure 3. (A) Anatomy of the hindbrain human derivatives highlighted in grey. (B) Depiction of a dorsal view of the mouse embryonic hindbrain with rhombomeres annotated (r1-r8). On the left side, positions of motor neuron pools (light grey) and their contributions to cranial nerves are shown in relation to *Hox* gene expression domains in the rhombomeres. *Hoxa1* is not expressed at this stage. On the right hand side, migratory streams of neural crest (dark grey arrows) into the pharyngeal arches are shown in relation to neural crest *Hox* gene expression. Darker shading of *Hox* expression domains represents higher expression levels. pr, pharyngeal arch; r, rhombomere. Adapted from Parker, Bronner and Krumlauf, 2016.

Key players of the gene regulatory network (GRN) for early hindbrain development are *Egr2*, *vHnf1*, *Kreisler*, *Irx3/iro7*, *Cdx1*, and PG1 *Hox* genes. They display specific regionalized expression through *cis*-regulatory elements that directly interpret the gradients of signaling molecules. In this regard, hindbrain patterning lies on the integrated role of retinoic acid (RA), Wnt and FGF signaling. For example, several retinoic acid response elements (RAREs) have been described and shown to receive direct input from the RA gradient to activate *Hoxa1*, *Hoxb1*, *Hoxb4*, *Hoxd4*, *Hoxb5*, *vHnf1* and *Cdx1* gene expression (Marshall *et al.*, 1994; Dupe *et al.*, 1997; Gould, Itasaki and Krumlauf, 1998; Studer *et al.*, 1998; Houle, Sylvestre and Lohnes, 2003; Hernandez, 2004; Pouilhe *et al.*, 2007; Nolte *et al.*, 2013; Ahn, Mullan and Krumlauf, 2014). In zebrafish, RA, Wnt and FGF systems collaborate to establish the shape of the RA gradient responsible for assigning positional identity through the regulation of the expression of the RA-degrading enzyme *cyp26a1* (Begemann *et al.*, 2001; Kudoh, Wilson and Dawid, 2002; White *et al.*, 2007; White and Schilling, 2008; Cai *et al.*, 2012; L. Zhang *et al.*, 2012; Schilling, Nie and Lander, 2012). This results in the regionalized expression of *HoxPG1*, *Egr2* and *MafB/Kreisler/val* (Maves, Jackman and Kimmel, 2002; Wiellette, 2003; Hernandez, 2004; Chomette, 2006; Labalette *et al.*, 2011, 2015; L. Zhang *et al.*, 2012).

Thus, RA signaling triggers a cascade of regulatory interactions that leads to the formation of tightly located stripes of gene expression, the borders of which progressively position the future rhombomeric boundaries (Manzanares *et al.*, 2002; Tumpel, Wiedemann and Krumlauf, 2009; Cermak *et al.*, 2011; L. Zhang *et al.*, 2012; Labalette *et al.*, 2015). Remarkably, the involved transcription factors are wired in a GRN via positive-feedback loops and reciprocal repressions. In the former case, positive-feedback loops allow the maintenance of gene expression (Tumpel, Wiedemann and Krumlauf, 2009; Bouchoucha *et al.*, 2013). As to reciprocal repressions, they are responsible for sharpening expression boundaries (Studer *et al.*, 1994; Wassef *et al.*, 2008).

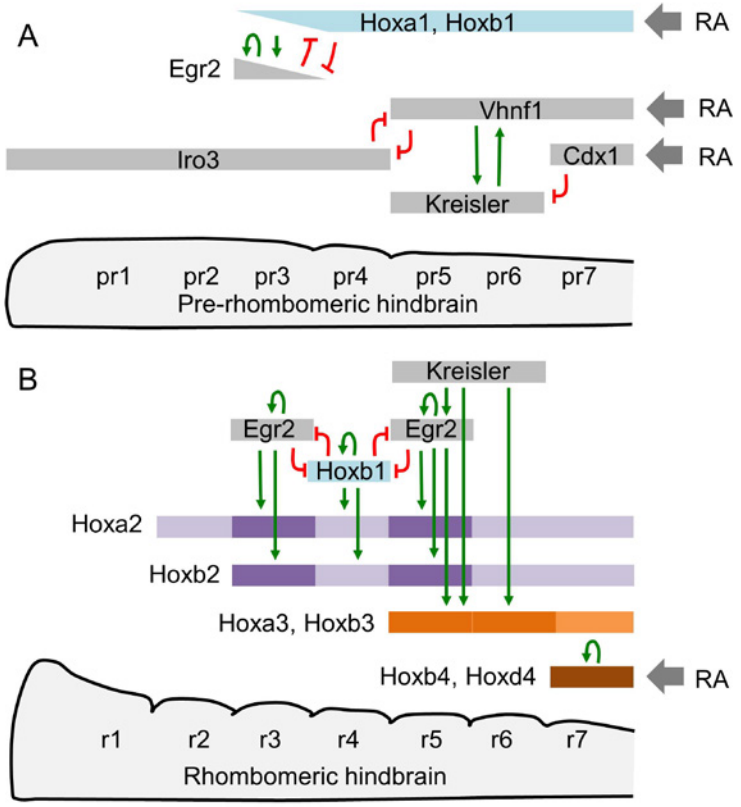

Figure 4. Regulatory interactions between *Hox* genes and other segmentally expressed genes in the developing hindbrain. (A) The network prior to the development of rhombomeres. Green and red arrows indicate positive and negative gene-regulatory interactions, respectively. Regulatory inputs from the retinoic acid (RA) signaling pathway (grey arrows) contribute to early gene expression domains that determine the boundaries of presumptive rhombomeres (pr1–pr7). *Egr2* and *Hox PG1* genes exhibit dynamic expression, with *Egr2* advancing posteriorly while *Hox PG1* genes retreat to the future r3–r4 boundary. *Egr2* represses *Hox PG1* genes and is repressed by them indirectly, via Nlz factors. (B) The network at the appearance of morphological rhombomeric bulges (r1–r7). Gene expression borders coincide with segmental boundaries. Darker shading indicates domains with higher expression levels. *Hoxa1* is not expressed at this stage. Adapted from Parker, Bronner and Krumlauf, 2016.

The first transcription factors that are expressed during the AP partitioning of the hindbrain are the rostrally expressed up to r4/r5 *Irx3* (mouse) or *iro7* (zebrafish) and the *vHnf1* caudal domain, which split the hindbrain in two domains according to the r4/r5 boundary, as these two factors interact via mutual repression (Wiellette, 2003; Lecaudey *et al.*, 2004; Aragón *et al.*, 2005; Sirbu, 2005; Jimenez-Guri and Pujades, 2011). These domains are further subdivided by the activity of *Hox* PG1 genes, *Egr2* and *MafB/Kreisler/val*. The anterior boundary of *Hoxb2* expression maps to the r2/r3 junction and contributes to the maintenance of r4, whereas *Hoxb3* anterior border marks the r4/r5 boundary, and *Hoxb4* maps to the r6/r7 junction. Members from *HoxPG* 2, 3, and 4 have anterior boundaries that map to the r2/r3, r4/r5, and r6/r7 boundaries, respectively. *Hoxa1* and *Hoxb1* display a posterior expression that transitory extends to

r3/r4 and are required for the proper development of r4 and r5 (Rossel and Capecchi, 1999; Barrow, Stadler and Capecchi, 2000; McNulty, 2005; Wassef *et al.*, 2008; Makki and Capecchi, 2010). *Hoxa2,* the only *Hox* gene expressed in r2, is present all along the hindbrain up the r1/r2 boundary. Further refinement of the nested territories is provided by the transcriptional regulation of *Egr2*, which specifies r3 and r5 (Schneider-Maunoury *et al.*, 1993, 1997; Voiculescu *et al.*, 2001); and by *MafB/Kreisler/val*, which specifies r5 and r6 (Moens *et al.*, 1996). *Cis*-regulatory analyses have been addressed to shed light on the regulation of the hindbrain GRN. For example, *Hoxa2* and *Hoxb2* are directly upregulated by *Egr2* in r3 and r5 (Sham *et al.*, 1993; Nonchev *et al.*, 1996), and by *Hoxb1* in r4 (Nonchev *et al.*, 1997; Tumpel *et al.*, 2007). *Hox* genes, thus, are involved in defining segmental territories by way of the early expression of PG1 factors, whereas later expression of PG1-4 genes are responsible for refining segmental borders and conferring segmental identity during rhombomere formation (Parker, Bronner and Krumlauf, 2016).

Given that FGF and RA morphogen gradients establish the AP field of the hindbrain, the system must cope with cell variability in misreading the morphogen concentration. Hindbrain boundaries coincide with the anterior limits of expression of *Hox* genes, as well as the aforementioned (and other) transcription factors (McGinnis and Krumlauf, 1992; Krumlauf and Keynes, 1994). As consequence, the perturbation that this noise implies to the robustness of the system results in ragged gene expression boundaries. Hence, cells located at boundary interfaces must respond to instructive signals and cope with the intrinsic noise of the system. In this sense, precision in border formation is achieved via gene expression change due to cell plasticity (Trainor and Krumlauf, 2000; Schilling, Prince and Ingham, 2001) and by cell sorting through selective cell adhesion and repulsion (see 1.4.2. Hindbrain morphogenesis and segmentation; Cooke and Moens, 2002). Moreover, computational analysis indicates that fuzzy gene expression borders induced by fluctuations in RA signaling are sharpened in part by way of a switch of *Hoxb1/Egr2* expression in response to intracellular noise (L. Zhang *et al.*, 2012).

Thus, hindbrain patterning involves coordination between morphological segmentation and the generation of unique profiles of gene expression within each segment to provide specific molecular identity (Lumsden and Krumlauf, 1996; Gruss and Kessel, 1991; Galis, 1999; Lumsden, 2004), meaning that a tight and orchestrated dynamic coordination exists between hindbrain patterning and morphogenesis.

1.4.2. Hindbrain morphogenesis and segmentation.

Morphogenesis is the positioning and shaping of tissues and organs in the body. The control of cell and tissue movements relies on the interplay between intrinsic molecular signals and extrinsic constraints conditioned by the mechanical environment. The zebrafish hindbrain undergoes through a process of morphogenesis with three main morphological and behavioral constraints: i) neurulation, ii) keeping hermetic compartments and iii) spatial organization of neural progenitors and differentiated cells.

Vertebrate neurulation consists of a morphogenetic process by which a mirror-symmetric tube is built *de novo* from a neuroepithelial sheet. In vertebrates, neurulation can occur via two different strategies. The so-called “epithelium-wrapping” mode involves the folding of the epithelial sheet around pre-existing apical extracellular space as an interior lumen; this is the mode of neurulation in amniotes. On the other hand, the “lumen-inflation” mode proceeds by aggregating first epithelial cells to form a solid rod where subsequently the lumen will emerge. This strategy is characteristic of the head and trunk regions of teleost fish and lamprey (Davidson and Keller, 1999; Colas and Schoenwolf, 2001; Lowery and Sive, 2004). Thus, during zebrafish neurulation, cells from the neural plate undergo cell intercalation through a process of convergent extension, which precedes the invagination of the neural plate to form a neural keel that eventually will generate a neural rod with two coherent hemispheres (Kimmel *et al.*, 1995; Tawk *et al.*, 2007; Guo *et al.*, 2018).

Noteworthy, the neural keel and rod stages are characterized by neural progenitor divisions called C-divisions, which consist in depositing one daughter cell on either side of the midline (Kimmel, Warga and Kane, 1994; Papan and Campos-Ortega, 1997, 1999; Ciruna *et al.*, 2006). Indeed, more than 90% of neural plate cells undergo this midline-crossing C-division (Lyons, Guy and Clarke, 2003). Once located on either side of the neural rod, both daughter cells elongate in the apico-basal axis of the corresponding hemisphere (Tawk *et al.*, 2007). This oriented cell division has been shown to play an important role in the developing zebrafish neural tube. Indeed, the polarity protein Pard3 is localized at the cleavage furrow of dividing progenitors during neural rod formation, and subsequently mirror-symmetrically inherited by the two daughter cells (Tawk *et al.*, 2007). This allows the cells to integrate into opposite sides of the developing neural tube (Tawk *et al.*, 2007). In line with this, *pard3* knockdown morphants and mutants display a dramatic decrease of midline crossing C-divisions (Tawk *et al.*, 2007).

Mechanistically, recent data proposes a three-stage model for zebrafish neural rod formation in which three distinct groups of apically-located proteins undergo different roles in a timely order. Briefly, a first group of apical proteins establish apical adhesions between cells from opposing hemispheres and between cells within the same hemisphere, resulting in the stabilization of the midline (Guo *et al.*, 2018). After that, the apical adhesions between opposing cells from the adjacent hemispheres disappear, being this a prerequisite for the last step, based on the recruitment of Na^+/K^+-ATPases in the apical domain, which will result in the generation of the osmotic pressure responsible for the opening of the lumen (Guo *et al.*, 2018).

This highly dynamic scenario goes along several signaling and mechanical strategies that end up converging in the subdivision of the hindbrain into repeated morphological units, the rhombomeres, each with a distinct regional identity (Lumsden and Krumlauf, 1996). The expression pattern of the transcription factors that regulate segment identity unveil that rhombomeric segment borders are initially ragged but then sharpen up (Cooke et al., 2005; Irving et al., 1996; Kemp et al., 2009). Importantly, Eph family of receptor tyrosine kinases and their ephrin ligands are expressed in complementary presumptive hindbrain segments (Becker *et al.*, 1994), being EphA4, for example, a direct target of the patterning factor Egr2 in r3 and r5 (Theil *et al.*, 1998). In zebrafish, *ephA4* and *ephrinB3* are expressed in rhombomeres r3 and r5 and r2, r4 and r6, respectively; whereas *ephB4* and *ephrinB2a* are expressed in r2, r5 and r6 and r1, r4 and r7, respectively (Chan et al., 2001; Cooke et al., 2001; Xu et al., 1995; Xu and Wilkinson, 2013). It is well known that Eph/ephrin signaling underlie cell sorting of adjacent rhombomeres (Calzolari et al., 2014; Cooke et al., 2001, 2005; Kemp et al., 2009; Xu et al., 1995, 1999) as well as the induction of boundary cells (see 1.5. The hindbrain boundary cell population; Cooke et al., 2005; Terriente et al., 2012; Xu et al., 1995). Indeed, disruption of Eph/ephrin signaling and ectopic expression assays indicate that bidirectional interactions in compartment boundaries prevent cell mixing and sharpen the borders (Cooke et al., 2001; Xu et al., 1995, 1999).

In zebrafish, two mechanisms have been proposed to operate in parallel: repulsive interactions between ephrinB-expressing and EphA4-expressing cells at rhombomeric boundaries (Xu et al, 1995, 1999), and adhesive interactions between cells of the same cohort (Cooke et al, 2005; Kemp et al, 2009).

As proposed by Addison *et al*. (Addison *et al.*, 2018), the mechanisms recruited for managing hindbrain compartment coherence might depend on the level of Eph/ephrin signaling at their interface. When Eph/ephrin signaling is not yet strong enough, cells

intermingle between adjacent compartments and undergo identity switch through a non-cell autonomous mechanism (Schilling, Prince and Ingham, 2001; Wang *et al.*, 2017; Addison *et al.*, 2018). However, according to this model, when Eph/ephrin signaling is compelling enough, cells display the preferential adhesion properties that keep them sorted (Calzolari, Terriente and Pujades, 2014). As development proceeds, the Eph/ephrin-mediated cell sorting mechanism is reinforced by the specification of the hindbrain boundary cell population, which contains cable-like actomyosin structures located in the apical side of the cells responsible for generating tension in the hindbrain boundary cell population, and therefore elasticity (see 1.5.1. Hindbrain boundaries are mechanical barriers; Calzolari, Terriente and Pujades, 2014; Letelier *et al.*, 2018). Indeed, the neural epithelium is constricted at hindbrain boundaries, where actomyosin cable-like fibers are found. In line with this, experimental manipulations that block or increase MyoII activity in zebrafish alter cell shape and morphological constriction in hindbrain boundaries (Gutzman and Sive, 2010).

Box 2: Ephs and Ephrins.

Eph receptors comprise the largest family of receptor tyrosine kinases (RTKs), with fourteen receptors divided into two subfamilies — EphAs and EphBs. Importantly, their cognate ligands, the ephrins, are tethered to the cell surface, in contrast to other RTKs whose ligands are generally soluble, meaning that Eph/ephrin signaling depends on cell–cell contact (Taylor, Campbell and Nobes, 2017). Consequently, Eph receptors allow cells to sense their immediate surrounding cellular microenvironment and make appropriate behavioural decisions. In this sense, Eph receptors control whether two contacting cells are repelled by, or attracted to, each other (Taylor, Campbell and Nobes, 2017). As such, they play an important role in normal physiological processes, including embryonic tissue boundary formation and directional guidance of developing axons (Taylor, Campbell and Nobes, 2017).

The type A Eph receptors (EphA1–A8 and EphA10) bind to and activate type A ephrins (ephrin-A1–A5), and the type B Eph receptors (EphB1–B4 and EphB6) bind to and activate the type B ephrins (ephrin-B1–B3) (Taylor, Campbell and Nobes, 2017). Additionally, there is a degree of promiscuity between categories; for example, EphB2 can bind ephrin-A5, and EphA4 is able to bind ephrin-B ligands. There is also promiscuity of binding within categories, for example EphB2 is able to bind to ephrin-B1, -B2 and -B3 (Taylor, Campbell and Nobes, 2017).

Remarkably, Eph/ephrin signaling can occur in a bidirectional way. Signalling through the Eph receptor has been termed forward signalling, while signalling through the ephrin ligand has been termed reverse signalling (Taylor, Campbell and Nobes, 2017).

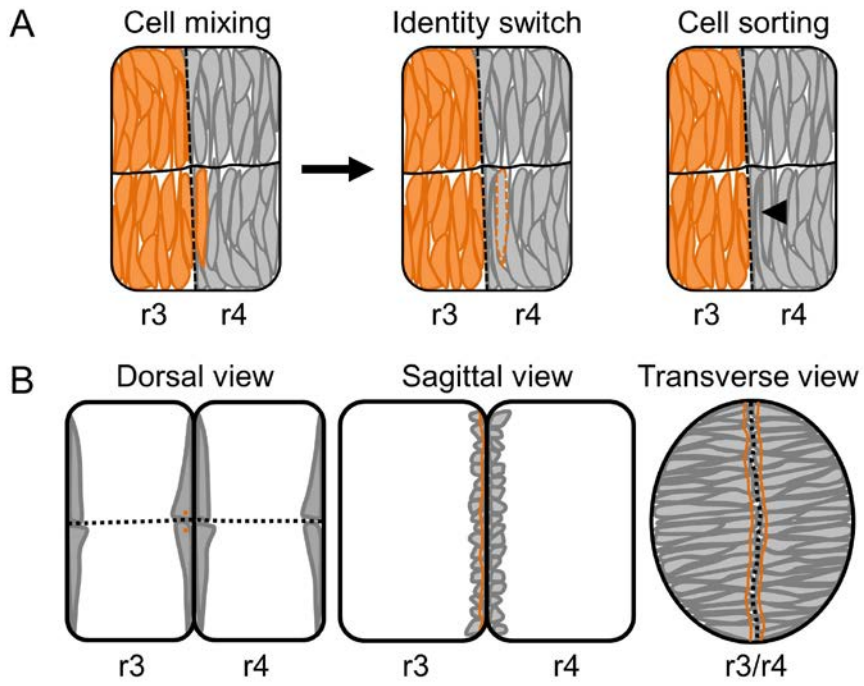

Figure 5. (A) Two strategies for managing cell mixing at rhombomeric interfaces. At earlier stages, before 12 hpf, cells can move across gene expression boundaries and switch their identity to adapt to the identity of their local neighbours (identity switch). Once Eph-ephrin signaling is well established, displaced cells are sorted back to the territory of origin. (B) Representation of the actomyosin cable-like structures in the hindbrain boundary cell population. Two adjacent rhombomeres are depicted. Actomyosin cables are represented as orange lines in transverse and sagittal views and as orange dots in the dorsal view. Hindbrain boundary cells are depicted in grey. Adapted from Dahmann, Oates and Brand, 2011 and Calzolari, Terriente and Pujades, 2014.

On top of this, the neurulation process proceeds with the formation of the lumen triggered at 18 hours post-fertilization (hpf) and consists in the opening of the neural tube at several points along its AP axis (Gutzman and Sive, 2010). These openings will grow in size until they all fuse in a final continuous ventricle at around 24 hpf (Gutzman and Sive, 2010). As the tube unfolds, the neuroepithelial cells within are engaged into active neurogenic and gliogenic programs and differentiated cells will accumulate in the ventral mantle zone with a stereotyped position and shape in a population-type dependent manner (Nikolaou *et al.*, 2009; Esain *et al.*, 2010; Gonzalez-Quevedo *et al.*, 2010). Remarkably, the opening of the tube implies a modification of the apico-basal coordinates of the tissue, since the most dorsal apical contacts initially positioned at the midline will now be in contact with the ventricle (see orange dotted line in Fig. 6).

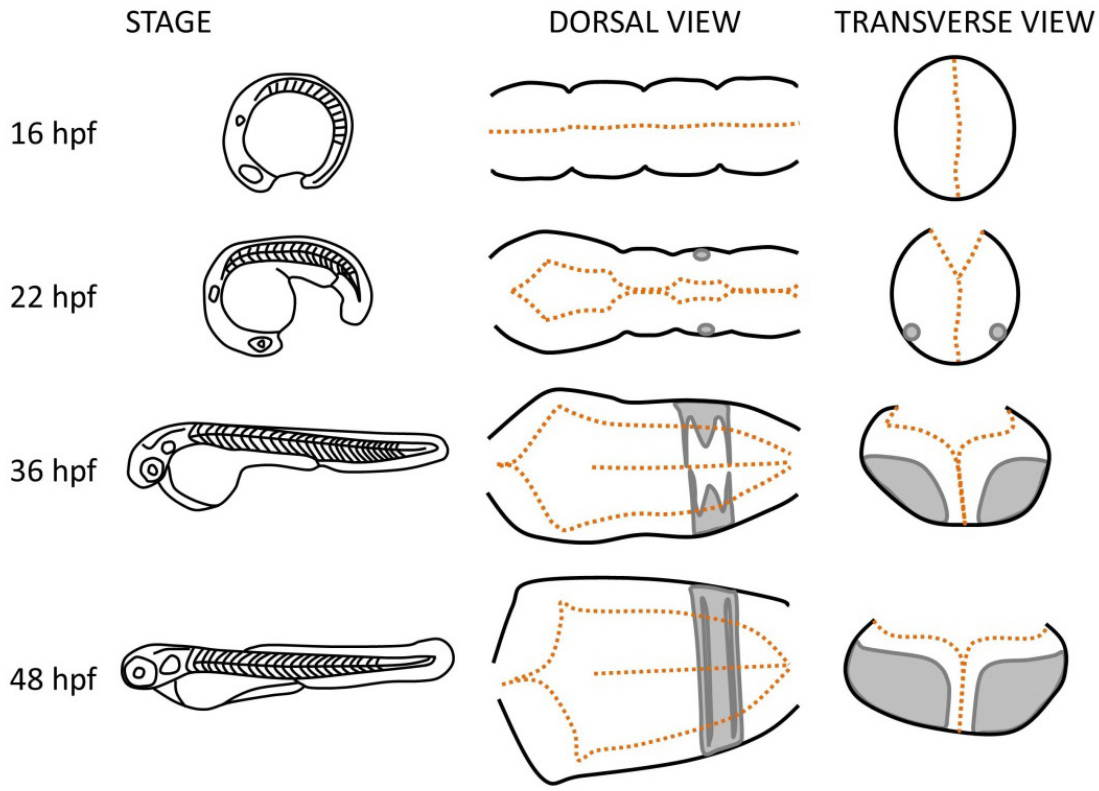

Figure 6. Morphogenetic progression of the formation of the ventricle and of the neuronal differentiated domains. The dashed orange line depicts the tube midline. Initially (16 hpf) the two hemispheres that compose the neural tube converge at a sealed midline. As embryogenesis proceeds, several holes are generated at different points of the midline (22 hpf) and grow until the entire ventricle is open (see, for example, 36 hpf), adapted from Gutzman and Sive, 2010. In parallel, differentiated neurons (grey domains) populate the tube mantle zone that will grow in size as development proceeds. The spatial distribution of these neuronal territories is stereotypical both in the AP and DV axes. Importantly, the growth dynamics of progenitor domain versus differentiated domain in the DV axis ends up confining the progenitors in the ventricular area (48 hpf).

On the whole, several constraints and behaviors scaffold the morphogenetic dynamism in the hindbrain, from neurulation to ventricle formation, encompassing all the developmental mechanisms operating for assuring segmental coherence. It is important to highlight the relevance of the temporal organization of these events not only in order and tempo, but also in its coordination with patterning and cell specification (neurogenesis and gliogenesis). Finally, we cannot fail to note that the hindbrain boundary cell population is the geographical coordinate where the refinement of compartments takes place. Not only that, but also other functions and behavioral specificities have been assigned to this intriguingly versatile cell population aside from being sharp border of gene expression (see 1.5. The hindbrain boundary cell population).

1.4.3. Hindbrain neurogenesis.

During the development of the central nervous system, neural progenitors differentiate to generate a wide variety of neuronal and glial cell types in different spatiotemporal coordinates. Importantly, the generation of differentiated cells has to be balanced with the neural progenitors that are maintained throughout development. In the zebrafish embryonic hindbrain, neurogenesis regulation is highly dynamic both in time and space.

In anamniotes, two main distinct neuronal populations are generated: primary and secondary neurons. Primary neurons are required for the coordination of the movements of the larvae soon after hatching (Appel, 2000) and they include *islet1/2*-positive primary motoneurons, *lim1*-positive primary interneurons and Rohon-Beard (RB) neurons that express *islet1/2* and *tlx3a*. Primary neurons arise from neurogenic regions, also called proneuronal domains, that are established as three longitudinal stripes along the AP axis in the dorsal ectoderm during the late gastrulation and early segmentation periods (around 12 hpf; Fig. 7). These proneuronal domains express bHLH proneural genes *neurog1* (Blader *et al.*, 1997; Bae, Shimizu and Hibi, 2005), *olig2* (Park *et al.*, 2002) and *neurod4* (Park *et al.*, 2003; Wang *et al.*, 2003), and a homeobox gene, *pnx* (Bae, 2003) in zebrafish, which elicit the transition from proliferative neural precursor cells to post-mitotic *huc*-expressing neurons (Kim *et al.*, 1996). Importantly, *neurog1*, *neuroD4* and *pnx* display a salt-and-pepper expression pattern within the proneural domain as expected from the Notch-mediated lateral inhibition mechanism (Box 1; Blader *et al.*, 1997; Kim *et al.*, 1997; Bae, 2003; Park *et al.*, 2003; Wang *et al.*, 2003). *olig2* is also expressed in the proneural domain that correspond to primary motoneurons (Park *et al.*, 2002). During zebrafish late gastrulation, two distinct expression profiles of *Her* genes have been described in the neural plate. *her2*, *her4*, *her12* and *hes5* are expressed in the three longitudinal proneural stripes (Fig. 7; Takke *et al.*, 1999), whereas *her9* and *her3* are expressed in the inter-proneuronal domains and they function as transcriptional repressors of neurogenesis (Bae, Shimizu and Hibi, 2005). Thus, the proneuronal domains are separated by the inter-proneuronal non-neurogenic domains.

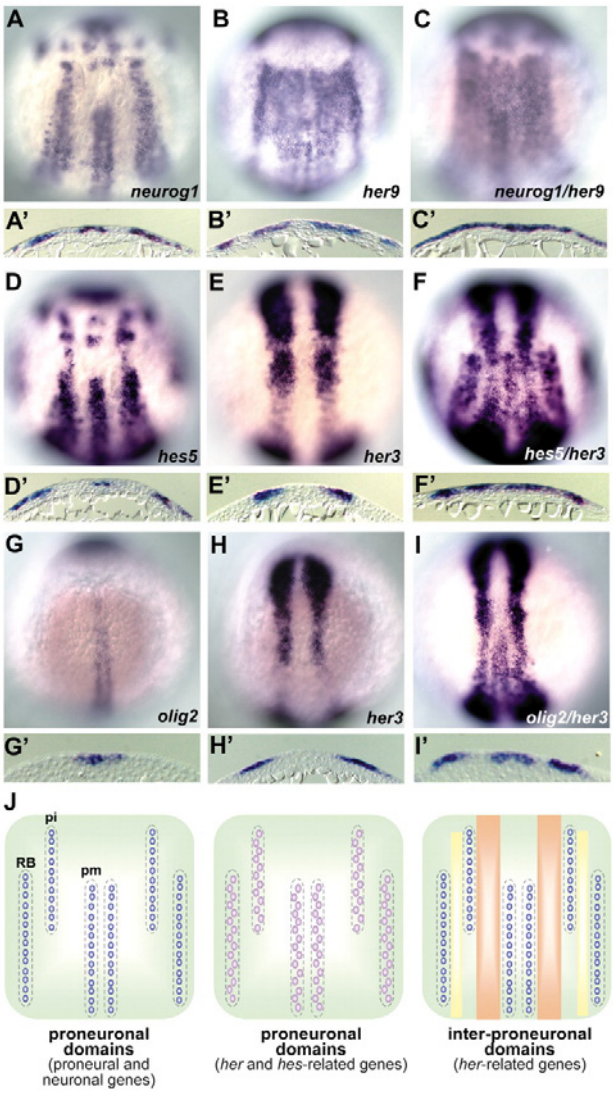

Figure 7. Expression of *Her* genes in the zebrafish posterior neuroectoderm. Dorsal views of the hindbrain and spinal cord region showing expression of *neurog1* (A, A′), *her9* (B, B′), *hes5* (D, D′), and *her3* (E, E′) at the one-somite stage, and *olig2* (G, G′) and *her3* (H,H′) at the three-somite stage. Co-staining with probes for *neurog1* and *her9* (C, C′, one-somite stage), *hes5* and *her3* (F, F′, one-somite stage), and *olig2* and *her3* (I, I′, three-somite stage). Schematic representation of expression profiles of the genes expressed in the proneuronal and inter-proneuronal domains (J). pm, primary motoneuron; pi, primary interneuron; RB, Rohon Beard neurons. *neurog1* and *olig2* are expressed in the proneuronal domains (indicated by purple circles, left). *hes5* and *her4* are also expressed in the proneuronal domains, but not in the cells that express proneural genes (indicated by pink circles, middle). *her3* is expressed between the primary motoneurons and interneurons (orange stripes), and *her9* is expressed in all the inter-proneuronal domains (orange and yellow stripes, right). Adapted from Bae, Shimizu and Hibi, 2005.

At early stages of hindbrain segmentation when morphological bulges are already present (18 hpf), the proneural genes *ascla/b* and *neurog1* are expressed in the dorsal and ventral half of each rhombomere, respectively, but are excluded from hindbrain boundaries (Amoyel *et al.*, 2005). Subsequently, by 24 hpf, proneural gene expression

acquires a stripped expression pattern that persists beyond 48 hpf. For instance, at 26 hpf *asclb* and *neurog1* are expressed in presumptive neuroblasts adjacent to rhombomere boundaries, namely the boundary flanking regions, with weaker expression throughout the medial zone but still absent in the whole boundary territories.

Accordingly, at 18 hpf, expression of the *delta* genes *deltaA* and *deltaD,* which are Notch ligands and downstream targets of proneural genes, and that of *p27Xic1-a,* an inhibitor of cdk that mediates cell cycle exit in neural progenitors, is extended along the rhombomeres, except in boundary cells (Fig. 8, Amoyel *et al.*, 2005). Later on, appears *neuroD4* downstream of proneural genes (Park *et al.*, 2003; Wang *et al.*, 2003) and, eventually, *huC/D* marking differentiating post-mitotic neurons in the mantle zone (Park *et al.*, 2000).

Importantly, in hindbrain segments, another layer of neurogenesis regulation is found at the level of the glycosyltranferase lunatic fringe (Lfng). Fringe glycosylates specific site of Notch extracellular domain post-translationally, and this glycosylation modulates the affinity of Notch towards its ligands: Delta binds more strongly to Fringe-modified Notch whereas the binding of Serrate is decreased (Panin *et al.*, 1997; Moloney *et al.*, 2000). At early stages, *lfng* expression is high in alternating segments in the hindbrain (Leve *et al.*, 2001; Prince *et al.*, 2001; Qiu *et al.*, 2004). In this sense, *lfng* is expressed by progenitors in neurogenic regions and downregulated in cells that have initiated neuronal differentiation (Nikolaou *et al.*, 2009). The expression of *lfng* does not require Notch activity, it is regulated downstream of proneural genes instead. The role of *lnfg* is to limit the amount of neurogenesis and to maintain progenitors. Surprisingly, *lfng* is upregulated downstream of proneural genes but its role is to limit differentiation. In this regard, the proposed model suggests a feedback loop downstream of proneural genes, which, by promoting Notch activation, *lfng* maintains the sensitivity of progenitors to lateral inhibition of their differentiation either by *trans*-activating Notch and/or by blocking *cis*-inhibition of Notch by Delta (Nikolaou *et al.*, 2009). Importantly, *lfng* is not the only fringe expressed in the developing hindbrain. As a matter of fact, *rfng* is expressed in hindbrain boundaries but the relationship between this fringe and Notch remains elusive (see 1.5. The hindbrain boundary cell population).

On the whole, these previous studies describe that neurogenesis in the hindbrain becomes confined to boundary flanking regions and does not occur in the central region of each segment and compartment boundaries (Fig. 8; Cheng *et al.*, 2004; Amoyel *et al.*, 2005).

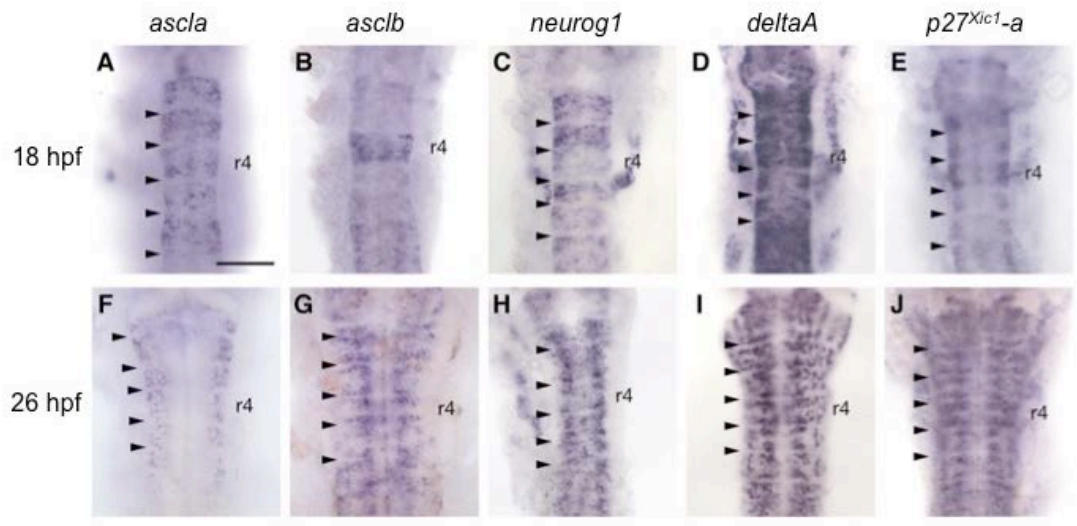

Figure 8. Patterns of neurogenesis in the zebrafish hindbrain. Expression of *asha* or *ascl1a* (A, F), *ashb* or *ascl1b* (B, G), *neurog1* (C, H), *deltaA* (D, I) and *p27xic1-a* (E, J) at 18 hpf (A-E) and 26 hpf (F-J). At both these stages expression is excluded from boundaries (arrowheads). At 18 hpf, transcripts for these genes are detected in all segments. At 26 hpf, stripes of proneural, *deltaA* and *p27xic1-a* gene expression occur adjacent to hindbrain boundaries (*asha*/*ascl1a* is expressed in stripes adjacent to boundaries by 30 hpf). Scale bar: 100 μm. Adapted from Amoyel *et al.*, 2005.

Differentiating neurons are first detected at the center of rhombomeres at 16 hpf, subsequently in other non-boundary regions, and only begin to be seen at rhombomere boundaries at 22 hpf (Trevarrow, Marks and Kimmel, 1990). A subset of neurons located at segment centers express *fgf20*, which is a critical activator of FGF receptor (Fig. 9; Gonzalez-Quevedo *et al.*, 2010). This event triggers a downstream cascade responsible for the precise spatial regulation of neurogenesis in the hindbrain. Thus, neurogenesis inhibition in the center of the rhombomeres is mediated by FGF receptor activation, which upregulates genes including *fgfr2*, *erm*, *sox9* and *cyp26*, downstream of which the gliogenic programme is triggered (Esain *et al.*, 2010).

What initially is a wide field of neurogenesis extended all along the AP extension of the hindbrain except in boundary regions, progressively becomes restricted to zones adjacent to hindbrain boundaries in such a way that by 48 hpf there is an absence of neuronal differentiation in the center of the rhombomeres (Fig. 9; Gonzalez-Quevedo *et al.*, 2010).

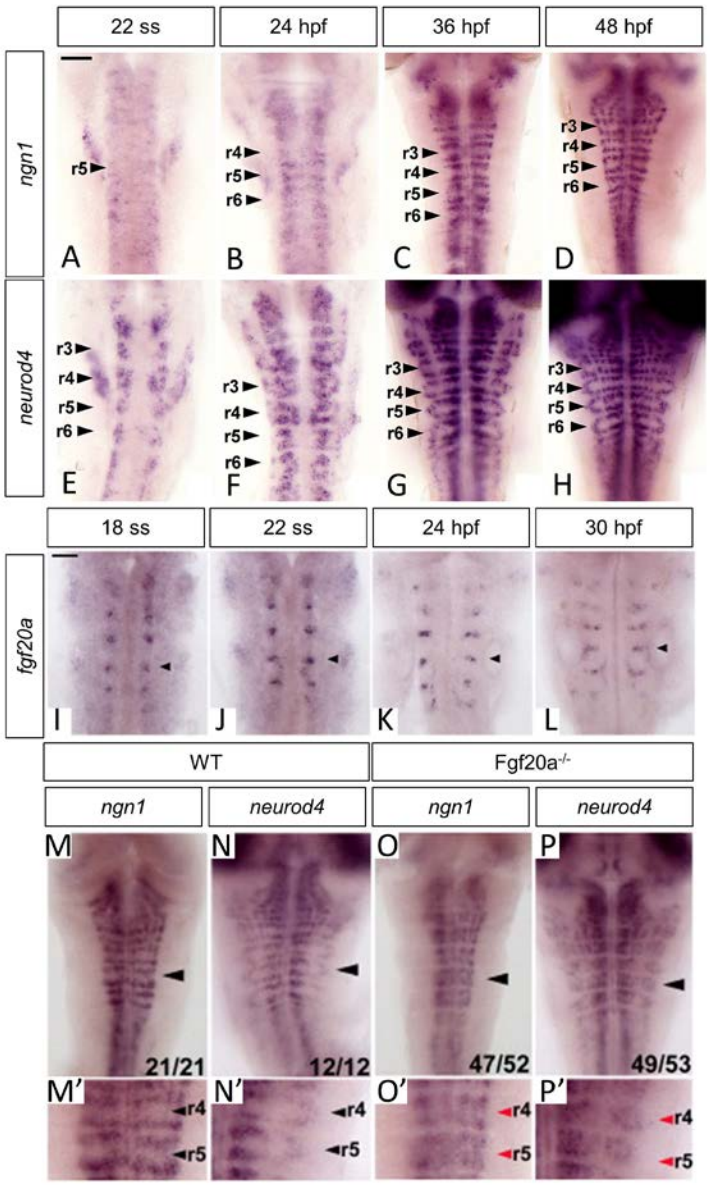

Figure 9. *fgf20a* is required for inhibition of neurogenesis in segment centers. Time course of *neurog1* (A–D) and *neurod4* (E–H) expression from 22 somites to 48 hpf. Arrowheads indicate segment centers. (I-L) Time course of *fgf20a* expression at 18 somites (I), 22 somites (J), 24 hpf (K), and 30 hpf (L). Black arrowheads point at the center of r5 (I-L). *In situ* hybridization of wt (M, N) or *fgf20a* homozygous embryos (O, P). M′–P′ show higher-power images of M–P. *fgf20a* mutant embryos have ectopic neurogenesis in segment centers, detected by *neurog1* (M and O) and *neurod4* expression (N and P). Red arrowheads indicate ectopic neurogenesis in segment centers (O′ and P′). Scale bar: 50 μm for A-P, 20 μm for M′–P′. Adapted from Gonzalez-Quevedo *et al.*, 2010.

As development proceeds, neurons further specify by maturing into a neurotransmitter phenotype. Importantly, neurons that share neurotransmitter phenotype are clustered in stripes that extend along the AP hindbrain of zebrafish larvae. Indeed, the transmitter stripes contain cell types ordered by age as well as structural and functional properties (Kinkhabwala *et al.*, 2011). Interestingly, the neuronal processes appear to be ordered

by age in such a way that processes of older ventral neurons are located dorsally in the neuropil, whereas the dorsal younger neurons have processes located more ventrally in the neuropil (Kinkhabwala *et al.*, 2011). Remarkably, this common structural plan is also functional, since this pattern is tied to behavior given that neurons are recruited along the axis of a stripe as the speed of a motor behavior increases (Kinkhabwala *et al.*, 2011).

In the late 1980s and early 1990s it was shown that a distinct cell population is present at the interface between adjacent rhombomeres (Lumsden and Keynes, 1989; Guthrie and Lumsden, 1991). Further research has shown that hindbrain boundaries are indeed territories devoid of proneural gene expression even under conditions of Notch inhibition (Cheng *et al.*, 2004; Baek *et al.*, 2006). Nevertheless, neurogenesis is detected in hindbrain boundaries upon Notch pathway-related gene dowregulation such as *rfng* in zebrafish (Terriente *et al.*, 2012) or *Hes/Her* genes in mouse (Baek *et al.*, 2006). Indeed, recent findings show that Sox2-postive boundary progenitors are able to giving rise to differentiated neurons in chick hindbrain (see 1.5.3. Hindbrain boundaries are progenitor pools; Peretz *et al.*, 2016). However, what keeps this population in the progenitor state when the rest of the hindbrain is engaged into active neurogenesis and how this cell population transits from being devoid of proneural gene expression to neuronal precursors are questions still to be addressed.

1.4.4. Evolutionary perspective.

In the adult brain, the hindbrain-derived structures are responsible for automatized functions that are basic for survival. Thus, unsurprisingly, this is the most conserved brain territory through evolution. As a matter of fact, a defining feature governing brain development of gnathostomes (vertebrates with jaw) is a highly conserved gene regulatory network that integrates regionalized expression of *Hox* genes with hindbrain segmentation (Parker, Bronner and Krumlauf, 2016). In line with this, segmental patterns of *Hox* expression are found in zebrafish, striped bass and dogfish (Prince *et al.*, 1998; Scemama *et al.*, 2002; Scemama, Vernon and Stellwag, 2006; Oulion *et al.*, 2011) and deep sequence conservation of segmental *Hox* enhancers has been demonstrated (Scemama *et al.*, 2002; Tumpel *et al.*, 2002, 2006).

Cephalochordates, represented by the amphioxus, and urochordates, represented by Ciona, are invertebrate chordates that lack compartmentalization between the prosencephalon, the mesencephalon and the rhomencephalon (Bertrand and Escriva, 2011; Lemaire, 2011). Nevertheless, some neuronal populations and molecular

markers such as *Hox* genes display comparable nested expression pattern to the vertebrate hindbrain (Fig. 10; Holland *et al.*, 1992; Wada, Garcia-Fernàndez and Holland, 1999; Jackman, Langeland and Kimmel, 2000; Knight *et al.*, 2000; Jackman and Kimmel, 2002). Moreover, as in vertebrates, amphioxus *Hox* genes are responsive to RA signaling and are involved in the specification of motoneurons (Schubert *et al.*, 2006; Koop *et al.*, 2010). However, despite the fact that non-vertebrate chordates display patterned *Hox* gene expression along the body axis, key regulatory elements from jawed vertebrate *Hox* clusters are not conserved in amphioxus or ascidians (Manzanares *et al.*, 2000; Schubert *et al.*, 2006; Natale *et al.*, 2011). Moreover, amphioxus hindbrain is not overtly segmented, and appears to lack vertebrate-like segmental expression domains for homologues of *Kreisler*, *Egr2* and *Ephrins* (Fig. 10; Knight *et al.*, 2000). Interestingly, the allocation of motoneurons is in register with the iterated expression domains of some genes such as *Islet*, *Egr2*, *Shox*, *Mnx*, *Err* and *Foxb*, indicating that the hindbrain is pseudosegmented, possibly regulated by signals from the underlying somites (Jackman, Langeland and Kimmel, 2000; Ferrier *et al.*, 2001; Mazet and Shimeld, 2002; Jackman and Kimmel, 2002; Bardet *et al.*, 2005). Thus, segmental patterning of neurons in the neural tube is present in the amphioxus, but the establishment of a bona fide segmented hindbrain may indeed have arisen in the vertebrate lineage.

The agnathans (jawless fish), comprised by the lampreys and the hagfish, are at the base of the phylogenetic vertebrate tree, meaning that they represent the only extant vertebrates that diverged earlier than cartilaginous fish. Importantly, agnathans are the first vertebrates with segmented rhombomeric gene expression (Fig. 10; Kuratani *et al.*, 1998; Horigome *et al.*, 1999). Lamprey *Hox* genes display transient offset segmental expression domains, implying that the lampreys hindbrain, as in gnathostomes, is composed of identifiable rhombomeric segments with an underlying *Hox* code (Parker, Bronner and Krumlauf, 2016). Indeed, a study on the gene networks involved in the segmentation of the hindbrain indicates that many of the *cis*-regulatory elements that control the expression of *Hox* genes and their targets are functionally conserved between mice and lampreys (Parker, Bronner and Krumlauf, 2014). For example, *Kreisler* or *Egr2a* regulatory regions from diverse gnathostomes drive segmental reporter expression in the lamprey hindbrain and require the same transcriptional inputs (Parker, Bronner and Krumlauf, 2014). In conclusion, the coupling of *Hox* gene expression to segmentation of the hindbrain via *Egr2* and *Kreisler* is an ancient vertebrate trait that evolved before the agnathans/gnathostomes split (Parker, Bronner and Krumlauf, 2014). Further evidence for the conservation of hindbrain segmentation

GRN comes from comparative genomics studies, which identified a number of lamprey conserved non-coding elements containing HOX, PBX and MEIS binding-site motifs that drive segmental reporter expression in the hindbrain (Parker *et al.*, 2011, 2014; Grice *et al.*, 2015). Furthermore, previous studies have shown that *Egr2* homologue (*LjEgr2*) is expressed in r3 and r5 in the developing lamprey hindbrain (Murakami, 2004; Jimenez-Guri and Pujades, 2011) and that *LIM*-homeodomain and *Pax* genes reveal highly similar hindbrain patterning between agnathans and gnathostomes (Osorio, Mazan and Rétaux, 2005).

Lampreys have reticulospinal neurons involved in swimming behavior and a rhombomeric organization of RSNs exists (Murakami, 2004). For example, Mauthner cells are present in r4, which indicates that the r4-specific GRN responsible for the generation of this neuronal type is conserved in the vertebrate lineage tree (Murakami, 2004). However, developmental differences exist between agnathans and gnathostomes. In gnathostomes, the motor nuclei of the cranial nerves are generated in correspondence with rhombomeres and each motor root innervates a single branchial arch (Lumsden and Keynes, 1989; Murakami, 2004). However, in lampreys, neuronal specification in the trigeminal and facial motor nuclei of the cranial nerves is not in register with hindbrain boundaries (Murakami, 2004). The trigeminal-facial boundary corresponds to the anterior border of *LjHox3* expression in the middle of r4 (Fig. 10). Interestingly, upon exogenous RA application, the *LjHox3* expression domain and branchiomotor nuclei are rostrally shifted whereas no obvious repatterning of rhombomeric segmentation or reticular neurons is detected (Murakami, 2004).

In conclusion, in lampreys (agnathans), subtype variations of motoneuron identity along the AP axis are not constrained by hindbrain segmentation. On the light of these observations, tissue segmentation and motor nuclei neuron specification have been proposed as two genetic programs working in parallel during hindbrain development. Regarding the emergence of hindbrain pattern formation and segmentation, studies in invertebrate chordates such as the amphioxus reveal the ancestral role of RA in driving nested *Hox* expression along the AP axis. However, analyses in the sea lamprey show that hindbrain segmentation in accordance to an underlying *Hox* code was an ancestral feature of vertebrates. Interestingly enough, most hindbrain-related genes in vertebrates have orthologs in amphioxus (Wada, Garcia-Fernàndez and Holland, 1999; Jackman and Kimmel, 2002; Schubert *et al.*, 2006), which paves the way towards hypothesizing that it is not necessarily their genes that conditioned evolutionary innovations, but most probably it is the way genes are deployed in GRNs. On the whole, the basic architecture of the vertebrate hindbrain originated very early, indeed,

the origin of the AP organization could be dated to the time of an amphioxus-like ancestor. Interestingly, the hemichordate acorn worm *Saccoglossus kowalevskii* expresses *Otx*, *Hox* and *Pax* genes along the AP axis in a vertebrate-like pattern, suggesting that ancient signaling centers for AP patterning already existed in an early deuterostome (Lowe *et al.*, 2003; Pani *et al.*, 2012). Noteworthy, the evolution of cell-lineage restricted compartments seems to have been a later event emerging already in the vertebrate lineage.

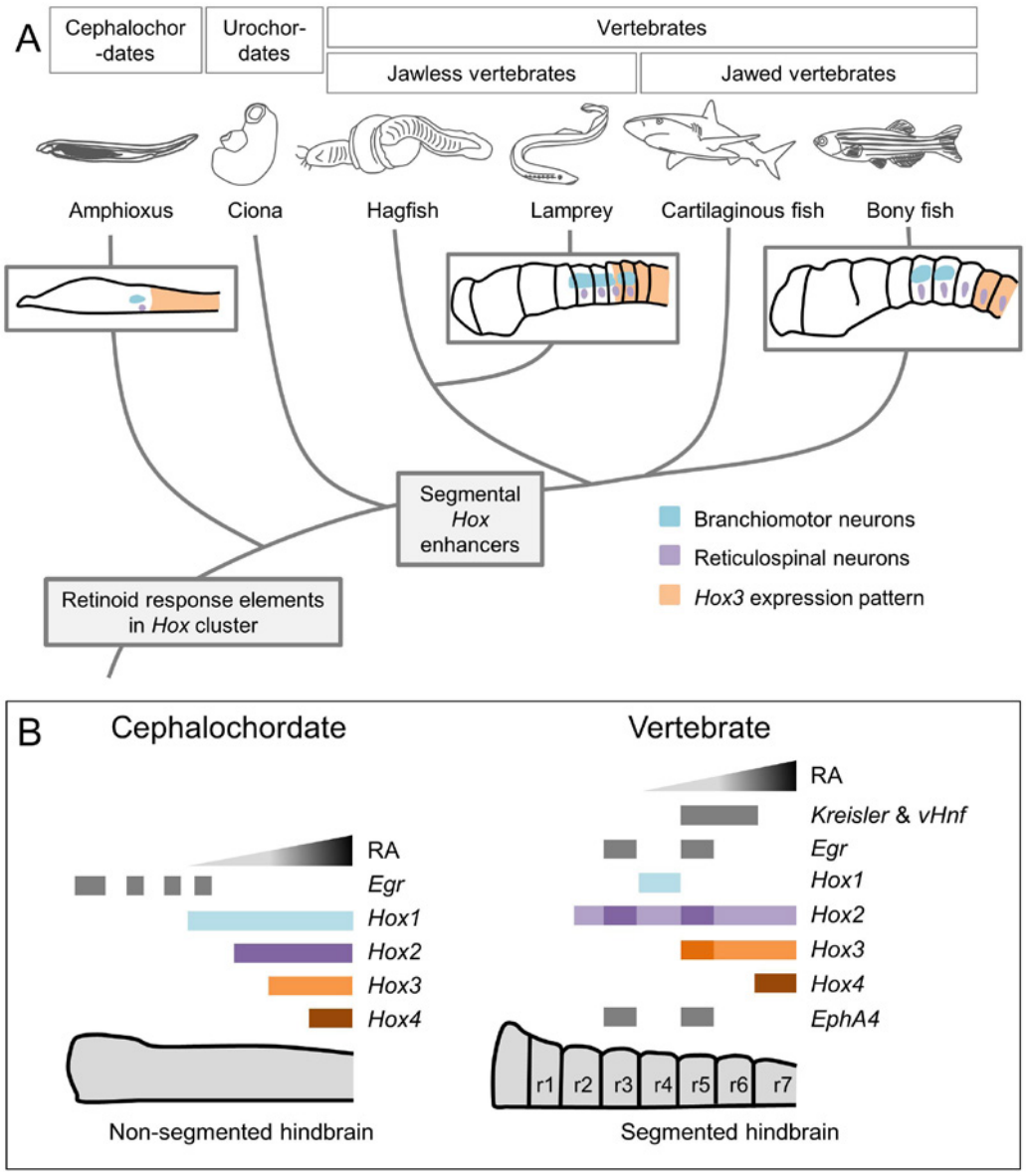

Figure 10. (A) Hypothetical scenario for the hindbrain evolution. Grey boxes are the derived characters in the hindbrain developmental plan recognized to each segment of the evolutionary lineage. White boxes display the distribution of reticulospinal neurons, motoneurons and *Hox3* gene expression pattern in the hindbrain of the corresponding lineage. (B) Comparison of hindbrain gene expression between vertebrates and invertebrate chordates. Patterns are aligned with the segmented vertebrate hindbrain and its unsegmented homologue in cephalochordates. Adapted from Murakami, 2004 and Parker, Bronner and Krumlauf, 2016.

1.5. THE HINDBRAIN BOUNDARY CELL POPULATION.

Compartment establishment through segmentation is a crucial event in hindbrain development (Keynes and Lumsden, 1990; Lumsden, 1990). Once the gene expression borders are established after patterning determinants have instructed the AP coordinates along the hindbrain and Eph/ephrin signaling is active at rhombomere interfaces, the hindbrain boundary cell population (BCP) is specified between adjacent rhombomeres in register with gene expression borders. In zebrafish embryonic hindbrain, the first morphological boundaries to appear are r3/r4 and r4/r5 and they do it between five to seven somite stage (around 12 hpf; Fig. 11), whereas the last one appears at about 17 hpf (Fig. 11; Moens *et al.*, 1998). However, boundary markers start being detected from 16 hpf onwards (Fig. 11; Cheng *et al.*, 2004).

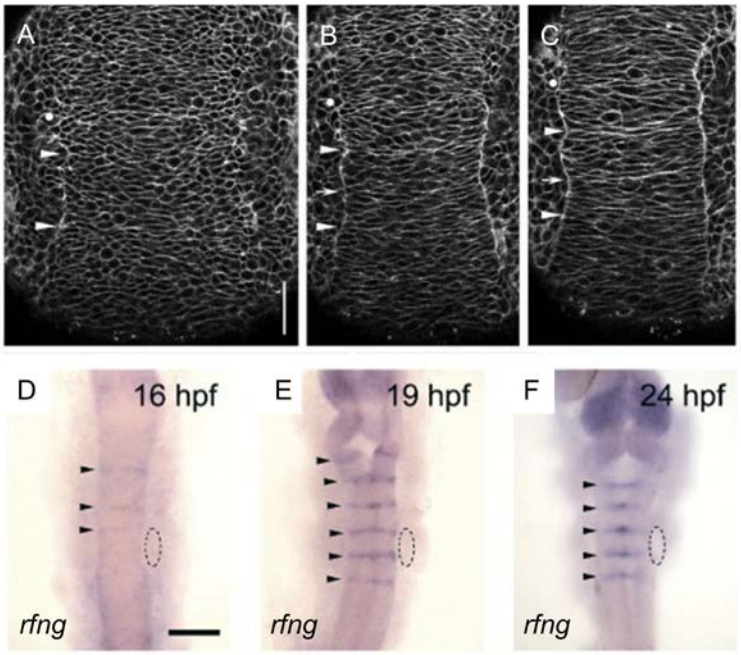

Figure 11. Time-lapse analysis of rhombomere boundary formation. (A-C) Confocal time-lapse images of a single BODIPY-stained embryo at approximately 6 somites (12 hpf; A), 8 somites (13 hpf; B) and 10 somites (14 hpf; C), in dorsal view with anterior to the right. During this interval, the r5/r6 boundary becomes visible (arrow) between the r4/r5 and r6/r7 boundaries (arrowheads). In all panels, the r3/r4 boundary is marked by a dot, the r4/r5 and r6/r7 boundaries by arrowheads, and the r5/r6 boundary by an arrow. Scale bars = 50 μm. In situ hybridizations were carried out to detect transcripts of (D-F) *rfng*, from 16 hpf to 24 hpf as indicated. Arrowheads indicate rhombomere boundaries and a dashed circle indicates the otic vesicle. Adapted from Moens *et al.*, 1998 and Cheng *et al.*, 2004.

Noteworthy, most lineage restriction borders described both in vertebrates and insects are associated with signaling centers (Kiecker and Lumsden, 2005). In this sense, hindbrain boundaries display two main conserved functions from teleosts to mammals,

since they serve as both physical barriers and signaling centers. However, it is important to note that despite the fact that these functions are conserved, different molecular players are involved.

In chick, hindbrain boundary cells have enriched extracellular matrix (ECM) (Guthrie and Lumsden, 1991; Heyman, Kent and Lumsden, 1993; Heyman, Faissner and Lumsden, 1995), a fan-shaped morphology (Lumsden and Keynes, 1989; Heyman, Kent and Lumsden, 1993), slow proliferation rate and reduced interkinetic nuclear migration (Guthrie, Butcher and Lumsden, 1991), enriched expression of *Fgf3* and *Pax6* (Sela-Donenfeld, Kayam and Wilkinson, 2009; Prin *et al.*, 2014) and they are a Sox2-positive pool of neural progenitor cells (Peretz *et al.*, 2016). In line with this, slow proliferation rate has been also described for mouse hindbrain boundary cells (Baek *et al.*, 2006).

In zebrafish, hindbrain boundaries have enriched expression of *foxb1.2* (Fig. 12; Riley *et al.*, 2004), *sempahorins* (Cooke, Kemp and Moens, 2005; Terriente *et al.*, 2012), *wnt1* (Riley *et al.*, 2004), *rfng* (Fig. 12; Cheng *et al.*, 2004), *sgca* (Letelier *et al.*, 2018), *rac3b* (Fig. 12; Letelier *et al.*, 2018) and GTP-binding protein regulators such as *arhgap29b* and *rasgef1ba* (Fig. 12; Letelier *et al.*, 2018). Morphology wise, non-boundary hindbrain cells are spindle-shaped whereas boundary cells display a triangular shape with an enlarged apical or basal domain (Gutzman and Sive, 2010).

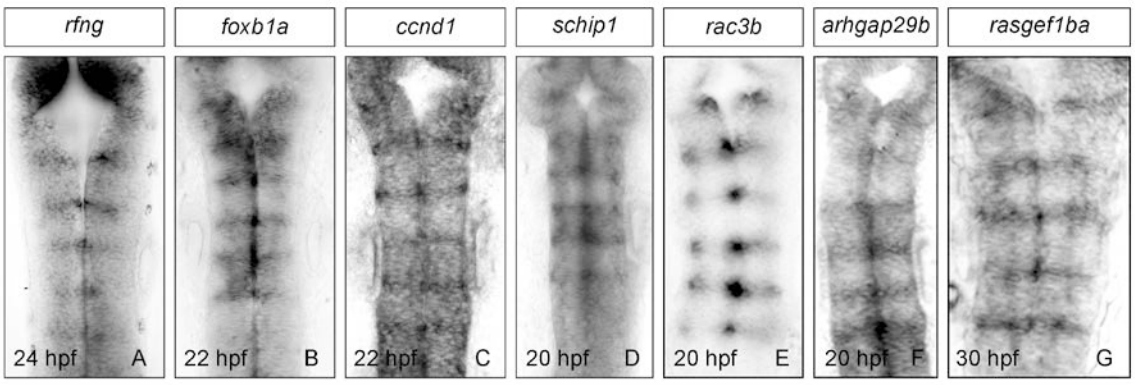

Figure 12. Genes expressed in hindbrain boundary cells. Hindbrain dorsal views of *in situ* hybridization for (A) *rfng*, (B) *foxb1a*, (C) *ccnd1*, (D) *schip1*, (E) *rac3b*, (F) *arhgap29b* and (G) *rasgef1ba*.

Little is known regarding the molecular mechanisms that regulate boundary cell specification. On the one hand, Eph/ephrin signaling is required for the expression of BCP markers (Cooke, Kemp and Moens, 2005) but whether ectopic Eph/ephrin interfaces can induce the specification of boundary markers is still to be addressed. On

the other hand, *rfng* is a good candidate to be involved in boundary cell maintenance because its expression is restricted to rhombomere boundaries (Cheng et al., 2004; Qiu et al., 2004) and, by glycosylating Notch, Rfng promotes Notch activation in other contexts (Irvine and Wieschaus, 1994). Interestingly, upon *rfng* downregulation, the boundary expression of *her9*, *sema3gb* and *sema3fb* decreases (Terriente *et al.*, 2012). However, whether *rfng* is a modulator of Notch activity in hindbrain boundaries remains elusive. Indeed, it has been suggested that Notch signaling is required for the maintenance of the boundary cell population but not for its specification: ectopic activation of DN-CSL results in the repression of BCP specification but activation of DA-CSL does not induce ectopic boundary cell fate (Cheng *et al.*, 2004). However, these experiments were carried out in injected embryos at the eight-cell stage or in *mind bomb* (*mib*) mutants, which display a strong Notch pathway deficiency (Jiang *et al.*, 1996) due to mutation of a ubiquitin ligase required for Delta ligand activity (Itoh *et al.*, 2003). In both cases, a constitutive interference with Notch signaling might be misleading the interpretation of the role of the pathway at the specific temporal window for BCP specification. To better address this issue, it will be important to bear in mind temporal coordinates such as Notch activity onset in different hindbrain territories. Thus, the role of Notch signaling pathway under conditional temporal windows and its effect on boundary cell specification and fate are questions in the field with room for further research.

In summary, the BCP is endowed with many molecular and architectural characteristics that differentiate them from the rest of hindbrain neuroepithelial cells. This uncanny cell population is specified at the interface between adjacent rhombomeres; nevertheless, the BCP not only demarks the gene expression limits in the hindbrain, but they do display several biological functions during the embryonic development of the posterior brain. Indeed, hindbrain boundaries are i) elastic mechanical barriers, ii) signaling centers and iii) progenitor pools.

1.5.1. Hindbrain boundaries are mechanical barriers.

The establishment of physically isolated tissues through cell segregation is essential for restricting cell mixing between adjacent territories upon cell proliferation. Cortical tension provided by actomyosin structures at compartment boundaries plays a fundamental role in keeping cells sorted in several systems, from *Drosophila* to vertebrates (Major and Irvine, 2005, 2006; Monier *et al.*, 2010; Calzolari, Terriente and Pujades, 2014; Terriente and Pujades, 2015). In the hindbrain interhombomeric boundaries, the Eph/ephrin signaling pathway instructs cell sorting by means of cell

adhesive properties modulation (see Box 2). Nevertheless, as development proceeds, this cell population assembles a specific actomyosin cable-like structure that provides the tissue with elastic properties required for restricting cell intermingling (Calzolari, Terriente and Pujades, 2014; Letelier *et al.*, 2018).

These actomyosin structures are present along the DV axis of hindbrain boundaries and are located in the apical side of both hemispheric cell rows that integrate the neural tube (Calzolari, Terriente and Pujades, 2014). The assembly of these cable-like structures is downstream of Eph/ephrin signaling and is RhoA-GTPase-dependent (Calzolari, Terriente and Pujades, 2014). The main challenge to compartment stability is cell division: dividing cells, upon mitotic rounding, incur in the adjacent territory and challenge the stability of the interhombomeric interface, which acts as an elastic mesh that bars these incurring cells from the adjacent compartment (Fig. 13; Calzolari, Terriente and Pujades, 2014). Hence, actomyosin structures assembly in hindbrain boundaries is necessary for generating tension at compartment interfaces keeping rhombomeric cell lineage restriction (Calzolari, Terriente and Pujades, 2014; Letelier *et al.*, 2018).

We have recently described that the evolutionary origin of the actomyosin-based cell-sorting mechanism depended on the cooption of critical genes to a novel regulatory block. The small-GTPase *rac3b*, which displays an enriched expression in hindbrain boundaries, is one of the players responsible for assembling the actomyosin cables (Letelier *et al.*, 2018). Interestingly, *rac3b* is located in chromosome 12 in synteny with *rfng* and *sgca*, both also expressed in hindbrain boundaries (Thisse *et al.*, 2004; Skromne *et al.*, 2007). This *rac3b*/*rfng*/*sgca* regulatory cluster emerged in Ostariophysi superorder upon the generation of a new *cis*-regulatory interaction by chromosomal rearrangement, resulting in the functional refinement of hindbrain segmentation (Letelier *et al.*, 2018).

1.5.2. Hindbrain boundaries are signaling centers.

Throughout evolution, compartment boundaries have served as signaling centers responsible for regulating and organizing gene expression in adjacent domains, controlling distinct fates and the dynamics of neurogenesis through the secretion of signaling factors (Kiecker and Lumsden, 2005). Two well-characterized organizing boundaries in the developing brain are the *Zona Limitans Intrathalamica* (ZLI), between the thalamic and prethalamic primordia, and the mid-hindbrain boundary (MHB), between the mesencephalon and the anterior hindbrain. The former expresses sonic

hedgehog (SHH), Wnts and fibroblast growth factors (FGFs) and regulates the specification of prethalamic and thalamic neurons (Scholpp, 2006; Guinazu *et al.*, 2007; Lim and Golden, 2007). The latter secretes Wnt1 and Fgf8 and is involved in midbrain development and cerebellum formation (Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Rhinn, Picker and Brand, 2006; Dworkin and Jane, 2013). In the hindbrain, interhombomeric boundary cells express signaling molecules such as FGF in mouse and chick or Wnts and Semaphorins in zebrafish that instruct the development of the adjacent rhombomeres.

During hindbrain embryogenesis, several members of the FGF superfamily and their receptors are expressed and act in pattern formation (Mahmood *et al.*, 1995; Marín and Charnay, 2000; Walshe and Mason, 2000; Hatch *et al.*, 2007; Lunn *et al.*, 2007; Weisinger, Wilkinson and Sela-Donenfeld, 2008; Aragon and Pujades, 2009; Weisinger *et al.*, 2010; Labalette *et al.*, 2011). In amniotes, FGF3 mediates the expression of molecules responsible for ECM integrity, neuronal differentiation and axonal organization at boundary cells.

On the other hand, in teleosts, Wnts seem to be the main signaling molecules expressed in hindbrain boundaries. The role of Wnt signaling in zebrafish hindbrain boundaries was initially associated to the regulation of neurogenesis in non-boundary territories, which had an effect on preventing the spreading of boundary cell identity out of boundary regions (Riley *et al.*, 2004; Amoyel *et al.*, 2005). However, further research unveiled that *wnt1* morphant embryos display extensive p53-dependent cell death and that the ectopic expression of boundary markers was associated to a p53-dependent non-apoptotic role of *puma* and *bax-a*, pro-apoptotic genes found to be required for hindbrain boundary marker expression (Gerety and Wilkinson, 2011). Thus, despite the fact that *wnt1*, *wnt8b* and *wnt3a* are enriched in zebrafish hindbrain boundaries, their functional relevance awaits further research.

During CNS development, neural cells are clustered and positioned in specific neurogenic and non-neurogenic niches. Interestingly, boundary cells express the semaphorins *sema3fb* and *sema3gb*, which are responsible for clustering *fgf20*-expressing neurons in rhombomeric centers (Fig. 13; Terriente *et al.*, 2012). The spatial organization of neurogenesis within hindbrain metameres in zebrafish has been proven FGF-mediated, since signaling from *fgf20a*-expressing neurons located at segment centers inhibit neurogenesis in the adjacent ventricular progenitor domain; as consequence, neurogenesis is confined to boundary flanking regions (Gonzalez-Quevedo *et al.*, 2010). Thus, despite the fact that we are still missing information

regarding hindbrain boundaries as signaling centers, we can already catch sight of how hindbrain segmentation along with the respective boundary populations is necessary to coherently organize the spatial distribution of neuronal niches.

1.5.3. Hindbrain boundaries are progenitor pools.

At early developmental windows of hindbrain development, when most of hindbrain neuroepithelial cells are engaged into neurogenic and gliogenic programs, hindbrain boundaries are kept undifferentiated (Amoyel *et al.*, 2005; Baek *et al.*, 2006; Gonzalez-Quevedo *et al.*, 2010). Furthermore, based on previous observations, it is hypothesized that neurogenesis is either delayed or does not occur in boundaries (Lumsden and Keynes, 1989; Guthrie, Butcher and Lumsden, 1991; Kahane and Kalcheim, 1998; Trokovic *et al.*, 2005).

On one hand, mouse hindbrain boundaries express high levels of *Hes1*, a neurogenesis repressor-type bHLH gene, and do not express proneural bHLH genes (Baek *et al.*, 2006). Upon *Hes* genes downregulation, proneural bHLH genes are ectopically expressed in hindbrain boundaries, which result in ectopic neurogenesis and in the impairment of the organizer role of hindbrain boundaries (Baek *et al.*, 2006). Thus, high levels of *Hes1* expression are required for keeping hindbrain boundaries as neuron-free zones and organizing centers. Interestingly, the *Hes1*-otholog *her9* is enriched in zebrafish hindbrain boundaries (Radosevic *et al.*, 2011), whether *her9* is involved in maintaining these neuroepithelial cells undifferentiated awaits further research.

On the other hand, in chick hindbrain there is a restriction of Sox2-positive cells in the boundaries as development proceeds and two subpopulations have been described: i) Sox2-positive cells that provide proliferating progenitors to adjacent rhombomeres, and ii) Sox2-positive cells that differentiate and give rise to neurons that will end up located in the boundary mantle zone (Fig. 13; Peretz *et al.*, 2016).

On the whole, two different strategies, not necessarily mutually exclusive, involved in keeping hindbrain boundaries in the progenitor state at early stages have been described in different model organisms. Nevertheless, as development proceeds, these cells seem to acquire the capacity of becoming neurogenic. How boundary progenitors fine-tune proliferative behavior and neurogenesis inhibition and induction according to the developmental temporal window remains to be uncovered.

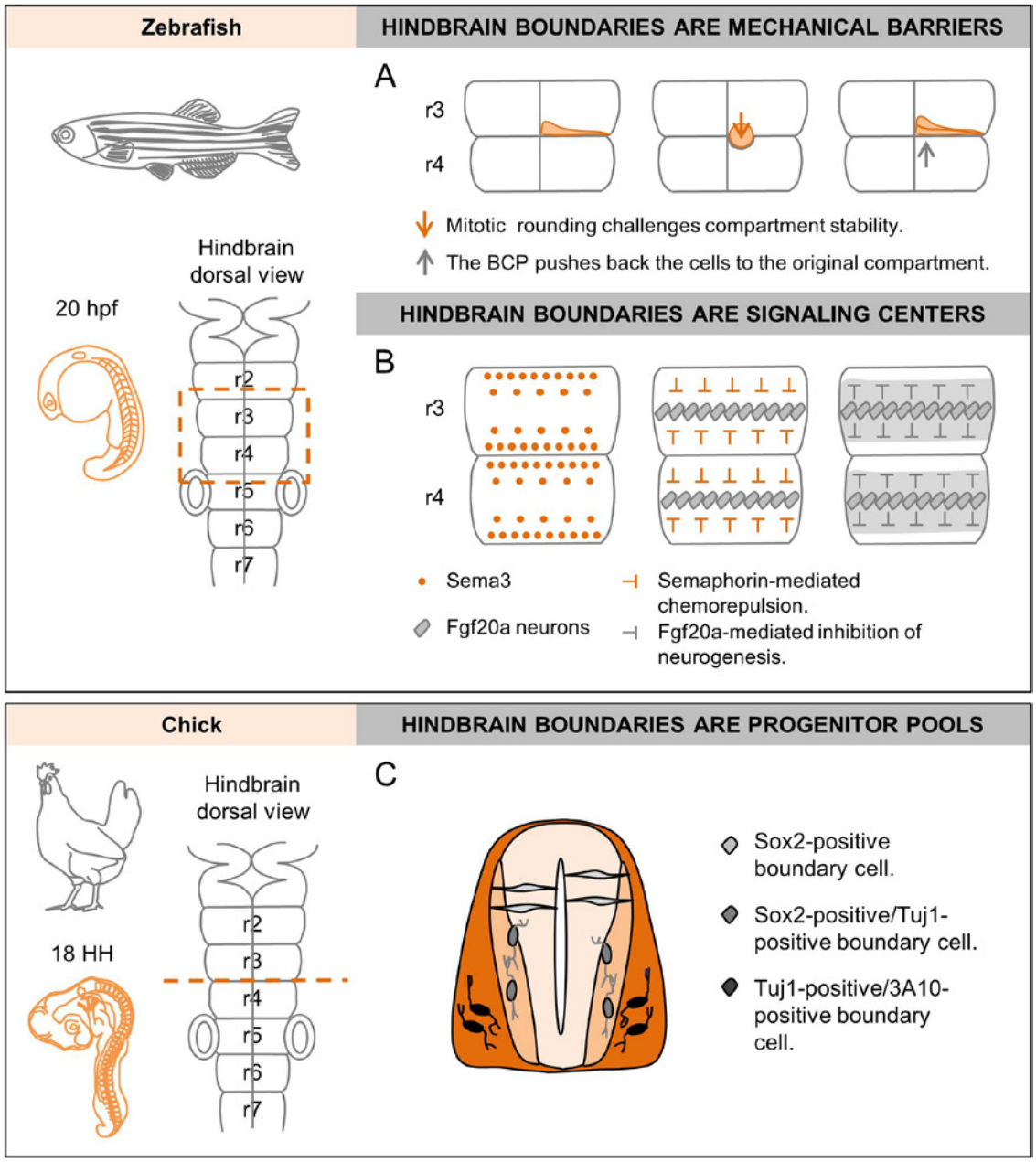

Figure 13. Functions of the hindbrain boundary cell population. (A) Hindbrain boundary cells are mechanical barriers. Dividing cells challenge the stability of the boundary, which is responsible for pushing back incurring cells to the compartment of origin. (B) Hindbrain boundary cells are signaling centers. Boundary-expressed Sema3 instructs the positioning of *fgf20a*-expressing neurons in rhombomere centers, which inhibit neurogenesis in segment centers, confining it to boundary flanking regions. (C) Hindbrain boundary cells are neuronal progenitors. Transverse view of the boundary r3/r4 at st. 18 chick hindbrain. According to the current model, boundaries consist of slow dividing Sox2-positive/Transitin-positive/GFAP-positive progenitors. Hindbrain neural differentiation occurs on the ventricular-to-mantle axis as cells lose progenitor markers and acquire neural markers (Tuj1/3A10) during migration to mantle zone. Adapted from Terriente *et al.*, 2012, Calzolari, Terriente and Pujades, 2014 and Peretz *et al.*, 2016.

1.6. YAP AND TAZ IN DEVELOPMENT AND MECHANOTRANSDUCTION.

Yes-associated protein (YAP) and its homolog WW domain-containing transcription factor (WWTR1 or TAZ) are transcriptional co-activators acting as an integrator nexus for multiple prominent pathways that play key roles in the control of cell division, differentiation and cell death in numerous tissues, including the nervous system (Hansen, Moroishi and Guan, 2015). YAP and TAZ regulation is best understood under the scope of Hippo kinase cascade, initially identified through genetic mosaic screens for suppressors of tissue overgrowth in *Drosophila melanogaster* (Oh and Irvine, 2010; Pan, 2010). Thus, Hippo signaling cascade is important for controlling organ size and tissue homeostasis through the regulation of cell proliferation, apoptosis and tissue regeneration. Not surprisingly, deregulation of the pathway has been implicated in varieties of cancers and diseases (Plouffe, Hong and Guan, 2015).

1.6.1. YAP and TAZ regulation and the hippo core.

The core components of the Hippo pathway, the kinase Hippo (Hpo, or MST1 and MST2 in vertebrates), the kinase Warts (Wts, or LATS1 and LATS2 in vertebrates) and the effector Yorki (Yki, or YAP and TAZ in vertebrates), are highly conserved from *Drosophila* to mammals (Hilman and Gat, 2011; Sebé-Pedrós *et al.*, 2012). Despite the conservation of the core players, it is interesting to note that the upstream regulators of the pathway seem to be divergent (Hansen, Moroishi and Guan, 2015). A representative example is Dachs, an essential regulator of Wts by Ds-Fat signaling in *Drosophila* (Cho *et al.*, 2006), which is not conserved in vertebrates (Bossuyt *et al.*, 2014). Importantly, the initiating signals of the pathway are multiple and include mechanical forces, cellular stress, cellular polarity and cell-cell contact (Pan, 2007; Genevet *et al.*, 2009; Hamaratoglu *et al.*, 2009; Dupont *et al.*, 2011; Zhao *et al.*, 2011; Halder, Dupont and Piccolo, 2012; Gumbiner and Kim, 2014; Hansen, Moroishi and Guan, 2015). As consequence, YAP and TAZ have emerged as key players in sensing mechanical cues and transducing them into cell-specific transcriptional programs (see 1.6.3. Mechanobiology of YAP and TAZ; Panciera *et al.*, 2017).

Hippo kinase cascade activation results in the phosphorylation of YAP and TAZ, which inhibits their nuclear import either by triggering their degradation or by retaining them in the cytoplasm. Thus, YAP and TAZ co-regulators shuttle between the cytoplasm and the nucleus where they interact with the DNA-binding TEA domain family members 1-4 (TEAD1-4, Scalloped (Sd) in *Drosophila*) to control the expression of their targets

(Vassilev *et al.*, 2001; Wu *et al.*, 2008; Zhang *et al.*, 2008; Zhao *et al.*, 2008). The mode of action is very similar between *Drosophila* and vertebrates (Fig. 14). The upstream kinases of the cascade (Hippo or MST1/2) heterodimerize with the adaptor protein Salvador 1 (SAV1 in vertebrates). As a result, there is an enhancement of MST1/2 kinase activity and a facilitation of the interaction between MST1/2 and LATS1/2, the following kinases in the cascade (Tapon *et al.*, 2002; Callus, Verhagen and Vaux, 2006). MST1/2 phosphorylate and activate MATS/MOB1, which binds to the autoinhibitory region of Wts/LATS1/2, enabling its phosphorylation and hence its activation (Chan *et al.*, 2005; Praskova, Xia and Avruch, 2008). Activated Wts/LATS1/2 phosphorylates and inactivates the main effectors of the pathway, Yki/YAP/TAZ, by cytoplasmic retention and eventually ubiquitination and degradation (Huang *et al.*, 2005; Zhao *et al.*, 2007; Lei *et al.*, 2008; Oh and Irvine, 2008; Liu *et al.*, 2010; Zhao Li, L., Lei, Q. and Guan, K. L., 2010; Yu *et al.*, 2014). In this regard, phosphorylation of different serines may trigger different responses. For example, phosphorylation of human YAP at serine 127 results in binding to 14–3–3 proteins and cytoplasmic retention (Basu *et al.*, 2003; Zhao *et al.*, 2007, 2010), whereas phosphorylation of YAP at serine 381 leads to its ubiquitination and proteolytic degradation (Zhao *et al.*, 2010).

On the other hand, when Wts/LATS1/2 is inactive, Yki/YAP/TAZ are not phosphorylated and can translocate into the nucleus, bind to Sd/TEAD and initiate expression of target genes regulating proliferation, differentiation and apoptosis (Kanai *et al.*, 2000; Dong *et al.*, 2007; Oh and Irvine, 2008; Ren, Zhang and Jiang, 2010). Thus, Yki/YAP/TAZ regulate the expression of genes associated to cell cycle control such as genes coding for proteins involved in DNA replication and repair, cyclins and their regulators, mitotic kinases and factors required for completion of mitosis (Nicolay *et al.*, 2011; Mizuno *et al.*, 2012; Kapoor *et al.*, 2014; Zanconato *et al.*, 2015). In addition, Yki/YAP/TAZ also indirectly control cell proliferation through the induction of transcriptional regulators of the cell cycle (Kapoor *et al.*, 2014; Zanconato *et al.*, 2015). Yki/YAP/TAZ target genes also comprise *Ctgf*, *Cyr61*, *Axl* and anti-apoptotic genes of the *Bcl2* and *IAP* families. Importantly, these genes may explain YAP/TAZ-driven chemoresistance (Lai *et al.*, 2011; Lin *et al.*, 2015; Schoumacher and Burbridge, 2017) and protection against apoptosis (Johnson and Halder, 2014; Zanconato, Cordenonsi and Piccolo, 2016).

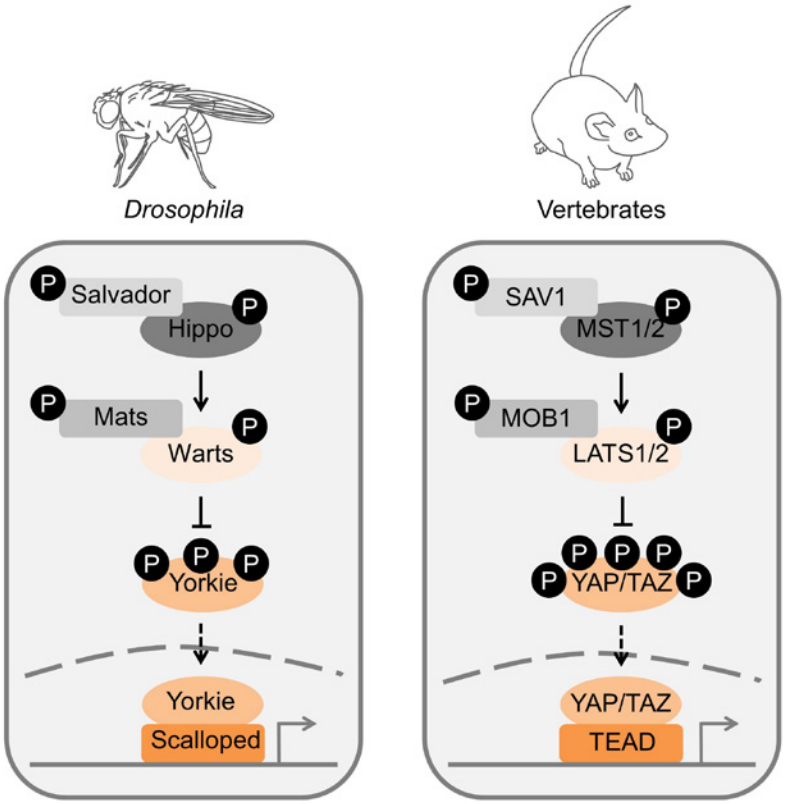

Figure 14. Overview of the Hippo signaling pathway in *Drosophila* and vertebrates. Hippo signaling is initiated by a variety of upstream stimuli. Activation of Hpo/MST1/2 leads to subsequent phosphorylation of Wts/LATS1/2. Wts/LATS1/2 negatively regulates the Hippo pathway effectors Yki/YAP/TAZ. Unphosphorylated Yki/YAP/TAZ translocates into the nucleus where it interacts with its Sd/TEAD transcription factors to upregulate the transcription of a variety of genes. In contrast, phosphorylation of Yki/YAP/TAZ by Wts/LATS1/2 leads to its cytoplasmic sequestration by 14-3-3 proteins and degradation. Adapted from Taha, Janse van Rensburg and Yang, 2018.

1.6.2. YAP and TAZ activity and the development of the nervous system.

The development of the nervous system requires a tightly controlled balance between expansion of neural progenitors and differentiation. Disruption of this equilibrium results in multiple abnormalities and disorders of the nervous system.

During normal development, the Hippo pathway modulates progenitor and stem cell behavior. For example, *Yap* is highly expressed in cultured embryonic stem cells and is required for self-renewal and suppression of differentiation (Lian *et al.*, 2010). Another paradigm for YAP-mediated cell self-renewal is found in mouse intestine, where *Yap* expression is restricted to progenitor cells. Transgenic mice displaying *Yap*

overexpression in the intestine exhibit significant expansion of undifferentiated progenitor cells in the crypt, which then undergo differentiation upon reduction of *Yap* expression (Cai, Zhang and Zheng, 2010). Furthermore, *Sav1* knockout impairs the growth arrest of epithelial progenitors during embryonic development in epidermis and intestine by preventing the activation of MST1 and thus inducing a significant decrease in LATS and YAP phosphorylated forms (Lee *et al.*, 2008). Last but not least, modulation of YAP or the MST1/2 kinases in mice can alter organ size (Dong *et al.*, 2007; Song *et al.*, 2010).

The molecular mechanisms that restrict neural progenitor expansion and the role of YAP/TAZ activity during brain development are poorly understood. In the developing nervous system, YAP/TAZ regulate progenitor expansion and/or fate decisions in a context-dependent way. In the developing chicken spinal cord, YAP regulates progenitor cell number via TEAD-mediated *CyclinD1* expression and inhibition of differentiation by suppressing *NeuroM* (Cao *et al.*, 2008). Thus, YAP over-expression induces the expansion of neural progenitors and inhibits neuronal differentiation (Cao *et al.*, 2008).

YAP regulates neural progenitor cell numbers not only in the developing neural tube of chick, but also in *Xenopus* neural plate (Gee *et al.*, 2011) and mouse hippocampus (Lavado *et al.*, 2013). In the latter case, the tumor suppressor neurofibromatosis 2 (NF2; merlin) limits the expansion of neural progenitor cells (NPCs) by inhibiting YAP/TAZ (Lavado *et al.*, 2013). Indeed, NF2/Merlin is a potent upstream inducer of the Hippo pathway serving as a scaffold for the core Hippo kinases at cell-cell junctions in epithelial cells (Lallemand *et al.*, 2003; Yin *et al.*, 2013). Importantly, neurofibromatosis type 2 (NF2) is an autosomal dominant disorder characterized by the development of nervous system tumors caused by inactivating mutations of the gene NF2/Merlin (Baser et al., 2003). Thus, NF2/Merlin mouse mutants display an expanded neural progenitor cell population in the hippocampus and the cortical hem (the hippocampal organizer) (Lavado *et al.*, 2013). Remarkably, brain tissues lacking NF2 display enhanced nuclear localization of YAP/TAZ and upregulation of their target genes (Lavado *et al.*, 2013)

Despite the fact that YAP/TAZ play a role in modulating progenitor proliferation in certain territories of the developing nervous system, they can be involved in other functions. For example, in the mouse neocortex, YAP in neural stem cells is involved in neocortical astrocytic differentiation and, interestingly, it also plays a role in differentiated astrocyte proliferation (Huang *et al.*, 2016).

As shown by the genetic characterization of human developmental eye disorders, Yap and TEAD play also a role in eye development. Loss-of-function mutations in *Yap1* can result in autosomal dominant coloboma and a mutation within the YAP-binding domain of TEAD1 causes Sveinsson's chorioretinal atrophy (SCRA), an autosomal dominant loss of retinal pigmented epithelium (RPE), choroid, and photoreceptors radiating from the optic nerve head (Fossdal et al., 2004; Williamson et al., 2014). However, the mechanisms underlying these defects remain unknown.

In the neonatal mouse retina, YAP protein is detected within the progenitor cell nuclei and its activity promotes cell proliferation and, upon RNAi inhibition of YAP, progenitor proliferation is reduced and differentiation promoted (H. Zhang *et al.*, 2012). Interestingly, *in vitro* assays unveil that a reciprocal inhibitory interaction between bHLH proteins and YAP may contribute to the overall regulation of neurogenesis, since proneural bHLH proteins can antagonize YAP function, both at the level of *Yap* expression and via the LATS1/2 kinases. Moreover, YAP can prevent cell cycle exit induced by proneural bHLH proteins (H. Zhang *et al.*, 2012).

During zebrafish eye development, YAP/TAZ-TEAD signaling is mainly involved in cell fate acquisition since YAP activity is crucial for optic vesicle progenitors to become RPE progenitors, as shown by the lack of RPE cells in *yap* mutants (Miesfeld *et al.*, 2015). Importantly, optic vesicle proliferation and apoptosis are unaffected in these mutants (Miesfeld *et al.*, 2015).

Thus, in the nervous system YAP/TAZ act as modulators of fate choice and neural progenitor cell number by controlling proliferation. However, although abundant evidences clearly establish the importance of YAP/TAZ-activity in nervous system development, the impact of the mechanical microenvironment, described as one of the major upstream conditioners of YAP/TAZ-activity, on the developmental role of these transcription factors remains largely unaddressed.

1.6.3. Mechanobiology of YAP and TAZ.

Mechanobiology addresses how mechanical stimuli are transformed into a biological response through the activation of genetic programmes. Living cells perceive physical stimuli through several mechanosensitive molecules at the cell membrane including integrins, stretch-activated ion channels, G protein coupled-receptors and growth factor receptors. Once a mechanical cue is sensed, the intracellular molecular process that transforms it into a biological response is called mechanotransduction (Martino *et al.*, 2018).

Remarkably, the propagation of external mechanical cues and cell-generated forces is conveyed by the regulation of cytoskeleton tension (Discher, Janmey and Wang, 2005; Martino *et al.*, 2018). However, the molecular processes that link mechanical forces and the expression of mechanosensitive genes that allows the cell to adapt to the new conditions are still largely unknown (Dahl and Kalinowski, 2011; Martino *et al.*, 2018).

An example of how cells can respond to mechanical conditioning is given by reports in which cultured cells display specific responses to substrate stiffness such as the induction of cell proliferation in endothelial cells (Yeh *et al.*, 2012), airway smooth muscle cells (Shkumatov *et al.*, 2015) and dermal fibroblasts (Razinia *et al.*, 2017). Indeed, tissue stiffness is associated to diseased conditions and as a prognostic factor in cancer progression (Wei and Yang, 2016; Reid *et al.*, 2017; Martino *et al.*, 2018). Importantly, physical inputs not only impact on the proliferative behavior of the sensing cells, but also on cell fate decisions. As a paradigm, cell body confinement on micropatterned surfaces has been shown to control stem cell commitment to specific lineages. In agreement with this, single mesenchymal stem cells constrained on these surfaces commit to adipogenic fate, whereas osteoblastic lineage is primed when these cells grow on islands allowing cell spreading (McBeath *et al.*, 2004). Strikingly, recent data points towards a new model in which multiple types of mechanical inputs in a variety of cell types rely on the regulation of YAP and TAZ. Thus, YAP/TAZ mechanobiology has emerged as a recent branch of study in the field (Fig. 15; Gaspar and Tapon, 2014; Panciera *et al.*, 2017).

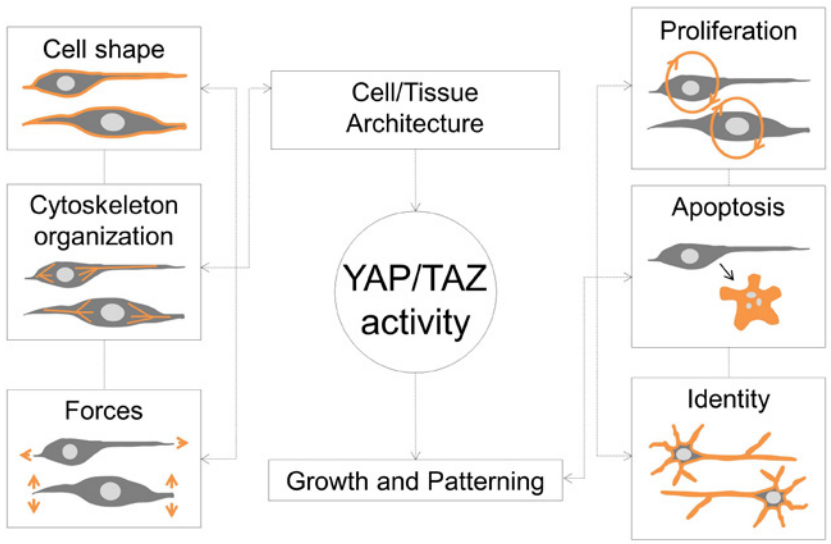

Figure 15. YAP/TAZ activity links mechanical stimuli and cell fate regulation. Upstream architectural features such as cell shape, cytoskeletal tension and tissue forces modulate YAP/TAZ activation. In its turn, YAP/TAZ activity target genes can be related to cell self-renewal, apoptosis inhibition and cell fate regulation.

Remarkably, the relationship between YAP/TAZ and the upstream Hippo core is context-dependent. In vertebrates, as well as in *Drosophila*, Wts/LATS proteins are the major regulators of Yki/YAP/TAZ (Lai *et al.*, 2005; Das Thakur *et al.*, 2010; Yin *et al.*, 2013; Rauskolb *et al.*, 2014; Sun, Reddy and Irvine, 2015; Vrabioiu and Struhl, 2015). The current convention establishes that the canonical signaling of Yki/YAP/TAZ regulation is LATS-dependent. In contrast, non-canonical signaling refers to LATS-independent regulatory scenarios. This distinction has proven useful in the context of mechanotransduction, since both types of strategies operate in the actin cytoskeleton-based regulation of YAP/TAZ activity (Wada *et al.*, 2011; Dupont *et al.*, 2011; Zhao *et al.*, 2012; Aragona *et al.*, 2013; Reginensi *et al.*, 2013; Feng *et al.*, 2014; Low *et al.*, 2014; Sorrentino *et al.*, 2014, 2017; Taniguchi *et al.*, 2015; Das *et al.*, 2016; Hu *et al.*, 2017).

The ability of YAP and TAZ to respond to mechanical stimuli highlights the central role of these transcriptional co-activators as mechanotranducers and mechanoeffectors, the biological effects of which are specific for cell type and mechanical stress (Panciera *et al.*, 2017). Nevertheless, a cross-cutting characteristic in the modulation of the activity of the pathway relies on the dynamic cellular localization of the Hippo network components (Sun and Irvine, 2016).

Cell-cell junctions and apicobasal polarity serve not only as the scaffold for tissue integrity and polarity, but also as a regulatory platform for YAP/TAZ signaling (Fig. 16). Many proteins that are found at tight junctions in mammals are conserved in *Drosophila*, organism in which many of the proteins classified as upstream activators of Hippo signaling, including Dachsous (Ds), Fat, Ex and Merlin (Mer), localize near this marginal zone or subapical region (McCartney *et al.*, 2000; Ma *et al.*, 2003; Sun, Reddy and Irvine, 2015). A paradigm for apicobasal polarity modulation of YAP/TAZ activity is found in the apical crumbs complex (CRB), which binds to YAP/TAZ and favors their cytoplasmic retention (Varelas *et al.*, 2010). In addition, the idea that the membrane is the subcellular compartment where Hippo cascade is activated was further supported by functional approaches that forced the membrane localization of overexpressed Hpo, Mats or Wts, which resulted in the increased activity of these kinases (Hergovich, Schmitz and Hemmings, 2006; Ho *et al.*, 2010; Deng *et al.*, 2013; Yin *et al.*, 2013). Basal to the tight junctions, cadherin-mediated cell-cell adhesion occurs at adherens junctions in both *Drosophila* and vertebrates. These sites of cell attachment are connected to the actomyosin cytoskeleton through catenins and associated proteins. Interestingly, the stability of the actin cytoskeleton has a key influence on YAP and TAZ mechanotransduction (Fig. 16).

The dynamism of actin filaments sustains cell movements, cell shape and provides the cortical tension necessary for maintaining cell-cell and cell-matrix contacts. F-actin arrangement and behavior depend on a wide array of actin binding proteins (ABPs) encompassing actin nucleators, depolymerizing factors, actin-bundling proteins and actin-crosslinking proteins (Winder, 2005; Michelot and Drubin, 2011). These ABPs can impact on the phosphorylation state of Hippo core kinases, on Yki/YAP/TAZ subcellular localization and on the expression of Yki/YAP/TAZ target genes. On the whole, capped or destabilized F-actin promotes sequestration of YAP in the cytoplasm, whereas barbed-end polymerization favors YAP activity.

Thus, sparse, flat and spread cells exhibit strong stress fibers and nuclear YAP, which promotes TEAD-dependent proliferation. Conversely, at high cell densities, when cells are round and compact, YAP is excluded from the nucleus and proliferation is suppressed (Fig. 17; Wada *et al.*, 2011). Additionally, YAP/TAZ activity can also be regulated independently from cell-cell contacts, since cell shape and extracellular matrix rigidity can induce YAP/TAZ-mediated proliferation (Fig. 17; Aragona *et al.*, 2013). Briefly, cells plated on large and stiff substrates display high ROCK- and non-muscle-myosin-II-mediated cytoskeletal tension, which results in YAP/TAZ activation. Conversely, cell rounding and reduced adhesive area conditioned by softer or smaller substrates, cause cytoplasmic retention and inhibition of YAP/TAZ (Dupont *et al.*, 2011; Aragona *et al.*, 2013). In agreement with this, the experimental depletion of capping and severing proteins, such as Cofilin-1 and CapZ, induce F-actin reorganization and stress fiber formation ultimately turning on YAP/TAZ activity (Aragona *et al.*, 2013). The relationship between cell shape, F-actin and YAP/TAZ activity has been addressed in many other contexts such as hepatic stellate cell conversion to myofibroblasts during liver fibrosis (Caliari *et al.*, 2016; Martin *et al.*, 2016; Lachowski *et al.*, 2017), proliferation of basal layer skin keratinocytes (Elbediwy *et al.*, 2016; Li *et al.*, 2016), activation and proliferation of cancer fibroblasts (Calvo *et al.*, 2013), self-renewal and chemoresistance of breast cancer stem cells induced by glucocorticoids (Sorrentino *et al.*, 2017) and others (for review see Totaro, Panciera and Piccolo, 2018).

At the molecular level, focal adhesion components and GTPases seem to be critical for modulation of YAP/TAZ activity (Dupont *et al.*, 2011; Wada *et al.*, 2011; Zhao *et al.*, 2012; Aragona *et al.*, 2013; Reginensi *et al.*, 2013; Totaro, Panciera and Piccolo, 2018). Indeed, integrin-dependent YAP/TAZ activation may be mediated by the F-actin modulators Rho-guanadine exchange factor beta-PIX, the small GTPase Rac1 and its effector p21 activated kinase (PAK) (Fig. 16; Sabra *et al.*, 2017; Sero and Bakal, 2017). Moreover, RhoA, an activator of F-actin and actomyosin contractility, regulates

YAP/TAZ activity independently of LATS when cells grow on a stiff substrate (Dupont *et al.*, 2011). It must also be noted that several hormones and growth factors act as metabolic signals through G-protein-coupled receptors (GPCRs) that can modulate YAP/TAZ activity by way of activating or inhibiting Rho-GTPases (Meng, Moroishi and Guan, 2016; Santinon, Pocaterra and Dupont, 2016). GPCRs ligands such as lysophospholipids sphingosine 1-phosphate (S1P), lysophosphatdic acid (LPA), thrombin, estrogens and acetylcholine link YAP/TAZ activation with RhoGTPase-dependent F-actin cytoskeleton (Fig. 16; Yu *et al.*, 2012; Feng *et al.*, 2014; Meng, Moroishi and Guan, 2016; Santinon, Pocaterra and Dupont, 2016). On the other hand, glucagon or epinephrine trigger YAP/TAZ repression through cAMP/PKA-dependent inhibition of RhoA-GTPase (Yu *et al.*, 2012, 2013).

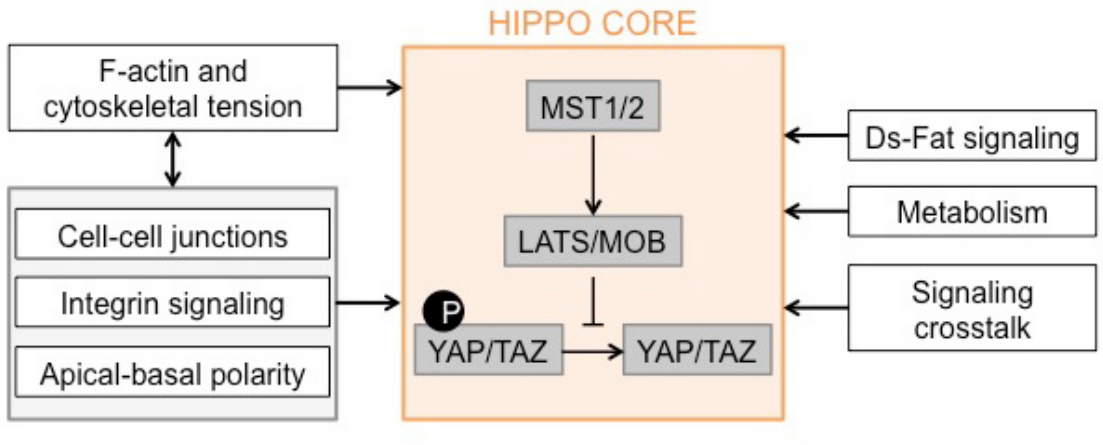

Figure 16. Activation of core Hippo kinases. Several upstream factors influence the activation of these core Hippo kinases and YAP/TAZ. Upstream biochemical cues include Ds-Fat signaling; crosstalk from other major signaling pathways including EGFR, Wnt, BMP, Hedgehog, Notch and GPCR; integrin signaling; cellular metabolism; and metabolic pathways. Upstream physical cues include F-actin levels, tension in the F-actin cytoskeleton, and mechanical stress experienced at cell junctions and focal adhesions. Adapted from Misra and Irvine, 2018.

However, a major question that remains unanswered in the field is how, mechanistically, signaling information generated by the F-actin cytoskeleton triggers YAP/TAZ subcellular localization and activation. Two possibilities are that i) angiomotin family proteins (AMOTs) act as mechanical mediators between the cytoskeleton and YAP/TAZ, and ii) proteins of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex interact with F-actin. Regarding the former, AMOTs directly bind and potentially inhibit YAP/TAZ (Zhao *et al.*, 2011; Gaspar and Tapon, 2014). As to the LINC complex, it has been proposed that increased nuclear permeability mediated by LINC-regulated nuclear stretching could facilitate YAP/TAZ entry in the nucleus (Elosegui-Artola *et al.*, 2017).

Epithelial cells are mechanically coupled to each other at adherens junctions and cytoskeletal tension is recruited in numerous biological contexts, how the distinct mechanisms of YAP/TAZ regulation through cytoskeleton control cell fate decisions *in vivo* during different developmental, physiological and pathological scenarios awaits further research.

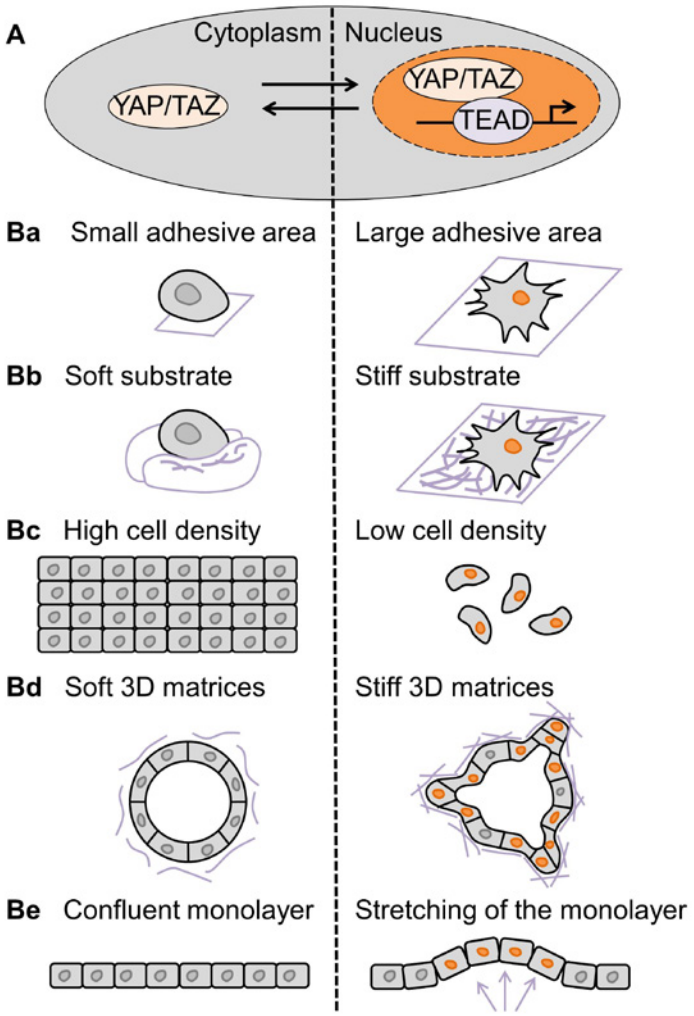

Figure 17. Schematic representations of mechanical stimuli influencing YAP and TAZ subcellular localization and activity. (A) When YAP and TAZ are mechanically activated (orange), they translocate to the nucleus, where they interact with TEAD factors to regulate gene expression. (B) Schematics illustrating how different matrix, geometry and physical conditions influence YAP and TAZ localization and activity: the left panels show conditions in which YAP and TAZ are inhibited and localized to the cytoplasm, whereas the right panels show conditions that promote YAP and TAZ nuclear localization (indicated by orange coloured-cell nuclei). Small adhesive area, soft substrate, high cell density, soft 3D matrices and cell confluent monolayers (Ba – Be left column) display YAP and TAZ cytoplasmic retention/degradation, whereas large adhesive areas, stiff substrate, low cell density, stiff 3D matrices and stretching forces induce YAP and TAZ nuclear subcellular localization (orange nuclei, Ba – Be right column). Adapted from Panciera *et al.*, 2017.

1.7. INTRODUCTION ENDNOTE.

The hindbrain offers an evolutionary conserved scenario with dynamic temporal and spatial distribution of cells during morphogenesis. Hindbrain boundaries, specified at the interface between adjacent rhombomeres, are cell populations endowed with unique characteristics when compared to the rest of hindbrain cells. On the one side, zebrafish hindbrain boundaries are kept undifferentiated at early stages when most hindbrain progenitors are engaged into neurogenic and gliogenic programs (Esain *et al.*, 2010; Gonzalez-Quevedo *et al.*, 2010). On the other side, it is important to note the characteristic mechanical juncture in the BCP. Hindbrain boundary cells display specific triangular cell shape (Gutzman and Sive, 2010) and they contain an apically-located cable-like cytoskeletal arrangement involved in avoiding cell intermingling between cells from different compartments, being the forces generated by mitotic rounding in the boundary vicinities the main challenge to cell-lineage restriction (Calzolari, Terriente and Pujades, 2014; Letelier *et al.*, 2018). Thus, BCP biology and its tentacular mechanical framework establish a mindset in which hypothesizing a potential relationship between progenitor biology and tissue microenvironment was tempting at the very least.

CHAPTER 2: OBJECTIVES.

The present work was aimed at delving deeper into the behavioral characteristics of the BCP and at shedding light on the largely unknown fate of this cell population. Importantly, these objectives were investigated under the scope of mechanobiology with YAP/TAZ-TEAD activity as the molecular hinge candidate between the mechanical environment in hindbrain boundaries and the particular behaviors displayed by this cell population.

Thus, the specific issues addressed are:

[1] To characterize the onset of expression of boundary markers during hindbrain development and its spatial relationship to the adjacent rhombomeres.

[2] To address the proliferative capacity of hindbrain boundary cells.

[3] To uncover YAP/TAZ activity in hindbrain boundary cells.

[4] To unveil the role of YAP/TAZ-TEAD activity in the hindbrain boundary cell population as a sensor and effector of mechanical stimuli.

[5] To reconstruct the YAP/TAZ-TEAD-active boundary cell lineage aiming at the:

> [5.1] Characterization of the proliferative capacity of the YAP/TAZ-TEAD-active boundary cell population.
>
> [5.2] Generation of a spatiotemporal map of the YAP/TAZ-TEAD-active boundary cell derivatives.

[6] To reveal the fate of YAP/TAZ-TEAD-active hindbrain boundary cells.

[7] To investigate the role of YAP/TAZ-TEAD activity in hindbrain boundary cells as modulators of progenitor biology and regulation of cell fate.

We tackle these questions in zebrafish embryos because they allow us to combine genetic tools with *in vivo* high-resolution imaging.

YAP/TAZ-TEAD ACTIVITY LINKS MECHANICAL CUES TO SPECIFIC CELL FATE DURING HINDBRAIN SEGMENTATION

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CHAPTER 4: GENERAL DISCUSSION.

ON TISSUE GROWTH, PROGENITOR MAINTENANCE AND MECHANOTRANSDUCTION IN HINDBRAIN BOUNDARIES.

4.1. ON HINDBRAIN BOUNDARIES GROWTH AND FORM.

During embryogenesis, tissues acquire specialized forms and functions. The role of mechanical forces and cell behavior in the regulation of tissue architecture is poorly understood. Specifically, how cell progenitor expansion is in register with tissue shape maintenance and how a final tissue shape is isotropically conserved during embryonic development are questions awaiting further research.

In this work we describe the architecture of the hindbrain boundaries, a structure that in the AP axis occupies two cell rows. These cell rows extend as sheets in the DV axis. We also show that each cell sheet has the identity of the corresponding rhombomere. Interestingly, the physical stability of the interface, both as a gene expression border and as segment delimitation, is maintained until around 36 hpf. After that, the interface between hindbrain compartments becomes ragged again. It is tempting to hypothesize that compartment interfaces become fuzzy because the specialization degree of each compartment is such that cell mixing does not represent a challenge for the integrity and the coherence of the rhombomeres anymore. We cannot fail to note that this opens up a temporal framework for questioning up to what stage physical compartmentalization is operative in the hindbrain.

Chick and mouse hindbrain boundaries display a reduced cell proliferation rate when compared to the rest of hindbrain cells (Guthrie, Butcher and Lumsden, 1991; Baek *et al.*, 2006). Remarkably, in this project we demonstrate that zebrafish hindbrain boundaries contain proliferative cells even at early stages of hindbrain segmentation. Thus, whether zebrafish boundary cells display different proliferation rate than non-boundary cells is a question to be addressed. Moreover, it does not go unnoticed that boundary cell proliferation might imply a challenge for the stability of the boundary tissue bilayer, since new daughter cells have to be incorporated in each of the two corresponding layers in a finite apical space. This opens up the question of how are morphogenesis and cell proliferation coupled in hindbrain boundaries. One possibility is that the medio-lateral growth of the ventricular boundary zone can accommodate new progenitor cells both in time and space. On the other hand, it might happen that the medio-lateral growth is not in register with cell proliferation, namely, that new incorporating daughter cells exceed the spatial capacity of the boundary ventricular domain, which would imply that either cells loose volume or that they differentiate and leave the ventricular zone in order to populate the boundary mantle region. The latter option is less likely given the spatiotemporal distribution of neurogenic and gliogenic capacities at the early stages of hindbrain segmentation, with no proneural or gliogenic

specification genes expressed in hindbrain boundaries (see 1.4.3. Hindbrain neurogenesis; Cooke, Kemp and Moens, 2005; Esain *et al.*, 2010; Gonzalez-Quevedo *et al.*, 2010).

In many tissues, the ontogenetic growth rate decreases as development proceeds (West, Brown and Enquist, 2001; Ricklefs, 2006). Interestingly, so it is the case for the growth rate in hindbrain boundaries as highlighted by the proliferative behavior switch of YAP/TAZ-TEAD-active cells. These cells actively proliferate from the moment TEAD activity is triggered around 22 – 26 hpf until 20 hours later when proliferation ceases. Indeed, in this work we describe YAP/TAZ-activity as the mechanism regulating proliferative behavior in hindbrain boundaries and hence, as the molecular pathway that enables cell proliferation to occur as long as these effectors are active in the tissue. In line with this, since YAP and TAZ are found in the nucleus of stem cells and progenitors, they have been proposed as stemness determinants (Panciera *et al.*, 2017).

One of the overriding aims in developmental neuroscience is to understand how cell behavior and morphogenesis are coupled in the making of the brain. Bringing a whole-system comprehension of hindbrain growth coupled to segmentation will require a deeper understanding of anatomical rearrangements, cell proliferation capacity changes and cell volume and morphology dynamics (see 6.1. Segmentation of single cells and embryonic territories).

4.2. HINDBRAIN BOUNDARIES AS NEURONAL PROGENITOR POOLS.

The balance between proliferation versus differentiation is of fundamental importance in the shaping of the overall architecture and function of the nervous system. During the first decade of the 2000s, significant advances were made in the identification and characterization of the two major types of neural progenitors, highlighting new insights into population-specific cell division modes, regulatory requirements and neurogenic capacities.

In this regard, progenitor pools and proneural clusters are the two types of neural progenitors that operate in the developing nervous system of both mouse and zebrafish (Bae, Shimizu and Hibi, 2005; Baek *et al.*, 2006; Stigloher *et al.*, 2008). Proneural clusters are proliferative progenitor populations that readily engage into early neurogenesis. These progenitors display oscillating expression of Notch target genes of the Enhancer of split (*E(spl)*) family (*her* in zebrafish, *Hes* in mammals) and are

singled out by lateral inhibition (Stigloher *et al.*, 2008). On the other hand, progenitor pools consist of neuroepithelial cells located at neural tube boundaries, which often act as signaling centers (Kiecker and Lumsden, 2005). Importantly, partially different E(spl) transcription factors are responsible for the maintenance of these progenitors that, in contrast to proneural clusters, undergo delayed neurogenesis. Not only progenitor pools display different *her*/*Hes* genes, but also these are expressed at stable and high levels in a Notch-independent manner (see Table 1; Geling, 2003; Baek *et al.*, 2006).

Table 1. Comparison between proneural clusters and progenitor pools.

Characteristic	**Proneural cluster**	**Progenitor pool**
Territory	Compartment	Boundary
Neurogenesis	Early	Late
Notch dependency	Dependent	Independent
***Her*/*Hes* genes**	Oscillating expression *Hes1/her6* *Hes5/her4*	Stable expression *her3* *Hes7/her5* *Hes1/her6* *Hes4/her9* *her11*

In this work we show that YAP/TAZ-TEAD activity is triggered at approximately 22 - 26 hpf in a hindbrain boundary cell subpopulation and that this activity is already switched off by 48 hpf. Remarkably, photoconversion experiments show that YAP/TAZ-TEAD activity is switched on synchronously in hindbrain boundary cells, since all the cells expressing new TEAD-dependent green KAEDE at later stages post-photoconversion, also display the old photoconverted magenta KAEDE. Importantly, neuronal derivatives of YAP/TAZ-active boundary cells are present in the boundary mantle zone from around 40-48 hpf onwards, implying that these progenitors undergo indeed delayed neurogenesis. Note that boundary cells are prevented from neurogenesis during hindbrain segmentation (Cooke, Kemp and Moens, 2005). However, as shown by the presence of boundary derivatives in the differentiated domain, at a given developmental period neurogenesis is either derepressed or triggered most probably preceded by YAP/TAZ-TEAD activity downregulation. In line with this, repression of YAP and TEAD leads to premature neuronal differentiation in chick spinal cord (Cao *et al.*, 2008).

This observation opens up the question of how boundary cells are maintained refractory to neurogenesis at early stages. Interestingly, boundary cells express high levels of *her9* (Fig. 18), a *her* gene characteristic of progenitor pools (Bae, Shimizu and Hibi, 2005; Radosevic *et al.*, 2011). Preliminary results indicate that *her9* expression in hindbrain boundaries is Notch-independent in contrary to non-boundary *her9* (Fig. 18).

In agreement with this, *Hes1*, the mouse ortholog for *her9*, is enriched in mouse hindbrain boundaries and is responsible for repressing proneural bHLH gene expression (Baek *et al.*, 2006). Hindbrain boundary delayed neurogenic capacity and *her9* behavior within these cells suggest indeed that this cell population might meet the requirements of progenitor pools. Considering that *her9* is a crucial inhibitor of neurogenesis in this type of neural progenitors (Bae, Shimizu and Hibi, 2005; Radosevic *et al.*, 2011), current work in our group is focused on unveiling the role of this *E(spl)* gene in the modulation of hindbrain boundary neurogenic capacity.

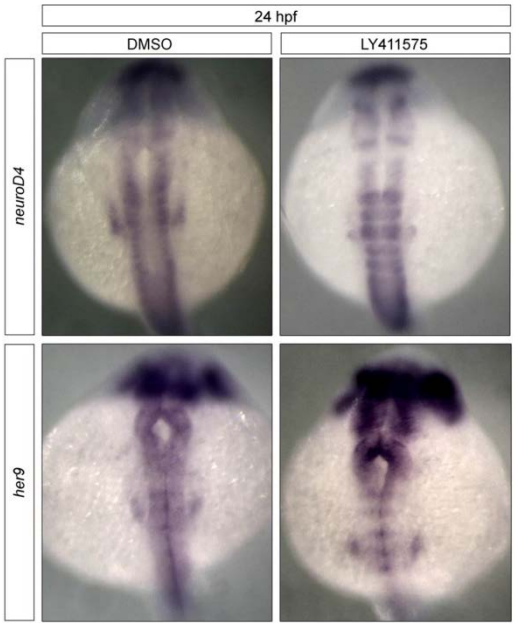

Figure 18. Neurogenesis inhibition and *her9* expression in hindbrain boundaries are Notch-independent. Upon 10 hours treatment of LY411575, a cell-permeable γ-secretase inhibitor that blocks Notch activation, hindbrain boundaries are not driven into neurogenesis as shown by the absence of *neuroD4* gene expression in the boundaries. Moreover, *her9* gene expression is not downregulated in hindbrain boundaries upon Notch inhibition.

Despite the fact that *her9* could be responsible for hindbrain boundary progenitor maintenance in a Notch-independent manner at earlier stages (between 14 – 36 hpf), in this work we demonstrate that YAP/TAZ-TEAD-active boundary cells are Sox2-positive progenitors that end up undergoing neuronal differentiation. Thus, Notch activity must be triggered at some point in hindbrain boundary cells prior to neuronal specification (around 36 – 40 hpf). This scenario unveils a framework in which Notch and YAP/TAZ-TEAD activity overlap. Interestingly, the crosstalk between YAP/TAZ and Notch signaling is a focus of intense research in the field (Totaro *et al.*, 2018). For example, Notch signaling is downstream of YAP/TAZ activation during intestinal repair after inflammation (Taniguchi *et al.*, 2015). Hence, upon tissue damage, interleukin-mediated activation of YAP/TAZ upregulates Notch signaling in intestinal stem cells

promoting regeneration (Taniguchi *et al.*, 2015). Furthermore, Notch ligand expression mediated by YAP/TAZ activity in a given cell triggers Notch signaling activation in the neighboring cells. For instance, JAG1 expression induced by YAP/TAZ activity turns on Notch signaling to promote differentiation of neural crest progenitors into smooth muscle cells during the development of the arterial wall (Manderfield *et al.*, 2015). Moreover, YAP mechanotransduction can be triggered upon contraction of muscle fibers, inducing JAG2 and turning on Notch signaling in neighboring satellite cells, which prevents their differentiation (Esteves de Lima *et al.*, 2016). On this account, the physical microenvironment is translated into YAP/TAZ activity that orchestrates spatial control of self-renewal versus differentiation of progenitor cells (Totaro, Panciera and Piccolo, 2018). Thus, considering the temporal scale of hindbrain boundaries YAP/TAZ activity, the late triggering of neurogenesis in this population and the potential role of *her9*, it is tempting to propose that hindbrain boundaries appear as a scenario where to further explore the potential connection between YAP/TAZ modulation and Notch signaling regulation in the control of cell fate decisions.

We should not omit that, since early neuronal specification is inhibited in progenitor pools, cell divisions in these neural progenitors are symmetric and self-renewing (Homem, Repic and Knoblich, 2015). On the contrary, proneural clusters are characterized by undergoing asymmetric neurogenic divisions, transit amplifying divisions or terminal divisions that end up leading to indirect or direct neuronal specification (Homem, Repic and Knoblich, 2015). Importantly, proneural genes are not expressed in hindbrain boundaries at least prior to 40-48 hpf (Cooke, Kemp and Moens, 2005; Gonzalez-Quevedo *et al.*, 2010). How cell division mode behavior scaffolds the balance between cell self-renewal and differentiation in the hindbrain, and how boundary neurogenic capacity is temporally and spatially profiled are questions still poorly understood. In this regard, high-temporal resolution *in vivo* imaging and topological analysis of boundary division modes within the tissue considering asymmetry determinants distribution, might provide new insight on division mode behavior in the context of the whole boundary and spatiotemporal hint for the onset and allocation of neurogenic capacity in this population. The lab is currently exploring these possibilities.

Not only cell division mode requires an exquisite coordination in the formation of the nervous system, but also precursor proliferation and cell cycle exit pose a regulatory layer in the making of tissues and organs. Indeed, tissue- and/or stage-specific expression profiles of key components of the cell cycle machinery might tip the balance towards either cell differentiation or proliferation (Hardwick and Philpott, 2014). From

yeast to humans, cyclins and cyclin-dependent kinases drive cell cycle progression and cell division. Cyclins were coined such name due to their variable protein concentration in a cyclical fashion during cell cycle progression. Interestingly, the expression of the *CyclinD1* (*ccnd1*) mRNA is enriched in zebrafish hindbrain boundaries from the earliest stages of hindbrain segmentation (Fig. 19). Nonetheless, at 48 hpf the expression levels in hindbrain boundaries is equivalent to the rest of the hindbrain (Fig. 19). *ccnd1,* as the rest of D-type cyclins, promotes progression from G1 to S phase in dividing cells through the activation of Cyclin-Dependent Kinases 4 or 6 (CDK4/6) and the sequestration of CDK inhibitors like CDK inhibitor 1B (CDKN1B or P27KIP1) (Kozar and Sicinski, 2005). As a result, CDK2 activity is enhanced, retinoblastoma proteins are inactivated and DNA replication is triggered. Importantly, the expression and requirement of the D-type cyclins during development is tissue specific (Ciemerych *et al.*, 2002). In Cao et al. (Cao *et al.*, 2008), *Yap* transcriptionally regulates *CyclinD1* in chick spinal cord and its overexpression avoids cell cycle exit and hence a reduction of the neurogenic potential, as consequence, the *NeuroM/NeuroD4* domain is reduced (Cao *et al.*, 2008). However, our results show that the temporal enrichment of *ccnd1* expression in hindbrain boundaries occurs before YAP/TAZ-TEAD activity onset (Fig. 19).

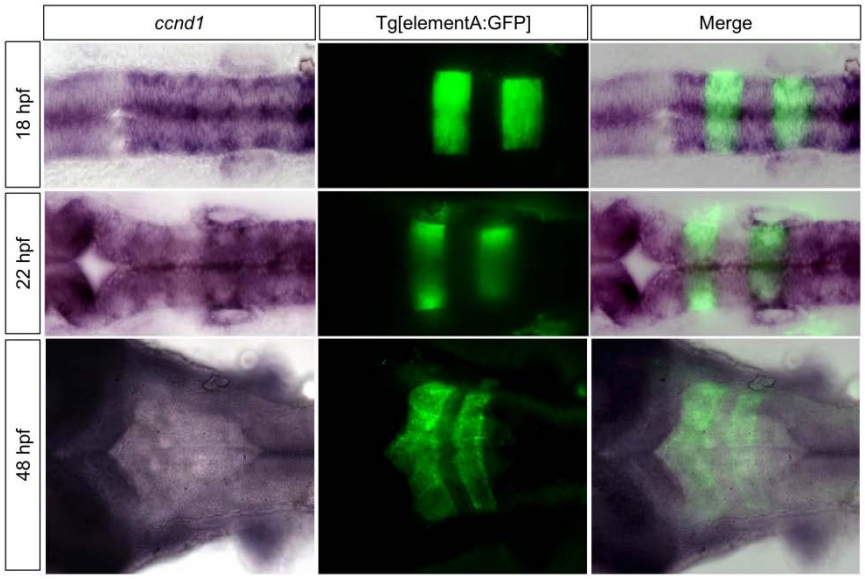

Figure 19. Dynamic analysis of the *ccnd1* gene expression profile in the embryonic hindbrain. *ccnd1* is expressed in the whole hindbrain and enriched in hindbrain boundaries at early stages after morphological bulges appear (18 hpf and 22 hpf). However, at 48 hpf *ccnd1* is no longer enriched in hindbrain boundaries.

On the whole, *cyclin D1* overexpression shortens cell cycle length in cultured cells (Quelle *et al.*, 1993), inhibits neuronal differentiation (Lobjois *et al.*, 2004; Cao *et al.*,

2008) and can be transcriptionally downstream of *Hes1* (the mouse ortholog for the zebrafish *her9*) as shown in cultured mouse E11.5 telencephalic neural progenitors (Baek *et al.*, 2006). However, the impact of *ccnd1* on hindbrain segmentation and on the ratio proliferation/differentiation in the boundary cells has not been directly assessed. Thus, aiming at addressing the role of *ccnd1* in the maintenance of the boundary population cell self-renewal versus differentiation, we generated a *ccnd1* mutant line by way of TALEN-mediated genome edition (see 6.2. TALEN-mediated genome edition in zebrafish).

During the development of the central nervous system, neural progenitors initially divide symmetrically to expand their pool and switch to asymmetric neurogenic divisions at the onset of neurogenesis (Paridaen and Huttner, 2014). This process involves various regulatory mechanisms such as cell division mode, transcriptional control and signaling pathways (Paridaen and Huttner, 2014). In this work we show that YAP/TAZ-activity modulates proliferation in hindbrain boundary cells and that these cells upon downregulation of YAP/TAZ-activity end up differentiating. Thus, one of the most overriding questions regarding neurogenesis modulation in the boundary cells are how neurogenesis is inhibited in hindbrain boundaries and, later on, what molecular regulatory scaffold is responsible for triggering neuronal specification.

4.3. BOUNDARY CELL LINEAGE, NEURONAL IDENTITIES AND EVOLUTIONARY IMPLICATIONS.

Currently, we can already catch glimpse of hindbrain boundary functions (Terriente *et al.*, 2012; Calzolari, Terriente and Pujades, 2014; Peretz *et al.*, 2016) and of molecular markers enriched in this population (Cheng *et al.*, 2004; Riley *et al.*, 2004; Terriente *et al.*, 2012; Letelier *et al.*, 2018). However, little is known regarding hindbrain boundary cell behavior and fate. In this work we describe the dynamics of hindbrain boundary progenitor behavior according to cell division, number of progeny of a founder cell, spatial location and differentiation status. We took advantage of high-temporal resolution *in vivo* imaging in order to reconstruct the lineage tree of YAP/TAZ-active hindbrain boundary cells.

Since we pursued a clonal analysis strategy based on random nuclear staining upon heat-shock induction, we obtained a partial depiction of hindbrain boundary developmental history and missed a comprehensive whole-tissue view. In addition, it has to be considered that tissue growth and hindbrain morphogenesis impact on the spatial cell coordinates and therefore on the whole boundary architecture. As

consequence, we still lack a precise description of the distribution and behavior of boundary cells in the context of the whole boundary. Future work is focused on establishing an experimental pipeline for whole-boundary *in vivo* imaging with cellular resolution and a temporal window encompassing the behavioral switches described in this work for the YAP/TAZ-active boundary cells, as well as the elusive neuronal specification of boundary cells (see 6.3. *In vivo* hindbrain imaging by using single plane illumination microscopy (SPIM)).

In this work we demonstrate that the YAP/TAZ-active boundary domain gives rise to neuronal derivatives located in the boundary mantle zone. However, a description of what are the neuronal types that derive from boundary progenitors remains elusive. We also describe a pool of YAP/TAZ-active cells that once TEAD activity is already switched off, are kept in the ventricular domain as non-dividing progenitors. Regarding the neuronal derivatives, the topological distribution of these differentiated cells might serve as a landmark, meaning that this positional information can be cross-referenced with neuronal markers the expression of which is already known in the hindbrain. This will allow us to interrogate the system in the search for the functional role of these neurons in the posterior brain.

Uncovering the clonal relationship between differentiated cells in the boundary mantle zone and the boundary progenitors will shed light on cell lineage hierarchies and temporal states. Temporal coordination between different developmental processes is key for tissue organization, patterning and morphogenesis (Ebisuya and Briscoe, 2018). Since hindbrain boundaries are signaling centers, mechanical barriers and progenitor pools, temporal control of the unfolding of these roles must be tightly regulated. Thus, the hindbrain boundary population might undergo a tinkering strategy (Jacob, 1977) and pose an evolutionary block opportunistically rearranged and available to serve different biological needs at different and/or overlapping developmental windows. Our lab is currently setting up a genetic cell ablation system subject to temporal control, the so-called ATTAC system (Weber *et al.*, 2016), for hindbrain boundary cell caspase-mediated apoptotic induction. In this sense, through conditional genetic cell ablation we aim at interrogating boundary cellular function requirements at different developmental stages.

Furthermore, in evolutionary terms, it is tempting to hypothesize that both YAP and TAZ can independently prompt TEAD activity as a mechanism of robustness and mutual backup, since by independently downregulating YAP and TAZ, we show that both transcriptional co-activators are involved in triggering TEAD-dependent

transcription. Indeed, hindbrain compartment boundaries seem to be a relevant population when it comes to developmental fitness since redundant enhancers have been found to back each other up in the stabilization of the boundary as a mechanical barrier (Letelier *et al.*, 2018). Thus, hindbrain boundaries have skillfully provided new answers to novel challenges in the development of the posterior brain.

4.4. MECHANOTRANSDUCTION AND HIPPO PATHWAY REQUIREMENTS IN HINDBRAIN BOUNDARY CELLS.

The transcriptional co-activators YAP and TAZ integrate diverse upstream signals to control cell fate decisions and regulate organ growth (Sun and Irvine, 2016). In this work we provide evidence that mechanical cues are sensed in hindbrain boundaries and transduced into cell proliferation modulation. Furthermore, by way of cell lineage reconstruction, we show that the temporal window when boundary cells are engaged into proliferation coincides with the presence of YAP/TAZ activity. Additionally, boundary cell proliferative capacity is compromised upon YAP/TAZ downregulation. GFP perdurance allowed us to keep track of these cells after the offset of the activity, which unveiled that most of the YAP/TAZ-active boundary cells give rise to differentiated neurons positioned in the boundary mantle zone.

Most probably, the specific mechanical microenvironment in hindbrain boundaries (Gutzman and Sive, 2010; Calzolari, Terriente and Pujades, 2014; Letelier *et al.*, 2018) is responsible for confining the activity of YAP and TAZ in these hindbrain territories, keeping boundary cells in the progenitor state while the rest of hindbrain cells are engaged into active neurogenesis and gliogenesis. The influence of cytoskeletal integrity on YAP/TAZ-activity first came from observations that mutations in *Drosophila* that result in an accumulation of F-actin could be linked to increased Yki activity (the homolog of YAP and TAZ), which also occurs in mammalian cells (Fernandez *et al.*, 2011; Sansores-Garcia *et al.*, 2011; Aragona *et al.*, 2013). Here, we show that cytoskeletal integrity is needed for YAP/TAZ-activity onset; however, once YAP and TAZ are already active in hindbrain boundary cells, mechanical cues are no longer required for YAP/TAZ-TEAD activity, suggesting the existence of a positive feedback.

Previous results show that Rac3b is a modulator of cytoskeletal organization in hindbrain boundaries (Letelier *et al.*, 2018). In this work, we show that activation of Rac3b-DN in hindbrain boundaries impedes YAP/TAZ-TEAD-activity. However, ectopic Rac3b-CA expression in non-boundary territories does not trigger ectopic YAP/TAZ-TEAD-activity. Hence, one possibility is that the ectopic tension induced by Rac3b-CA

in small cell clones is not sufficient to reach the activation threshold for YAP/TAZ. A second possibility would imply that mechanical cues are needed but not sufficient to trigger the activation of YAP and TAZ in the hindbrain. Regarding the former experimental limitation, ongoing developments in the field of mechanobiology will be able to shed light on the issue. The main challenge relies on applying and adapting current techniques used for probed-force application on cultured cells (Elosegui-Artola *et al.*, 2017) or magnetic bead displacement of epithelial cells in *Drosophila* (D'Angelo *et al.*, 2017) to the spatiotemporal requirements of developing 3D tissues and organs.

On top of this, it is important to note that YAP/TAZ-TEAD-active cells represent a subpopulation of the whole boundary interface. It is appealing to propose that this observation could be linked to topological differences in tissue tension distribution or sensing. However, it is also noteworthy that the boundary domains devoid of TEAD activity do not display the classical boundary markers. For example, the most dorsal region of hindbrain boundaries, which correspond to the rhombic lip territory, expresses *atoh1a*, but does not display *rfng*, *sgca*, *rac3b* or TEAD activity. The regulation of dorsoventral/apicobasal differences in the boundary tissue remains elusive; however, they might entail the underpinnings of the spatial specificities of TEAD activity within hindbrain boundaries.

In this work we explore the interplay between cytoskeleton stability and YAP/TAZ-TEAD-mediated activity. Nevertheless, whether YAP/TAZ activity is regulated via LATS or via a non-canonical mechanism remains unexplored. The main LATS regulator is MST kinase. Recent studies have identified additional proteins that do not belong to the core of the pathway that scaffold and promote the interaction between different Hippo pathway players. One of this scaffolding proteins is Schip1, which was described in *Drosophila* as a protein that induces Hpo (MST) activation by binding to Expanded (Ex) and Tao-1 (Chung, Augustine and Choi, 2016). Briefly, Ex directly recruits Schip1, which is required for the localization of Tao-1 kinase to the cell membrane (Chung, Augustine and Choi, 2016). Tao-1, in its turn, will promote Hpo function by facilitating its phosphorylation (Chung, Augustine and Choi, 2016). Thus, Schip1 works as a Hippo pathway activator, implying a negative modulation of Yki. As consequence, *Schip1* mutant clones overproliferate in developing imaginal discs and loss of *Schip1* upregulates Yki target genes (Chung, Augustine and Choi, 2016).

Interestingly enough, *Schip1* expression is enriched in hindbrain boundaries at stages preceding the activation of YAP/TAZ-TEAD activity (Fig. 12). Thus, it is tempting to hypothesize that Schip1 might be an upstream negative regulator of YAP/TAZ

activation at the earliest stages of hindbrain segmentation. Future work addressing the functional relationship between Schip1 and YAP/TAZ activation in hindbrain boundaries will shed light on the Hippo requirements for TEAD-dependent transcription in the developing hindbrain.

CHAPTER 5: CONCLUSIONS.

[1] The hindbrain boundary cell population occupies two cell rows along the AP axis at rhombomeric interfaces. Boundary cells display rhombomeric markers; however, each cell row displays different markers corresponding to each of the two contacting compartments.

[2] In zebrafish, hindbrain boundary cells do proliferate and, contrary to other systems such as chick or mouse, no specific spatial distribution of proliferation capacity is detected.

[3] A hindbrain boundary cell subpopulation of Sox2-positive progenitors displays YAP/TAZ-TEAD activity from around 22 hpf up to 48 hpf.

[4] Both YAP and TAZ independently contribute to TEAD-dependent activity.

[5] Mechanical microenvironment is responsible for triggering YAP/TAZ-TEAD activity in hindbrain boundaries; however, it is not necessary for the maintenance of the activity.

[6] YAP/TAZ-TEAD-active hindbrain boundary cells do proliferate up to around 40-48 hpf, coinciding with YAP/TAZ-TEAD activity shutdown. At that time, there is a behavioral switch and proliferative capacity is lost in this cell population.

[7] YAP/TAZ-TEAD-active progenitors produce neuronal derivatives that allocate in the boundary mantle zone and express neuronal differentiation markers.

[8] YAP and TAZ control proliferative behavior but not cell survival in hindbrain boundary cells.

CHAPTER 6: TECHNICAL DEVELOPMENTS.

6.1. IMAGE SEGMENTATION OF SINGLE-CELLS AND EMBRYONIC TERRITORIES.

Tissue shape is often established at early stages of embryonic development. However, how cells interact and distribute to enable coordinated isotropic tissue scaling is not yet understood. As shown in this work, boundary cells are organized in a bilayer along the AP axis of the hindbrain. This architecture is transiently maintained as the boundary tissue grows. Nevertheless, how global changes in cell shape enable isotropic growth of the developing hindbrain interfaces remains to be addressed.

Unveiling the dynamics of proliferation rate changes and of volumetric rearrangements of boundary cells might shed some light on the relationship between single-cell characteristics and whole-tissue growth. Novel approaches for computational image analysis such as image segmentation are a resource with enormous potential for addressing the latter objective.

Image segmentation is the process of delineating cellular or other labeled boundaries, yielding their numbers, positions, geometries and volumes (Khairy and Keller, 2011). Several computational supports allow for either automatic or manual segmentation, such as ITK-Snap, which is an open-source image analysis software application that offers semi-automatic segmentation methods, manual delineation and image navigation (Yushkevich *et al.*, 2006). This tool was initially conceived for clinical image analysis; however, in our hands, it has proven useful for analyzing regular confocal 3D-stacks.

In order to deeper characterize cellular differences between hindbrain boundary and non-boundary cells, we resorted to ITK-Snap single-cell manual delineation. We made use of images from double transgenic Mü4127;Tg[CAAX:GFP] embryos at 18hpf. These embryos carry a rhombomeric marker that labels r3 and r5 in red, which allows for the positional identification of rhombomeric boundaries. In addition, these double transgenic embryos display GFP in the cell plasma membrane, which allows for the segmentation of cell contours (Fig. 20). Interestingly, preliminary results indicate that the cell volume of boundary cells is lower than in non-boundary cells (Fig. 20 A-C, n=15 segmented cells / territory, 1 embryo, p=0,004).

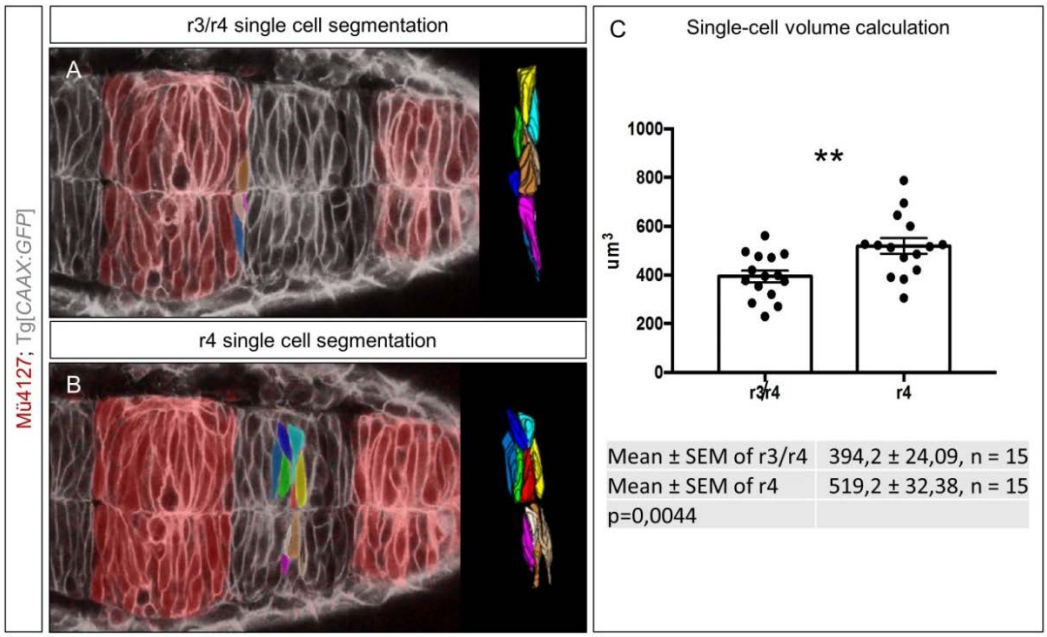

Figure 20. Single-cell volume comparison between hindbrain boundary and non-boundary cells. (A-B) Left panels display single Z-stacks of the hindbrain dorsal view with cell contours shown in grey and rhombomeres 3 and 5 in red. 2D-view of hindbrain boundary single-cell segmentations (A, r3/r4) and non-boundary single-cell segmentation (B, r4). Right panels display the 3D-reconstruction of all the segmented single-cells. (C) Plot depicting the single-cell volumes in r3/r4 and r4. r4 single-cell volume > r3/r4 single-cell volume, p<0,01. The unpaired t-test was used.

We also tested ITK-Snap potential for whole compartment segmentation. Aiming at deciphering rhombomere volumetric changes over time, we resorted to automatic segmentation of the stained r3 and r5 territories as provided by the Mü4127 transgenic line and obtained 3D-reconstructions for these entire embryonic territories (Fig. 21).

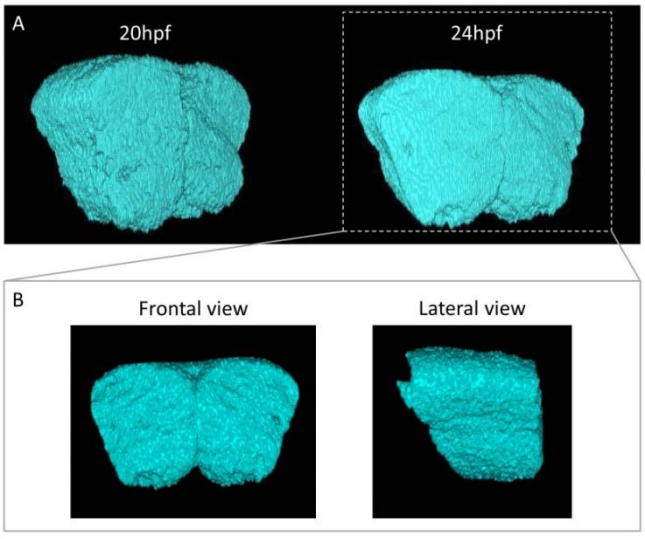

Figure 21. r5 automatic segmentation. (A) r5 whole 3D volume at 20 hpf and 24 hpf. (B) Frontal and lateral views at 24 hpf.

In conclusion, ITK-Snap functionalities have been set up for inquiring mechanistic questions regarding single-cell and whole-compartment shape and volume changes. Addressing single-cell segmentation analysis coupled to proliferative capacity changes over time will allow a better comprehension of cell dynamics in the scope of boundary cellular bilayer growth. In addition, tracing the complexity of rhombomeric anteroposterior, dorsoventral and mediolateral morphogenesis considering the increasing embryo size has the potential for a system-level understanding of the dynamic topography of whole-hindbrain growth.

6.2. TALEN-MEDIATED GENOME EDITION IN ZEBRAFISH.

How boundary cells are specified is an elusive question in the field. *foxb1a* (also called *mariposa*) is a gene coding for a DNA-binding protein, whose expression is enriched in hindbrain boundaries (Moens *et al.*, 1996). So far, this gene has been used as a boundary marker but nothing is known regarding its functional role. Since *foxb1a* is among the boundary markers that are first detected in this population (Fig. 12) and it codes for a DNA-binding protein, it was tempting to hypothesize its potential role in boundary identity specification. On the other hand, *ccnd1* expression is enriched in hindbrain boundaries (Fig. 19) and interestingly enough, this cyclin has been linked to neurogenesis inhibition in other systems (Baek *et al.*, 2006; Cao *et al.*, 2008). Aiming at generating tools for exploring the function of the aforementioned genes in hindbrain boundaries, we resorted to transcription activator-like effector nucleases (TALENs) for zebrafish genome edition.

TALENs utilize customizable DNA-binding domains that are engineered to recognize specific target DNA sequences. TALE specificity is determined by two hypervariable amino acids that are known as the repeat-variable diresidues (RVDs). Like zinc-fingers, modular TALE repeats are linked together to recognize contiguous DNA sequences. The TALE DNA-binding domain is fused to a nuclease that, upon DNA binding, will induce double-strand breaks (DSBs). As consequence, the repairing mechanisms of the cell will potentially introduce frame-shift mutations into genes, which can lead to their knockout (Gaj, Gersbach and Barbas, 2013).

Loss-of-function mutations were aimed at *foxb1a* and *ccnd1* loci. The genomic target site was specifically designed considering the disruption of the DNA-binding fork-head domain in *foxb1a* and the CDK-activating cyclin box in *ccnd1*. TALE repeat arrays were assembled following the Golden Gate TALEN assembly protocol originally described in (Cermak *et al.*, 2011), modified by the Voytas lab and available on the Addgene website.

https://www.addgene.org/static/cms/filer_public/98/5a/985a6117-7490-4001-8f6a-24b2cf7b005b/golden_gate_talen_assembly_v7.pdf

Target sites and the corresponding RVD sequences were chosen using the online tool MoJo Hand (http://talendesign.org/) in exon 1 and 2 of *foxb1a* and *ccnd1* genes, respectively. The array plasmids were fused to the Fok1 endonuclease in the GoldyTALEN backbone. After linearization, mRNAs were transcribed using the T3 mMessage mMachine Kit (Ambion by Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's instructions.

The two mRNAs corresponding to the left and right arms were then mixed in equal quantities and injected into embryos at the one-cell stage. 20–30 embryos at 48 hpf were collected from each clutch and gDNA was extracted. The target sites were amplified using primers generating a 350–600 bp long PCR product. Efficiency of the TALEN pair was estimated by digesting the PCR product with the restriction site present in the spacer of the target site. If a significant amount of uncut PCR product was observed, the rest of the injected embryos were further grown to adulthood.

The resulting mosaic adult fish were out-crossed and genomic DNA was prepared from 50 pooled embryos to identify potential mutations using the same PCR and digestion as described above.

F1 fish were finally genotyped by fin clipping. The uncut band (carrying the mutation) was further amplified for sequencing. Heterozygous carriers were incrossed and the progeny was raised to adulthood. Two different mutant lines *foxb1a*^{f5} (Fig. 22) and *ccnd1*^{c5} (Fig. 23) were generated using TALEN. Current work is focused on addressing the viability of adult homozygous and testing boundary specification phenotype.

The emergence of highly versatile genome-editing technologies has provided us with the ability to rapidly and economically introduce sequence-specific modifications into the zebrafish genome (Morata and Lawrence, 1975), opening up the door to targeted genome-questioning of the system of interest. Under the scope of this work, TALE Nucleases will allow us to address long lasting open questions in the field regarding hindbrain boundary cell identity specification.

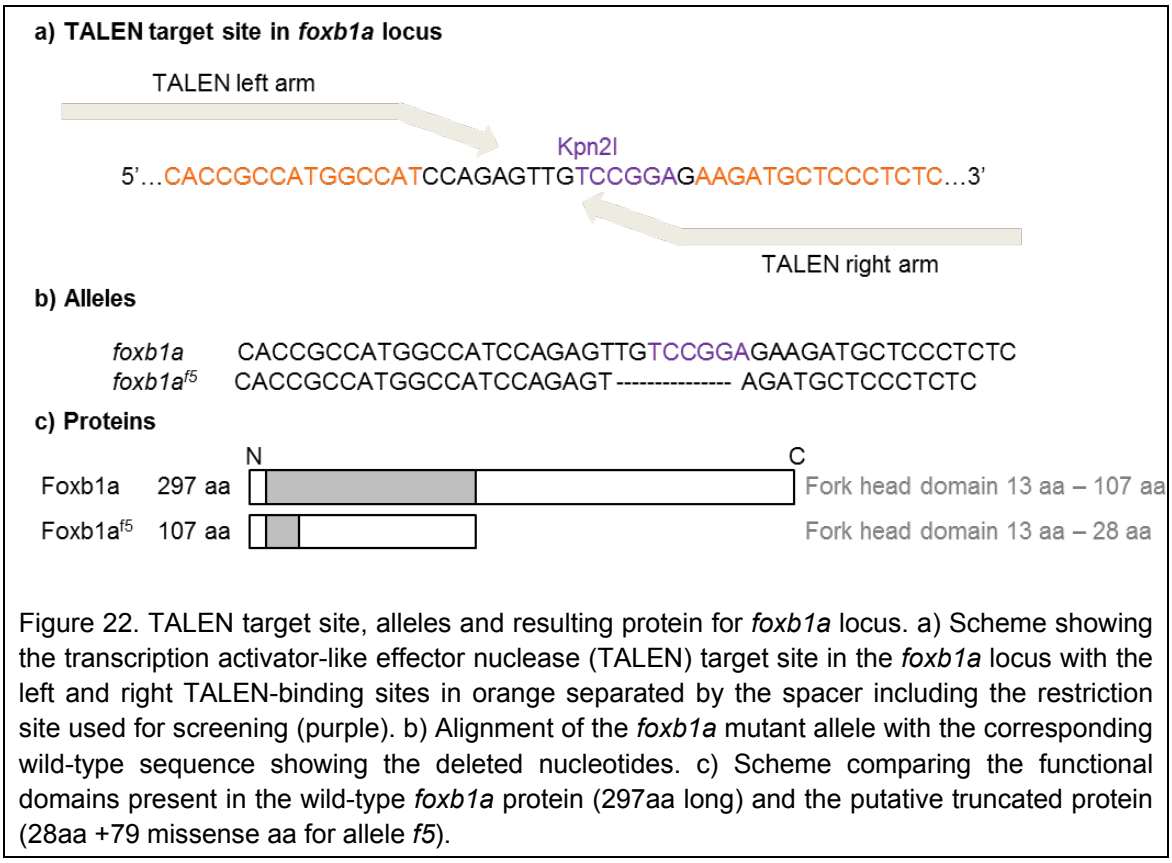

Figure 22. TALEN target site, alleles and resulting protein for *foxb1a* locus. a) Scheme showing the transcription activator-like effector nuclease (TALEN) target site in the *foxb1a* locus with the left and right TALEN-binding sites in orange separated by the spacer including the restriction site used for screening (purple). b) Alignment of the *foxb1a* mutant allele with the corresponding wild-type sequence showing the deleted nucleotides. c) Scheme comparing the functional domains present in the wild-type *foxb1a* protein (297aa long) and the putative truncated protein (28aa +79 missense aa for allele *f5*).

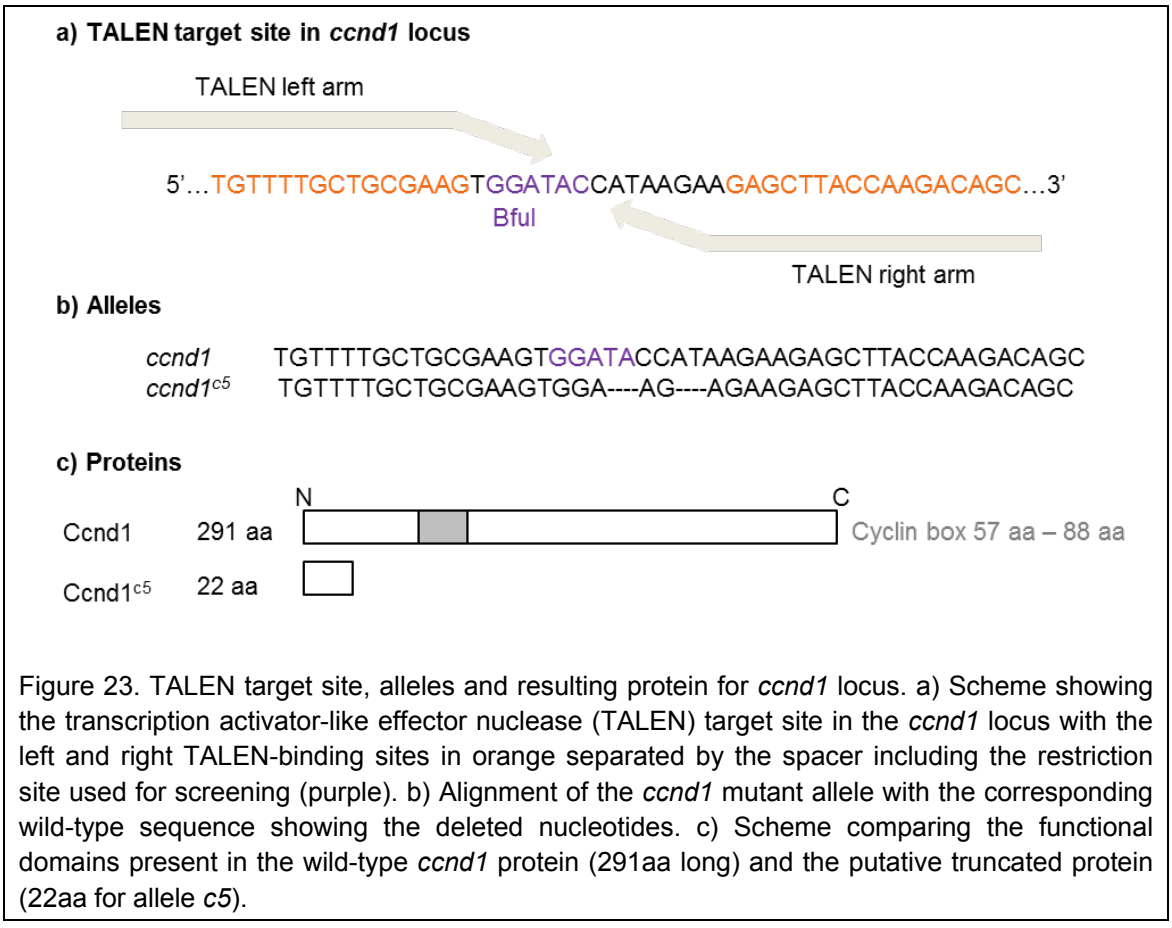

Figure 23. TALEN target site, alleles and resulting protein for *ccnd1* locus. a) Scheme showing the transcription activator-like effector nuclease (TALEN) target site in the *ccnd1* locus with the left and right TALEN-binding sites in orange separated by the spacer including the restriction site used for screening (purple). b) Alignment of the *ccnd1* mutant allele with the corresponding wild-type sequence showing the deleted nucleotides. c) Scheme comparing the functional domains present in the wild-type *ccnd1* protein (291aa long) and the putative truncated protein (22aa for allele *c5*).

6.3. *IN VIVO* HINDBRAIN IMAGING BY USING SINGLE PLANE ILLUMINATION MICROSCOPY (SPIM).

A major challenge in developmental biology is to explain how spatiotemporally controlled cell specification and differentiation occur alongside morphogenesis in the construction of functional organs. Recent developments in 4D-microscopy imaging and cell tracking tools permit now simultaneous measurements at high spatial-temporal coverage and resolution, and therefore the assessment of cell lineages and cell behaviors including displacements and proliferation (Amat et al., 2014; Blanpain and Simons, 2013; Faure et al., 2016; Keller, 2013; Li et al., 2015; Olivier et al., 2010; Truong et al., 2011). However, imaging at single-cell resolution in a highly dynamic morphogenetic scenario faces fundamental challenges. In this sense, the ultimate goal is to capture, simultaneously, the fast dynamic behavior of individual cells, as well as their system-level interactions and whole-tissue rearrangements over long periods of time. However, until now, long term *in vivo* imaging has been difficult to achieve in vertebrate embryos due to the challenges of deep-tissue imaging, and the need to improve spatial resolution and to increase temporal sampling.

The main challenge that has to be faced is the proper balance between the following parameters (Keller, 2013): i) high imaging speed, needed to facilitate effective temporal imaging of big live samples and to capture fast cellular processes such as mitotic divisions; ii) high spatial resolution, required to follow intracellular processes or to properly resolve structures in close contact such as two juxtaposed nuclei; iii) high signal-to-noise ratio, to obtain intelligible images suitable for robust automatized computational image analysis, iv) long observation periods, to encompass all the relevant developmental windows of a given forming tissue; v) comprehensive physical coverage, to follow developmental events on the whole-tissue level; an finally vi) low levels of light exposure, required to minimize photobleaching and phototoxicity. Importantly, species and tissue-dependent limitations also have to be considered, specially concerning spatial resolution.

We are working on overcoming these significant problems in order to optimize the study of progenitor cells in their native/modified environment, thereby providing quantitative insights into cell behaviour, including progeny numbers, clonal relationships and their location and differentiation status. For this we take full advantage of the experimental toolkit available for zebrafish and use a combination of genetics, functional studies and *in vivo* imaging technologies paired with image processing tools.

Reconstructing the lineage of cells is central to understanding how the wide diversity of cell types develops. Imaging speed, signal-to-noise ratio and photon-efficiency of point-scanning microscopy modalities (confocal and two-photon fluorescence microscopes) display fundamental limitations in resolving the spatio-temporal requirements for cell lineage reconstruction. Such limitations were partially overcome with the advent of single-plane illumination microscopy (SPIM), also known as light sheet fluorescence microscopy (LSFM; Huisken *et al.*, 2004; Khairy and Keller, 2011).

Light-sheet microscopy combines intrinsic optical sectioning with wide-field detection. Thus, in contrast to the point excitation approach in confocal microscopy, an entire micrometer-thin volume is illuminated from the side with a laser light sheet. After that, reporter molecules in this thin volume will emit fluorescence that will be collected in a single step with a camera-based detection system properly oriented to the light sheet angle (Amat and Keller, 2013; Keller, 2013). Fast 3D-imaging is carried out by displacing the sample through the light sheet or by quickly moving the light-sheet and detection optics (Amat and Keller, 2013). Thus, light-sheet microscopy provides reduced exposure of the specimen to the laser light and, as consequence; it offers three main advantages over conventional imaging approaches. Firstly, photo-bleaching and photo-damage effects are substantially reduced, since only the thin volume in the focus of the detection is illuminated (Icha *et al.*, 2017). In this sense, it is important to note that the physiological properties of the marked cells should not change. Hence, limiting phototoxicity by limiting illumination and fluorophore excitation to the focal plane is a prerequisite for obtaining reproducible quantitative data on biological processes (Icha *et al.*, 2017). Moreover, camera-based fluorescence detection allows for faster image acquisition, improving temporal resolution. In this sense, the speed bottleneck in light-sheet microscopy is conditioned by the performance of the camera and the electronics required for data transfer and storage. Finally, light-sheet microscopy provides high signal-to-noise ratio, owing to the long time that the laser stays on each pixel position arising from parallelized signal-detection (Amat and Keller, 2013).

Despite the fact that the zebrafish embryo is a suitable model for high-temporal resolution imaging, deep tissues such as the hindbrain still face the challenge of spatial resolution. In addition, as the embryo develops, the hindbrain becomes more densely packed and with a longer DV height, which dramatically affects the resolution of the most ventral areas of the neural tube. A potential approach to solve this compromise comes by the hand of the vertical sample mounting in light-sheet microscopy, which offers the possibility of multiview imaging that allows acquiring a series of data sets of the same volume along multiple angles. Each angle provides a different optimal view of

the same specimen that can be eventually merged into a final optimized reconstruction. However, in contrast to zebrafish tail, spinal cord or external tissues, the hindbrain grows right on top of the yolk, which limits the possible angle orientations for the light sheet to go through. In addition, abortion and scattering of the light become less pronounced as the light wavelength increases, therefore, the development of far red or near infrared fluorescent proteins should help to increase sensitivity and hence penetration depth (Shaner *et al.*, 2004; Chudakov *et al.*, 2010). Thus, spatial resolution of the ventral hindbrain territory is still a challenge that awaits further optimization.

Hindbrain cell density offers a major challenge when it comes to single-cell tracking. High cell packing implies that two or more cells in close vicinity might display partially overlapping nuclei that can be difficult to resolve by means of both automated algorithms and visual discrimination. This limitation is especially tangible for ubiquitous nuclear marker expression. Thus, in the short run, this limitation could be overcome by foregoing whole-hindbrain approaches and considering population-specific strategies. In this work, we resorted to the transgenic line Tg[4xGTIIC:d2GFP], which expresses d2GFP in a TEAD-dependent manner and in the hindbrain is exclusively active in the boundary cell population and the vessels, and we combined it with mosaic nuclear H2B:RFP expression. Ongoing work in the lab is focused on generating transgenic lines expressing H2B directly in the domain of interest, which will overcome the randomness of injected H2B mosaicism.

However, growth and morphogenetic movements transform the topologies of the different population domains at the same time cells proliferate and undergo specification. Thus, the foremost long-term goal has to consider a comprehensive whole-view capable for accounting on the dynamism of the system, in order to progress in filling the void between gene regulatory networks and tissue architecture. Previous work from the lab was focused on establishing an experimental pipeline that would allow for *in vivo* imaging of entire structures with cellular resolution during an extended period of time (Dyballa *et al.*, 2017). The work was aimed at generating data from the otic vesicle and an adaptation of the pipeline to the specificities of the hindbrain should consider the limitations described in this section (Fig. 24). Thus, the combination of cell lineage and cell behavior analyses *in vivo* will unravel the importance of morphogenesis in the control of spatial positioning of cells.

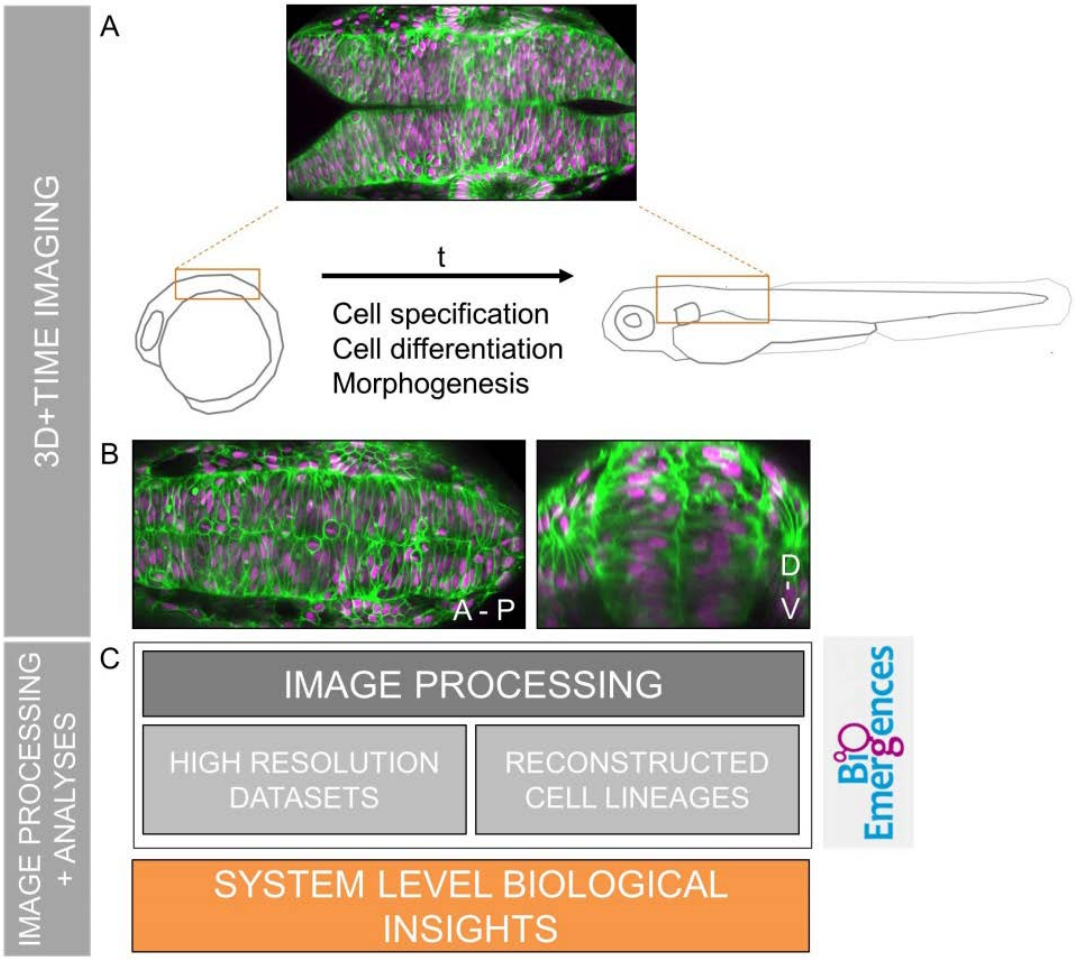

Figure 24. 3D+time image analysis pipeline. (A – B) Information about plasma membranes, nuclei and cell identities collected upon imaging of the whole AP and DV volume of the hindbrain of zebrafish embryos for several hours (16 - 72 hpf) under a light sheet microscope (3D+t SPIM imaging). (C) The acquired data is preprocessed to generate the high-resolution datasets to be launched in BioEmergences platform (Olivier et al., 2010; Faure et al., 2016) for cell center detection and automatic tracking. Data are validated, curated and analyzed using an ad-hoc strategy based on Mov-IT, a custom-made graphical interface (Faure et al., 2016), which offers the tools for segmentation and tracking of cells to accurately reconstruct their positions, movements and divisions. The high-resolution datasets and reconstructed lineages are used for qualitative and quantitative studies of the biological processes of interest. Adapted from Dyballa et al., 2017.

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APPENDIX: OTHER CONTRIBUTIONS

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