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## REVIEW

# Early Development of the Neural Plate: New Roles for Apoptosis and for One of its Main Effectors Caspase-3

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**Summary:** Despite its tremendous complexity, the vertebrate nervous system emerges from a homogenous layer of neuroepithelial cells, the neural plate. Its formation relies on the time- and space-controlled progression of developmental programs. Apoptosis is a biological process that removes superfluous and potentially dangerous cells and is implemented through the activation of a molecular pathway conserved during evolution. Apoptosis and an unconventional function of one of its main effectors, caspase-3, contribute to the patterning and growth of the neuroepithelium. Little is known about the intrinsic and extrinsic cues controlling activities of the apoptotic machinery during development. The BarH-like (Barhl) proteins are homeodomain-containing transcription factors. The observations in *Caenorhabditis elegans*, *Xenopus*, and mice document that Barhl proteins act in cell survival and as cell type-specific regulators of a caspase-3 function that limits neural progenitor proliferation. In this review, we discuss the roles and regulatory modes of the apoptotic machinery in the development of the neural plate. We focus on the Barhl2, the Sonic Hedgehog, and the Wnt pathways and their activities in neural progenitor survival and proliferation. *genesis* 53:203–224, 2015. © 2015 Wiley Periodicals, Inc.

**Key words:** organizer; BarH; diencephalon; development; Sonic Hedgehog; Wnt

## INTRODUCTION

The vertebrate nervous system is an extraordinarily complex structure. Its formation relies on the coordinated progression of genetic programs through time and space. The vertebrate nervous system emerges from the neural plate, a homogeneous layer of neuroepithelial cells. The understanding of how developmental programs are encoded and induced during establishment of the neural plate is at present a major goal in developmental biology. The first key steps in neural development are conserved during evolution, including patterning, growth, migration, and wiring mechanisms. Notably, core components of the gene regulatory networks (GRNs) that orchestrate development are evolutionary conserved. Programmed cell death (PCD) allows the efficient elimination of cells and is

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implemented through the activation of a molecular pathway conserved during evolution. Observations indicate new roles for apoptosis and its key executioners, the caspases, in neural development. Little attention has been devoted to understanding the roles and regulatory modes of the apoptotic cascade in neural morphogenesis.

### Early Events in Neural Plate Patterning and Growth

A highly complex process involving simultaneous and sequential steps subdivides the neural plate into distinct neural territories. Based on the morphological considerations in different species, embryological manipulations, and the expression pattern of regulatory genes, a conceptual model holds that the neural plate is divided into transverse and longitudinal segments that define a developmental grid generating distinct histogenic fields. Some fields become compartmentalized, specified by a unique pattern of gene expression and maintained by polyclonal cell lineage restriction. In the last two decades, elegant fate-mapping experiments have been undertaken in zebrafish (Woo *et al.*, 1995), *Xenopus* (Eagleson and Harris, 1990), and chick (Couly and Le Douarin, 1987; Figdor and Stern, 1993; Lumsden and Keynes, 1989) by several pioneering teams. Such fate map analyses prove that the position of the histogenic fields in the neural plate is a flat representation of their topological relationships in the mature neural tube. Indeed, in the neural plate, the primordia of the forebrain, midbrain, hindbrain, and spinal cord are all already established along the anterior-posterior (AP) axis of the neural plate (Heasman, 2006; Hoch *et al.*, 2009; Wilson and Houart, 2004). Therefore, a construction blueprint of the neural organization, and specifically that of the forebrain, is set up during gastrulation. At the stage of neural induction, when neural bulges have not yet appeared, the histogenic fields can be detected as spatially restricted domains of gene expression (Puelles and Rubenstein, 2003; Rubenstein *et al.*, 1994). The analysis of these patterning cues has unveiled some of the GRNs involved in the specification of neural territories (Beccari *et al.*, 2013). The correct development of a histogenic field depends on two critical biological processes that act in parallel and synchronously: the specification of neuroepithelial cells into distinct cell types, and the growth of the neuroepithelium. The precise coordination of growth and differentiation processes is necessary because disturbances in the balance between these two can be harmful to the developing organism (Dreesen and Brivanlou, 2007).

Initial regionalization of the neural tube relies on the synergistic action of at least five major signaling factors secreted by the axial mesoderm that signal in a vertical and planar manner (Niehrs, 2004; Stern, 2002).

The combinatorial activity of various secreted signals both induces the specific expression of identity genes and controls the growth of the different subregions of the central nervous system (CNS). The experiments performed mainly in amphibian, avian, and zebrafish embryos have established crucial roles for bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Hedgehog (HH), Wnt, and Nodal proteins in neural induction, neural proliferation, and in the establishment of the future dorsal-ventral (DV) and AP axes of the developing nervous system. The roles of these signaling pathways are highly conserved in vertebrates and invertebrates (Beddington and Robertson, 1999; Jessell and Sanes, 2000). The BMP inhibitors chordin, noggin, and follistatin, which are involved in the decision of ectodermal cells to become epidermal or neural cells, and Sonic Hedgehog (Shh), act as concentration-dependent morphogens to help regionalize the DV axis of the developing nervous system. Wnt signaling initially participates in the establishment of a crude AP axis and later, together with FGFs, participates in a more refined patterning of the anterior neural tube (Stern, 2005; Wilson and Rubenstein, 2000). Besides instructing cell fates, morphogens control the growth of the neural tissue, a process that depends on a delicate balance between the proliferation of neural progenitor cells and early programmed cell death (EPCD), primarily through apoptosis (Raff, 1998). BMPs, FGFs, Wnt, and Shh are all believed to be involved in controlling proliferation and apoptosis in the developing nervous system (Chenn and Walsh, 2002; Megason and McMahon, 2002; Mehlen *et al.*, 2005).

Small groups of specialized cells emerge as local sources of secreted patterning factors and hence act as "organizing centers." In 1924, the first "organizing" center was discovered by Hans Spemann and Hilde Mangold. They showed that the dorsal lip of the blastopore, if grafted into the ventral part of a host embryo, was able to induce a secondary axis containing a complete nervous system (De Robertis *et al.*, 2000; Niehrs, 2004; Stern, 2001). Since this discovery, this organizing center has been referred to as "Spemann organizer" and has been found in other model organisms; Hensen's node in the chick, the node in the mouse, and the shield in zebrafish. This primary organizer is the first to emerge during embryonic development at gastrulation and gives rise to the prechordal plate and the notochord, two tissues that send planar and vertical signals to the overlying prospective neuroepithelium (Niehrs, 2004; Stern, 2002; Wilson and Houart, 2004). The primary organizer induces the neuroectoderm, which gives rise to the neural plate in the dorsal ectoderm through the secretion of inhibitors of the BMP signaling pathway. In contrast, the epidermis forms in the ventral ectoderm, which is still exposed to the high levels of BMP signals (Harland, 2000). The recent studies rendered this

model of neural induction more complex by revealing the importance of crossinteractions with other signaling pathways such as Wnt and FGF (Stern, 2006). Secondary signaling centers emerge later in development. These signaling centers are located at molecular frontiers that separate territories and are of fundamental importance for the regionalization of the brain as they allow the structures of the CNS to refine their development (Cavodeassi and Houart, 2012; Echevarri *et al.*, 2003; Kiecker and Lumsden, 2012). The anterior neural ridge (ANR) borders the neural plate anteriorly at the interface between the non-neural ectoderm and the neuroectoderm. The ANR expresses Fgf8 and specifies the forebrain (Houart *et al.*, 1998). The *zona limitans intrathalamica* (ZLI) is defined by the alar plate expression of Shh, a secreted morphogen that mediates regionalization of the prethalamus anteriorly and the thalamus posteriorly (Juraver-Geslin *et al.*, 2014; Scholpp *et al.*, 2006; Vieira *et al.*, 2005). The midbrain-hindbrain boundary (or isthmus organizer) separates the anterior and posterior neural tube and secretes Wnt1 and Fgf8 (Crossley and Martin, 1995). Finally, the floor plate through the secretion of Shh, and the roof plate through that of Wnts and BMPs ligands also act as signaling centers (Fig. 5a) (Alexandre and Wassef, 2003; Cavodeassi and Houart, 2012; Echevarri *et al.*, 2003).

As morphogenetic gradients are essential for the outgrowth and patterning of the neural plate and tube, the number of cells producing these morphogens must be tightly regulated. At least for a signaling structure secreting Shh, the location, size, and shape of the signaling center determine the size, shape, and orientation of the target tissue (Agarwala *et al.*, 2001). This is also true for the neural plate whose size and shape are at least partially determined by the secretion of BMP inhibitors from the notochord (Inomata *et al.*, 2013; Khokha *et al.*, 2005) and for the ventral telencephalon, whose correct development relies on local antagonism of Wnt signaling and Fgf-8 secretion from the ANR (Houart *et al.*, 2002; Nonomura *et al.*, 2013). In both *Xenopus* and mouse, apoptosis is part of the system that controls the correct establishment of signaling centers during the rapid growth of the embryo (Nonomura *et al.*, 2013; Offner *et al.*, 2005).

Moreover the response to a morphogen can be cell specific and, during the period of regionalization, the growth rate of adjacent histogenic fields can vary. In amphibian, the diencephalic (caudal forebrain) primordium grows less compared to the telencephalic (rostral forebrain) primordium. Interestingly, this differential growth has been attributed to an unconventional activity of caspase-3, one of the main executioners of the apoptotic cascade. Caspase-3 acts as a proliferation brake by limiting Wnt signaling. Importantly, this role of caspase-3 in limiting *Xenopus* diencephalon growth does not depend on its apoptosis effector function

(Juraver-Geslin *et al.*, 2011). Taken together, these observations reveal important activities of the apoptotic machinery in early regionalization of the neural plate.

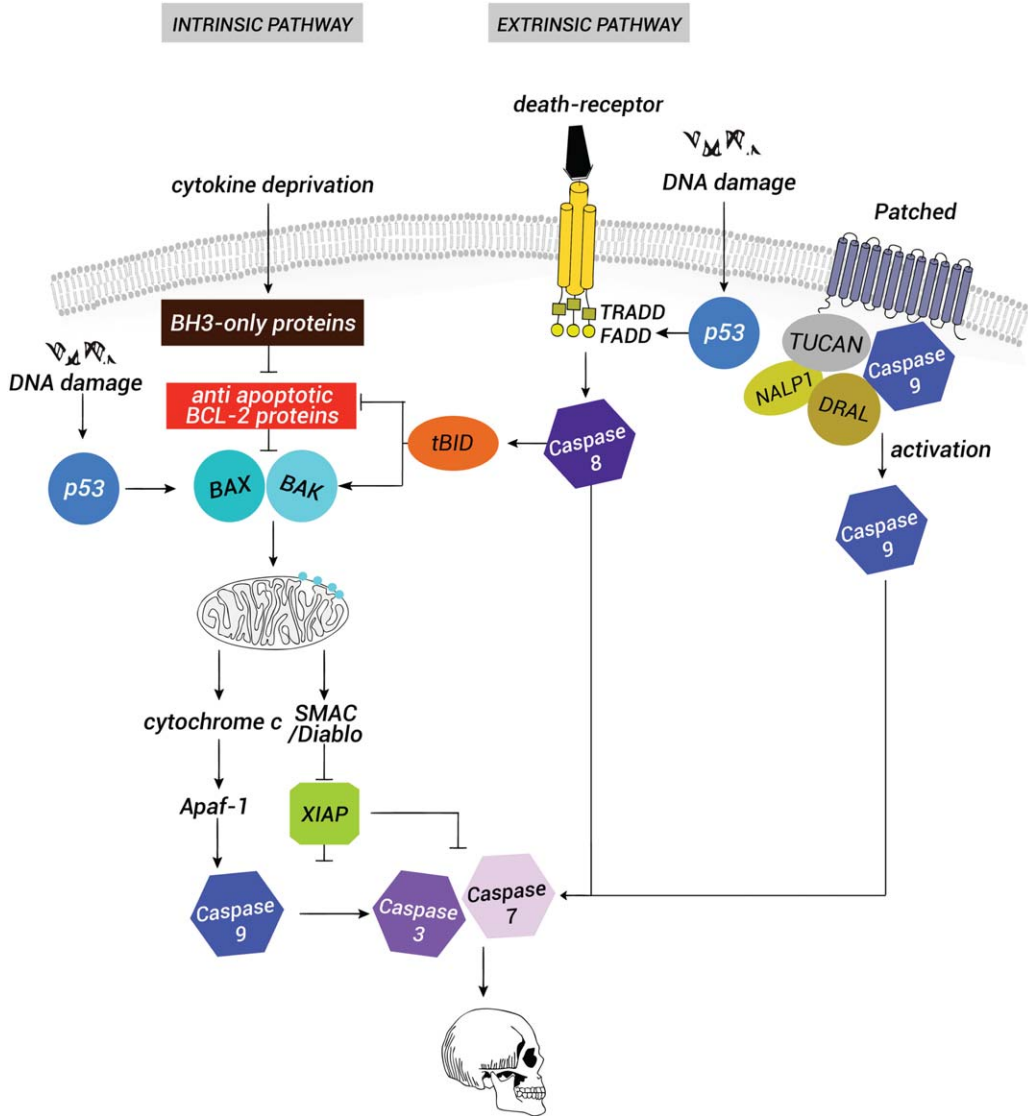
The homeodomain-containing genes are important target genes regulated by morphogens. They encode gene regulatory proteins and specify regional identity by regulating the “effector” genes, which influence cell proliferation, survival, differentiation, adhesion, shape, and migration (Rubenstein and Rakic, 1999). A family of such transcription factors, the Bar-class HD (Barh), contains two members, BarH1 and BarH2, which are well conserved in metazoans during evolution. In both *Xenopus* and mice, *barhl2* is expressed in the developing neuroepithelium from early gastrulation onward, at a time period overlapping with the initial steps of neural plate patterning (Fig. 2A) (Juraver-Geslin *et al.*, 2011, 2014; Offner *et al.*, 2005; Patterson *et al.*, 2000). The investigation of BarH proteins activities in nematode, *Xenopus*, and mice revealed the roles for these transcription factors in the developmental control of cell death and as cell type-specific regulators of caspase-3 nonapoptotic functions (Juraver-Geslin *et al.*, 2011; Li *et al.*, 2002, 2004; Nehme *et al.*, 2010; Offner *et al.*, 2005; Peden *et al.*, 2007; Schwartz and Horvitz, 2007). These activities are strictly dependent on the cellular context and their regulatory modes in vertebrates have not yet been elucidated.

In this review, we discuss the respective contributions of EPCD and the nonapoptotic functions of caspase-3 to early development of the neuroepithelium. We present the activities of BarH proteins in the regulation of cell survival. We examine the molecular mechanisms underlying the differential growth within the diencephalic field.

### Apoptosis and Caspase-3 Activities in Neural Plate Development

Most studies, to date, on the function of the apoptotic cascade in neural development have focused on the late developmental stages after cell differentiation. Here, we focus on EPCD, which affects proliferating cells during neurulation.

**Local apoptosis plays an important role in the formation of organizing centers.** PCD was originally documented in the nematode *Caenorhabditis elegans* and more than a decade of extensive work has led to a better understanding of the mechanisms driving cell suicide (Fuchs and Steller, 2011; Hyman and Yuan, 2012; Yi and Yuan, 2009). An emerging body of evidence demonstrates that the apoptotic signaling pathway is tightly regulated at different levels (Fig. 1). Current knowledge on the different stages of this cascade in vertebrates comes from the studies on the adult immune system. The key players in the regulation of this cascade are the proteins of the Bcl-2 (B-cell



**FIG. 1.** Scheme of the vertebrate apoptotic pathway. In a viable cell, the proapoptotic proteins Bax and Bak of the B-cell lymphoma 2 (Bcl2) family are repressed by the antiapoptotic proteins Bcl2, Mcl1 and Bcl-X<sub>L</sub>. Proapoptotic stimuli such as cytokine deprivation or endoplasmic reticulum stress trigger the induction of the BH3-only proteins. BH3-only proteins counteract the effect of Bcl2 and Bcl-X<sub>L</sub>, a process that leads to the release of Bax and Bak. Bax and Bak are then inserted and activated at the outer mitochondrial membrane where they promote the formation of pores. A key event in the induction of death is the release of cytochrome c and SMAC/Diablo from the mitochondrial intermembranous space to the cytoplasm. SMAC/Diablo inhibits the IAP proteins, whereas cytochrome c recruits APAF-1 to form the apoptosome. Caspase-9 is activated by the apoptosome that leads to the cleavage of the effector caspases, caspase-3 and caspase-7, and to irreversible death. In parallel to this intrinsic pathway, the stimulation of death receptors leads to the recruitment of the adaptor proteins such as TRADD and FADD through their death domain. Caspase-8 is then recruited to the membrane dimerizes and can directly activate caspase-3 and caspase-7. Crosstalk between the extrinsic and the intrinsic pathway depends on the caspase-8 cleavage of the BH3-only protein BH3-interacting domain death agonist BID. The product of this cleavage t-BID (truncated BID) inhibits Bcl2, Mcl1 and Bcl-X<sub>L</sub> and promotes the accumulation of Bax and Bak. Both pathways can be activated through p53 stimulation resulting from DNA damage. Another pathway is linked to the absence of the survival factor Shh that leads to the stimulation of the proapoptotic function of its receptor patched. In the absence of its ligand, patched forms a protein complex with the adaptor protein DRAL (downregulated in rhabdomyosarcoma LIM-domain protein) and one caspase recruitment (CARD)-domain containing protein, either TUCAN (family member, 8) or NALP (NLR family, pyrin domain containing 1). Apical caspase-9 is then recruited to this complex and activated by patched, which leads to activation of the effector caspases.

lymphoma 2) family, which include antiapoptotic proteins that allow survival (Bcl-2, Mcl1, Bcl-X specifically its long isoform Bcl-X<sub>L</sub>) and are different from those which play a proapoptotic role (Bim, Puma, or Bid)

(Happo *et al.*, 2012; Tsuchiya and Yamashita, 2011). Both types of Bcl-2 proteins are present in most cells and a balance between the ratio of pro-versus antiapoptotic proteins decides whether a cell lives or dies (Raff,



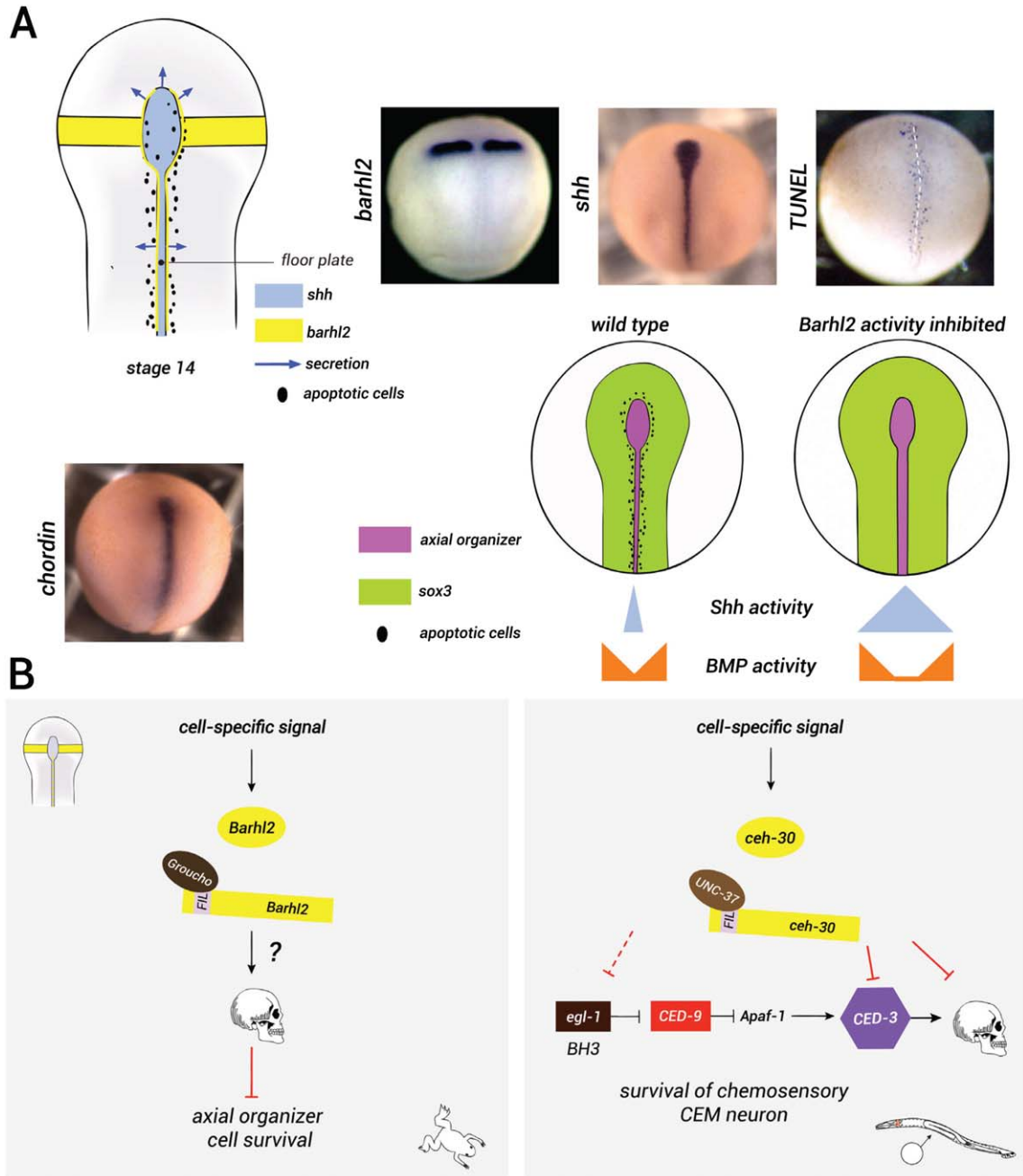
1992, 1998). The activation of the apoptotic cascade leads to the activation of a family of cysteine proteases, the caspases, which are very promiscuous enzymes that cleave their substrates at an aspartate residue. The activation of effector caspases such as caspase-3 and caspase-7 by proteolytic cleavage leads to self-activation of caspases and irreversibly to cell death by triggering degradation of the DNA and other vital cell components (Nicholson and Thornberry, 1997) (Fig. 1). From all these studies emerged, the concept that all animal cells contain the proteins required to execute the death program and that most developing cells, at least in higher organisms, require signals from other cells to avoid apoptosis (Raff, 1992, 1998).

EPCD mainly occurs during neurulation, neural crest formation, and eye induction and formation (de la Rosa and de Pablo, 2000; Kuan *et al.*, 2000; Yeo and Gautier, 2004). The targeted disruption of the effector molecules of the apoptotic pathway reveals the occurrence and magnitude of EPCD and its early roles during vertebrate CNS development. Specifically, disruption of caspase-3, caspase-9, and apaf-1, and specific ablation of cytochrome *c* apoptotic function, result in massive disorganization of the CNS structures (Kuida *et al.*, 1996; Nonomura *et al.*, 2013). Evidence in both mice and *Xenopus* suggests that, at these developmental stages, defects generated by the lack of apoptosis executioners are indirect effects. TUNEL analysis performed in different vertebrate species demonstrates that during the regionalization process apoptosis occurs in a developmentally controlled manner. During gastrulation and early neurulation, cells undergoing apoptosis are localized within the forming axial organizer, which we define as the notochord, the prechordal plate, and the floor plate (Fig. 2A) (Hensey and Gautier, 1998; Offner *et al.*, 2005; Yeo and Gautier, 2003). In *Xenopus*, interference with endogenous apoptosis interferes with correct signaling from the forming axial organizing center. The neural induction process is largely controlled by the secretion of anti-BMP signals from the Spemann organizer (Inomata *et al.*, 2013). The inhibition of apoptosis by overexpression of an antiapoptotic gene of the *bcl2* family increases both the size of the neural plate and the number of *chordin*- and *shh*-secreting cells from the notochord and floor plate (Offner *et al.*, 2005; Yeo and Gautier, 2003). It has, therefore, been argued that in this context EPCD is part of the system that controls both the size and the correct establishment of the axial organizer and indirectly accurate formation of BMP and Shh activities gradients within the neural plate (Fig. 2A) (Offner *et al.*, 2005). At later developmental stages, TUNEL-positive cells are detected at the border between the neural plate and the non-neural ectoderm (Juraver-Geslin *et al.*, 2011; Yamaguchi and Miura, 2013). In mouse, *apaf1*- and *caspase-9*-null embryos, the primary cause of brain mal-

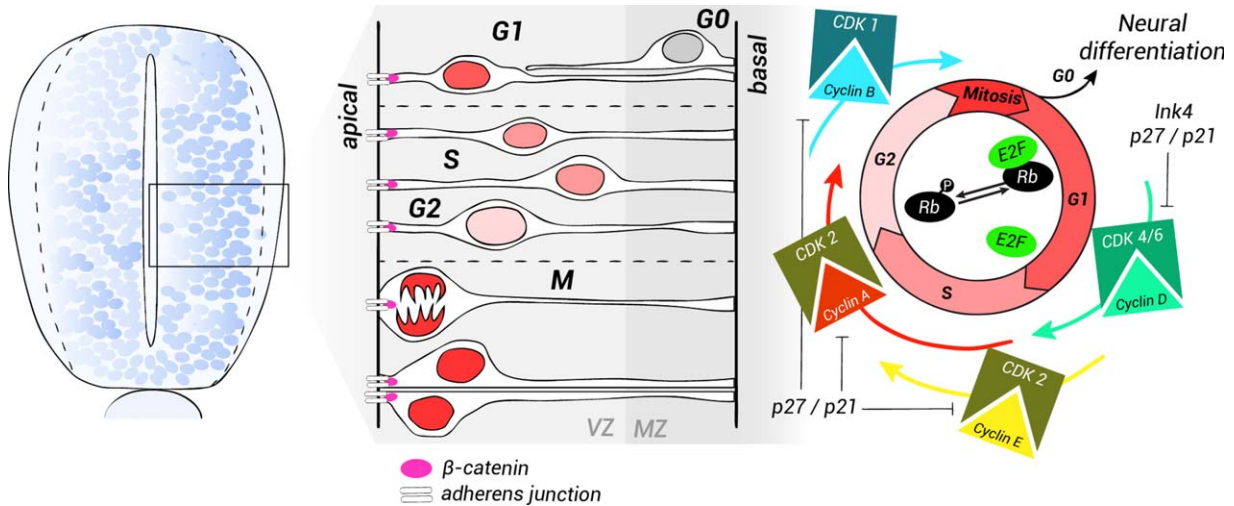
formations is a failure to complete cranial neural tube closure and the accumulation of nonproliferative FGF8-producing cells from the ANR. This defect in the elimination of FGF8-producing cells from the ventral part of the ANR by apoptosis generates an accumulation of the FGF8 protein and an increase in FGF8 signaling. This enhanced FGF8 signaling owing to apoptosis deficiency perturbs correct development of the ventral telencephalon (Nonomura *et al.*, 2013). Finally, during vertebrate limb development, the zone of polarizing activity (ZPA), a group of cells located at the posterior margin of the early limb bud, produces Shh, which acts as a morphogen gradient and progressively specifies anteroposterior digit identities in the wing. The ZPA acts as an organizing center and apoptosis similarly regulates the number of Shh-expressing cells in this region (Sanz-Ezquerro and Tickle, 2000). In this context, apoptosis indirectly contributes to establishing the proper distribution of anti-BMP (*chordin*), Shh, and FGF8 proteins and hence to correct patterning of the neural plate, the developing forebrain, or the limb. It is worth noting that signaling center cells and their neighboring cells differ in their susceptibility to morphogen-induced apoptosis (Nonomura *et al.*, 2013; Offner *et al.*, 2005; Sanz-Ezquerro and Tickle, 2000; Storm *et al.*, 2003).

The mechanisms regulating EPCD at these developmental stages are largely unknown. The previous studies demonstrated the importance of morphogens, specifically BMP, FGFs, and Shh that are actively involved in regulating apoptosis in the developing brain or limb (Aoto *et al.*, 2002; Furuta *et al.*, 1997; Sanz-Ezquerro and Tickle, 2000; Thibert *et al.*, 2003; Zou and Niswander, 1996). However, as the activation of PCD is dependent on cell lineage or cell location, it remains a challenge to determine whether the growth factors directly induce apoptosis or whether their ability to regulate cellular physiological status has an indirect effect on sensitivity to apoptosis (Mehlen *et al.*, 2005). In all cases, although the effector mechanisms controlling apoptotic processes appear to be shared between proliferating neuroepithelial cells and postmitotic neurons, the genetic controls for apoptosis induction probably differ in both types of cells (Kumar and Cakouros, 2004).

**An unconventional activity of caspase-3 limits growth in the anterior neural tube.** Disruption of the *caspase-3* gene in mice results in disorganized CNS structures associated with a brain overgrowth phenotype and hyperplasia of the ventricular and/or mantle zone. The first signs of these defects appear around E10.5 and cellular masses in the forebrain are expanded, in particular in the thalamus and the hypothalamus, two structures derived from the diencephalon (Kuida *et al.*, 1996). Two main routes to caspase activation have been described in vertebrates: activation of death domain receptors and induction of



**FIG. 2.** Barhl2: a regulator of EPCD in the neural plate. **(a)** Expression patterns of *barhl2*, *shh*, and *chordin* and pattern of endogenous apoptosis in *Xenopus* neurula embryos. The schematic representation of *barhl2*, *shh*, and endogenous apoptosis in the developing neural plate: At stage 14 *barhl2* is expressed in the caudal forebrain primordium as two bilateral strips and in cells bordering the floor plate and possibly the notochord (yellow in the scheme). The axial organizer, which we define as the notochord, the prechordal plate and the floor plate, emerge from the Spemann organizer and secrete anti-BMP factors (chordin) and Shh (lavender). The secretion is shown with arrows and cells undergoing apoptosis are shown as black spots. Whole mount in situ hybridization (ISH) using *barhl2*, *shh*, or *chordin* as probes and whole mount TUNEL staining as indicated. Representative neurula embryos are shown in dorsal view, anterior up. Cells undergoing endogenous apoptosis are marked by Sox3 (green), (after Offner *et al.*, 2005). **(b)** The BarH survival pathways in amphibians and nematodes. During neurulation, Barhl2 regulates EPCD. Barhl2 interacts with Groucho and induces cell death of axial organizer cells expressing the morphogens Shh and chordin through an unknown mechanism. In *C. elegans*, the Barhl2 orthologue, Ceh-30, regulates the survival of CEM chemosensory neurons in male organisms. In males, *ceh-30* is induced by cell-specific signals, and it interacts with UNC-37, the groucho orthologue, and acts as a transcriptional repressor of *ced-3* and *egl-1* genes.



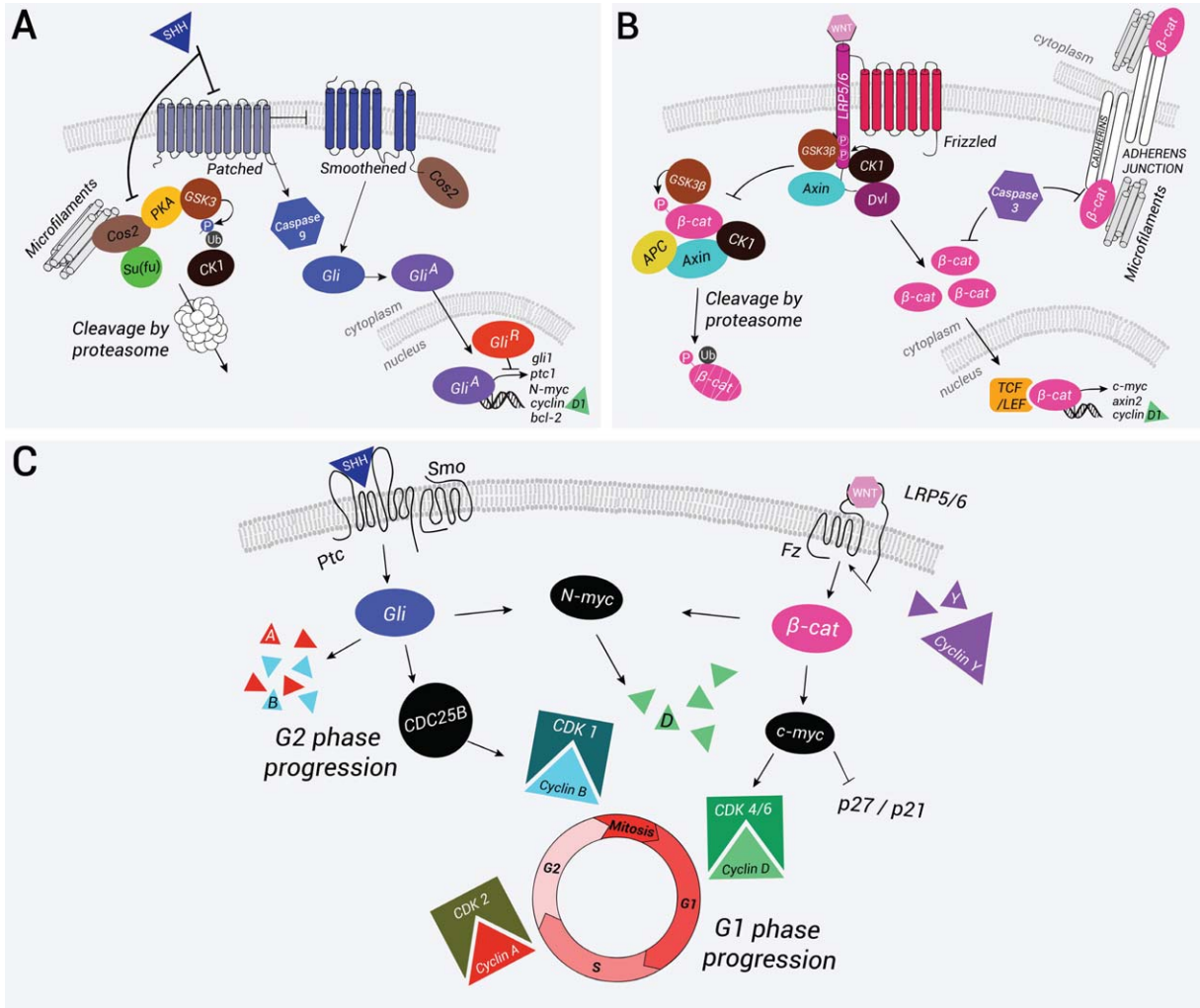
**FIG. 3.** Scheme illustrating cell-cycle dynamics in neural progenitors. The neuroepithelium is composed of a single layer of rapidly dividing NSCs and progenitors. As the cells adjacent to the lumen continue to proliferate, the neural progenitors migrate and form a second layer around the neural tube. This layer becomes progressively thicker and is called the mantle zone (MZ), whereas the germinal neuroepithelium is known as the ventricular zone (VZ), subsequently called the ependyma. Cells in the MZ differentiate into both neurons and glia. In the germinal neuroepithelium, neural progenitors undergo different phases of the cell cycle. The nucleus of proliferating cells undergoes a process of interkinetic nuclear migration that corresponds to each phase of the cycle. The nuclei move away from the apical surface during G1. DNA replication (S phase) takes place in the basal VZ. During G2, the nuclei migrate away from the apical ventricular zone where they undergo mitosis (M). After one cycle is completed, the cells choose either to re-enter the cell cycle or to exit and differentiate. Quiescent cells are found in the MZ. Progression through the cell cycle depends on the cyclin/CDK complexes. During G1, phosphorylation of the retinoblastoma complex (Rb) by cyclins D releases the transcription factor E2F and induces the expression of cyclins E. DNA synthesis during the S phase is followed by the verification of DNA integrity. The progression through mitosis depends on the cyclin B/CDK1 complex. The CDK inhibitors (CKI) p27/Kip1 and p21/Cip1 stop the progression through the cycle and favor exit from the cell cycle (G0).

BH3-only proteins, the proapoptotic members of the Bcl-2 family, that is the extrinsic and intrinsic pathways, respectively (Fig. 1). In the developing CNS, the Bcl-X<sub>L</sub> protein, the long isoform encoded by the *bcl-x* (*bcl211*) gene, and the myeloid cell leukemia 1 Mcl1 protein, act as the main antiapoptotic Bcl2 family members (Motoyama et al., 1995; Arbour et al., 2008; Hasan et al., 2013). The targeted disruption of Bcl211 (Bcl-X) causes a dramatic increase in apoptosis of immature neurons throughout the embryonic nervous system. When Bcl211 (Bcl-X) mutant mice are crossed with the caspase-3-deficient mice, the double mutation abrogates the ectopic cell death observed in postmitotic neurons, caused by the Bcl211 (Bcl-X) deficiency alone. Therefore, in these cells, caspase-3 acts downstream of Bcl-X<sub>L</sub> as an effector of cell death. In contrast, Bcl211 (Bcl-X) deficiency does not affect apoptosis of neural precursor cells and Bcl-X<sub>L</sub> protein levels are low in the forebrain ventricular zone. Mcl1 deficient neural progenitors undergo cell death as they commit to a neuronal fate and migrate away from the ventricular zone (Arbour et al., 2008). However overexpression of antiapoptotic proteins of the Bcl2 family, which protect cells against death, does not affect the growth of the forebrain (Martinou et al., 1994). In addition, Bax-deficient embryos have no signs of hyperplasia or malformations of the nervous system, whereas there is a marked hyperplasia of the embryonic nervous tissue in caspase-3-deficient mice. These results

indicate that, though downstream of Bax and Bcl-X<sub>L</sub> in the apoptosis of postmitotic neurons, caspase 3 has a unique function in regulating the size of the progenitor pool during early development, before neurogenesis begins (Kuan et al., 2000; Oppenheim, 2001; Roth et al., 2000).

In *Xenopus* embryos, overexpression of Bcl-X<sub>L</sub> decreases the rate of proliferating neuroepithelial cells undergoing apoptosis. However, in agreement with the observations in mice, these embryos do not exhibit any growth phenotype. In contrast, at similar developmental stages caspase-3-depleted embryos exhibit a forebrain hyperplasia. Indeed, these embryos have a greater number of neuroepithelial cells and this increase is associated with a higher rate of proliferation in cells where caspase-3 is absent. The analysis of apoptotic cell distribution, together with the analysis of the phenotype of Bcl-X<sub>L</sub>-overexpressing embryos compare with that of caspase-3-deficient embryos, demonstrates that the requirement of caspase-3 for normal growth of these brain regions involves its influence on cell proliferation rather than on apoptosis (Juraver-Geslin et al., 2011). In addition, *Xenopus* caspase-3-depleted embryos exhibit a localized disorganization of the neuroepithelium architecture, with a disturbance in nuclear migration interkinetic movements (Fig. 3). The proliferation and organization defects of caspase-3-depleted neuroepithelium are similar to those observed





**FIG. 4.** The Wnt canonical and the Shh pathways and their interactions. **(a)** Schematic representation of the Shh pathway. In the absence of Shh, patched (Ptc) inhibits smoothened (Smo) and prevents its translocation to the cell surface. Cos2 binding to the microtubules allows the formation of a protein complex with recruitment of GSK3, CK1 $\alpha$ , PKA, and Su(Fu). The protein complex phosphorylates the Gli proteins and targets their proteosomal degradation. Truncated Gli forms translocate to the nucleus where they repress target gene expression. In parallel, patched can recruit and activate caspase-9 a process that leads to cell death. Binding of Shh to Ptc initiates the pathway, alleviates repression of Smo, which is translocated to the membrane, and promotes cell survival. Cos2 binds to Smo and inhibits the phosphorylation of Gli proteins by releasing the different kinases. Full activation of the pathway is achieved by phosphorylation of the cytosolic C-tail of Smo by PKA and CK1. Full-length forms of Gli are translocated to the nucleus where they induce the expression of Shh target genes such as Gli-1, Ptc, N-Myc, Bcl-2, and cyclin D1. **(b)** The schematic representation of the Wnt canonical pathway. In the absence of a Wnt signal,  $\beta$ -catenin is phosphorylated and targeted for proteasome-mediated degradation by a protein complex containing GSK3- $\beta$ , CK1 $\alpha$ , APC, and Axin. After binding of the Wnt ligand to the receptors Frizzled (Fz) and LRP5/6, dishevelled (Dvl) binds to Fz and recruits the destruction complex at the cytosolic tail of LRP5/6. LRP5/6 tail is then phosphorylated by GSK3- $\beta$  and bound by Axin. In this context,  $\beta$ -catenin is stabilized and translocated to the nucleus where in association with TCF/LEF transcription factors it stimulates expression of the target genes *c-myc*, *axin2*, and *cyclin D1*. In parallel,  $\beta$ -catenin is present at the adherens junctions where it associates with cadherins and with the cytoskeleton, allowing adhesion between neighboring cells. Caspases, and specifically caspase-3, can destabilize the adherens junctions in part by inhibiting  $\beta$ -catenin association with E-cadherin. At note in vitro  $\beta$ -catenin is a substrate for caspases. **(c)** Crosstalk between the Wnt and the Shh pathways promotes cell-cycle progression. The Shh pathway mostly acts on the G2 and S phases through the regulation of phosphatase CDC25B expression. N-Myc is capable of regulating cyclin D1 expression, which allows progression through G1. In some cases, Shh also regulates the expression of late cyclins A and B. The Wnt canonical pathway also promotes G1 progression by inducing the expression of *c-myc*, which regulates cyclin D1 expression and inhibits p27/Kip1 and p21/Cip1. A feedback loop is regulated by cyclins Y, which phosphorylate the cytosolic tail of LRP5/6 to activate the Wnt pathway.

in mice expressing an activated form of  $\beta$ -catenin (Chenn and Walsh, 2002; Junghans *et al.*, 2005). Indeed, the amounts of total  $\beta$ -catenin and its active form are increased in the brain of caspase-3-depleted

*Xenopus* embryos. The increase in  $\beta$ -catenin is associated with the upregulation of downstream targets of  $\beta$ -catenin/T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) signaling, *axin2*, and the cell-cycle

regulator, cyclin D1 (*ccnd1*) (Fig. 4B).  $\beta$ -Catenin is known to participate in adherens junctions and to form a stable complex with the molecules of the cadherin family (Figs. 3 and 4B) (Redies, 2000; Stepniak *et al.*, 2009). The depletion of  $\beta$ -catenin through morpholino injection, or overexpression of glycogen synthase kinase 3  $\beta$  (GSK3- $\beta$ ) which inhibits  $\beta$ -catenin activation, partially rescues the proliferative and architectural defects observed in caspase-3-depleted embryos. Therefore, increased levels of total and active  $\beta$ -catenin probably account for the proliferative and neuroarchitectural defects observed in caspase-3-depleted embryos (Juraver-Geslin *et al.*, 2011).

$\beta$ -Catenin is one of the many substrates of caspase-3. When the apoptotic cascade is activated, rupture of cell polarity and loss of cell junctions are two of the harbingers of the death event (Strasser *et al.*, 2000). In the epithelial cells of *Drosophila*, drICE, the orthologue of caspase-3, directly interacts with Armadillo, the orthologue of  $\beta$ -catenin, and cleaves it at its DQVD peptide motif. This cleavage inhibits its interaction with E-cadherin (epithelial cadherin) and destabilizes adherens junctions (Kessler and Muller, 2009). The caspase cleavage site in the sequence of  $\beta$ -catenin is conserved in vertebrates. However, this ability of caspase-3 to cleave  $\beta$ -catenin is at odds with what is observed in the diencephalic primordium of *Xenopus* embryos depleted for caspase-3. In these embryos, depletion of either caspase-3 or  $\beta$ -catenin alters the attachment of cells in mitosis to the apical surface. The depletion of  $\beta$ -catenin partially rescues this defect in caspase-3-depleted embryos and vice versa (Juraver-Geslin *et al.*, 2011). It is therefore tempting to speculate that  $\beta$ -catenin is not the main substrate of caspase-3 in this context and that in caspase-3-depleted embryos, the equilibrium between membrane-bound  $\beta$ -catenin at the adherens junctions and cytosolic  $\beta$ -catenin is disrupted (Nelson and Nusse, 2004). Such disruption is known to dramatically alter the internal structure of the neuroepithelium (Kadowaki *et al.*, 2007).

In conclusion, observations in amphibians, chick, and mice indicate that local EPCD plays an important role in the formation of organizing centers. It coordinates the emergence and the removal of morphogen-producing cells both spatially and temporally, allowing the accurate growth of the developmental process. In addition, the apoptotic machinery contributes to the differential growth of neighboring histogenic fields, at least partly through caspase-3 unconventional activities. The similarities between the mouse caspase-3-deficient phenotype and that observed in caspase-3-depleted *Xenopus* embryos suggest that the role of caspase-3 in regulating the proliferation of neuroepithelial cells during development could be conserved at least in some vertebrates, irrespective of its role in apoptosis.

### **Barhl2: A Regulator of EPCD in the Neural Plate**

**Barhl2 regulates survival of axial organizer cells.** BarH1 and BarH2 are homeodomain-containing transcription factors of Bar-class HD (BarH) initially discovered in *Drosophila*. The paralogues known as Barh-like (Barhl) have subsequently been identified in fish (zebrafish, medaka), amphibians (*Xenopus*), birds (chicken), and mammals (mouse). In vertebrates, *barhl1* is the closest relative of *barhl2*, and phylogenetic analysis shows that Barhl proteins are orthologues of the *Drosophila* BarH and nematode CEH-30 and CEH-31 proteins and are well conserved in metazoans during evolution (Reig *et al.*, 2007; Schuhmacher *et al.*, 2011). During the embryonic development of vertebrates, *barhl1* and *barhl2* are predominantly expressed in the CNS and their expression patterns are distinct but partially overlap (Bulfone *et al.*, 2000; Colombo *et al.*, 2006; Patterson *et al.*, 2000). BarH genes are involved in diverse processes such as the acquisition of neural identity in the retina, the specification of commissural neurons in the spinal cord, and cell migration in the cerebellum and hindbrain (Chellappa *et al.*, 2008; Ding *et al.*, 2009; Li *et al.*, 2004; Mo *et al.*, 2004; Poggi *et al.*, 2004; Saba *et al.*, 2005; Jusuf *et al.*, 2012). Here, we focus on the activities of Barhl2 in early neural development.

In *Xenopus*, *barhl2* is normally expressed from the onset of gastrulation in two longitudinal stripes along the axial organizer (Fig. 2a). Barhl2 promotes apoptosis in the *Xenopus* neuroectoderm through a mechanism that cannot be attributed to an unspecific cellular stress response. Barhl2 GOF induces a decrease in the size of the neural plate that is rescued by inhibition of apoptosis. Reversibly, Barhl2 LOF generates a dramatic increase in neuroepithelium size that mimics BMP signaling LOF. Indeed, it has been shown that the proapoptotic activity of Barhl2 is essential during normal neural plate formation as it limits the number of chordin- (a BMP inhibitor) and Shh-expressing cells in the prospective notochord and floor plate, which act as organizing centers (Fig. 2a). Therefore, we proposed that *Barhl2* is part of a pathway that regulates EPCD in the neural plate. Barhl2-regulated cell death contributes to control both the size and the correct establishment of the axial organizer and indirectly, to the formation of the BMP and Shh activities gradient that control patterning and growth of the neural plate (Offner *et al.*, 2005). The determination of how Barhl2 regulates EPCD during development is unknown. BarH transcription factors are characterized by their homeodomain, and their FII domains located in the N-terminal region of the protein (Offner *et al.*, 2005). The BarH/Barhl proteins differ from other homeodomain-containing proteins in the presence of a tyrosine (Y) at position 49 in the homeodomain, which is commonly occupied by a phenylalanine (F). This residue is located in the third helix,

which is the recognition site for the DNA, suggesting a modification of the specific recognition site. In addition, Barhl proteins possess two highly conserved FIL domains in their N-terminal region that correspond to the engrailed homology 1 (EH1) motif, which is known to interact with the protein Groucho (*C. elegans* UNC-37, human transducin-like enhancers of split). Although in *Drosophila* barH genes contain one FIL domain, they contain two in vertebrates. It is believed that through the recruitment of Groucho, Barhl form a protein complex that binds at the transcription initiation site and inhibit transcription (Cinnamon and Paroush, 2008). Therefore, Barhl2 could act through transcriptional modulation of the survival pathways controlling apoptosis, specifically the Shh and/or the BMP pathway that are produced by axial organizer cells. Alternatively, Barhl2 could transcriptionally modulate the apoptotic developmental program.

**Barhl2 and the signaling pathways in neural plate cells survival.** At the end of gastrulation, the prechordal mesoderms, together with the notochord, are the primary sources of anti-BMP signals and of Shh. Several BMPs have been shown to promote apoptosis in developing embryos, specifically in the dorsal neuroepithelium and in the interdigital mesenchyme (Furuta *et al.*, 1997; Hanel and Hensey, 2006). In the limb, BMPs appear to affect PCD indirectly by regulating the secretion of FGFs from the apical ectodermal ridge, which directly act as a cell survival factor (Pajni-Underwood *et al.*, 2007). Overall, there is little genetic evidence that BMPs directly trigger apoptosis (Hegarty *et al.*, 2013). On the contrary, Fgfs ligands, and most specifically Fgf8, have been demonstrated to act on the survival of neural crest cells and cells of another brain signaling center, the ANR. The conditional or hypomorphic Fgf8 mutant mice exhibit defective craniofacial development with a significant increased in the rate of apoptosis in the ANR and in migrating neural crest cells (Abu-Issa *et al.*, 2002; Crossley and Martin, 1995). Similarly, mice defective in two transcription factors Foxi3 and Sp8 develop dramatic craniofacial malformation that have been associated to reduction in Fgf8 and Fgf17 expression in the neural crest cells and, in the case of Sp8-deficient mice, in the ANR (Edlund *et al.*, 2014; Kasberg *et al.*, 2013). Interestingly, the Wnt canonical pathway participates in the expression of Fgf8, Fgf3, and Fgf17 in the ANR. The conditional mutant mice for  $\beta$ -catenin exhibit a dramatic upregulation of apoptosis in the rostral head tissues. This increase in EPCD is associated to a striking diminution of FGF ligands' expression in the ANR and the adjacent frontonasal ectoderm. Reversibly, conditional gain-of-function of  $\beta$ -catenin signaling causes an upregulation of Fgf8 expression in the ANR, and in the entire facial ectoderm. These data argue that in the ANR, Fgf8 is a target gene regulated by  $\beta$ -catenin signaling and its loss

most probably account for the observed apoptosis in the head tissues (Wang *et al.*, 2011). Both secreted Wnt and Fgf ligands contribute to patterning of the posterior neural plate and could play a part in controlling axial organizer cell survival. However, among the multiple Fgf and Wnt ligands, none has been detected in the axial organizer and it is therefore unlikely that Barhl2 directly act on either the BMP, or the Wnt, or the Fgf pathways in controlling the survival of axial organizer cells. During neurulation, Shh is induced in the notochord, the floor plate, and anteriorly in the ventral neuroepithelium (Fig. 2a) (Placzek and Briscoe, 2005). The activation of the Shh pathway leads to an intracellular response that depends on the zinc finger of the Gli family of transcription factors. A balance between the production of activating forms (Gli<sup>A</sup>) and the repressor forms of Gli (Gli<sup>R</sup>) allows modulation of this response. In mammals, there are three Gli proteins (Gli, 1–3). The receiving system of the Shh signal is composed of two proteins: Patched (Ptc), a 12-transmembrane receptor, and smoothened (Smo), a protein with seven transmembrane domains. Ptc is a constitutive Shh pathway inhibitor. In the absence of its ligand, Ptc blocks the activity of Smo. When Smo is inhibited, Gli2 and Gli3 are phosphorylated, targeted to the proteasome, and then cleaved. The cleaved forms correspond to repressive forms (Gli<sup>R</sup>) and Gli3<sup>R</sup> is the primary transcriptional repressor of the Shh signaling pathway. The binding of Shh on Ptc lifts its inhibition of Smo, which abolishes the cleavage of Gli proteins. This activates the Shh target genes in full-length forms (Gli<sup>A</sup>). In mammals, Gli2<sup>A</sup> is the main transcriptional activator of Shh signaling (Fig. 4a) (Briscoe, 2009).

Shh regulates apoptosis in various ways in the neural plate. It acts as a cell survival signal (Charrier *et al.*, 2001; Chiang *et al.*, 1996; Litingtung and Chiang, 2000) but also has an apoptotic promoting activity as demonstrated in chick ventral neuronal precursors and floor plate cells (Oppenheim *et al.*, 1999). The analysis of Shh-defective mice reveals a massive neural cell death mostly in the ventral part of the neural tube (Chiang *et al.*, 1996). In chick embryos, experimental removal of the notochord and the floor plate—the sources of Shh signals—induced a massive apoptosis of the ventral neuroepithelial cells (Charrier *et al.*, 2001; Thibert *et al.*, 2003). Indeed, Shh regulates the apoptosis machinery and *bcl-2* is a target of the Shh pathway (Fig. 4a) (Bigelow *et al.*, 2004). The extra toes (Xt) mouse mutant carries a null mutation of the Gli3 gene. Very little apoptosis is detected in Shh-Gli3 double mouse mutants (Litingtung and Chiang, 2000), and a role for Gli3<sup>R</sup> as an effector of a Shh-regulated cell survival pathway has been suggested. Ptc, the Shh receptor, is a dependence receptor. Ptc induces apoptotic cell death of neuroepithelial cells unless Shh is present to block the signal (Thibert *et al.*, 2003). Importantly, Ptc



triggers caspase-9 activation and enhances cell death through a caspase-9 dependent mechanism (Figs. 1 and 4a) (Mille *et al.*, 2009).

In summary, Shh survival activity is restricted within a developmental time window and could participate in the survival of axial organizer cells, indirectly contributing to the size and shape of the neuroepithelium. The determination of whether Barhl2 modulates the cellular production of Shh and/or the response of neuroepithelial cells to this morphogene is unknown.

**Barhl2: a transcriptional regulator of the cell-death switch.** The studies in nematode suggest that the temporal and spatial regulation of PCD during development is primarily regulated by transcriptional control of the expression of cell-death execution machinery. In the nematode, the BH3-only protein EGL-1, a proapoptotic member of the Bcl-2 family, is the key effector of developmental cell death and is transcriptionally controlled (Nehme and Conradt, 2008). Interestingly, three *C. elegans* genetic screens performed independently highlighted the regulation of the apoptotic pathway by Barh proteins (Nehme *et al.*, 2010; Peden *et al.*, 2007; Schwartz and Horvitz, 2007). Indeed, the orthologue of Barh, CEH-30, blocks apoptosis to allow the survival of four specific chemosensory neurons in males (CEM cephalic companion neurons). These neurons undergo apoptosis in the hermaphrodite by inducing the expression of Egl-1, which activates the caspase-3 orthologue, Ced-3. In males, CEH-30 acts as a transcriptional repressor of *ced-3* and *egl-1* genes (Fig. 2b) (Nehme *et al.*, 2010). The expression of CEH-30 depends on both cell-specific and sex-determination signals. Specifically, the transcription factor Unc-86, a determinant of CEM fate, induces the expression of *ceb-30* in male bodies. In contrast, in hermaphrodites, Tra1-A, (the orthologue of Gli proteins) a determinant of sex, inhibits its expression. In *C. elegans*, the vertebrate *barhl1* and *barhl2* transgenes rescue the CEM survival defect of male CEH-30 mutants, showing that these genes encode proteins that retain the functions and target specificity of CEH-30 (Fig. 2b) (Schwartz and Horvitz, 2007).

As one may expect, the regulation of PCD in vertebrates appears considerably more complex. However, the Hox-dependent regulation of apoptosis seems to be a conserved mechanism during animal development. *Hoxa13* may be involved in eliminating cells located between the forming digits in the mouse limb bud (Stadler *et al.*, 2001). The targeted deletion of *barhl1* caused degeneration of mouse cochlear hair cells. The analysis of this phenotype showed that *barhl1* plays an essential role in the maintenance of cochlear hair cells, whereas it had little effect in the specification and differentiation of these cells (Li *et al.*, 2002). Furthermore, *barhl1* plays a role in the survival of cerebellar granule precursor cells and precerebellar neurons, as well as in

the radial migration of these cells (Li *et al.*, 2004). In vertebrates, Egl-1 is the orthologue of proteins that contain a single Bcl-2 homology (BH) domain, BH3, for example, Bid, Bim, Noxa, Puma, and Bmf, commonly known as the BH3-only proteins (Happo *et al.*, 2012). The proapoptotic activity of Barhl2 requires the presence of intact FIL domains, reflecting the fact that Barhl2 could act as a transcriptional repressor at these stages of development, (Muhr *et al.*, 2001; Offner *et al.*, 2005). It is therefore possible that, as in the nematode, Barhl2 directly or indirectly affects the decision of axial organizer cells to die by transcriptional upregulation of proapoptotic genes or, more probably by downregulation of antiapoptotic genes. However, the identity of such transcriptional targets has not been addressed.

In conclusion, although some transcription factors in vertebrates have been shown to participate in the control of PCD, their relevant targets remain largely unknown. One key question that has not been resolved so far is whether temporal and spatial control of apoptosis during vertebrate development is also orchestrated at the transcriptional level. In *C. elegans*, the transcription factors that turn the survival-death switch on and off are also involved in other pathways. Specifically, CEH-30, the Barhl orthologue, regulates caspase-3 activity (Nehme *et al.*, 2010; Schwartz and Horvitz, 2007).

### Developmental Features of the Caudal Forebrain: At Least Three Pathways to Control its Growth

A combinatorial process of growth, patterning, and morphogenetic movements in the neural plate allows the emergence of this three-dimensional complex structure: the mature CNS. The forebrain (telencephalon and diencephalon) is derived from the most anterior part of the neuroepithelium, the prosencephalic neural plate. The cerebral hemisphere, the largest part of the brain, emerges from the telencephalon and contains nuclei controlling perceptual, cognitive, and higher motor functions as well as emotion and memory. The diencephalon lies between the midbrain and the cortex. It principally generates the thalamus, which processes and distributes almost all sensory and motor information going to the cerebral cortex. Its key role is to integrate all the sensory information from the periphery (with the exception of smell) and to relay it to the cerebral cortex.

In the developing neural tube, the population of neuroepithelial cells is rapidly expanding. The cell cycle depends on growth of the cell and replication of its genetic material and includes four finely controlled phases: G1 (gap1), S (DNA replication), G2 (gap 2), and M (mitosis). The location of the nuclei of dividing cells in the pseudo-stratified neuroepithelial ventricular zone depends on their status in the cell cycle. This process is



called nuclear interkinetic migration. A short overview of the cell cycle and of the nuclear interkinetic migration is shown in Figure 3. Apart from the first divisions of the zygote, which are cell-autonomous, the division of any animal cell is dependent on extracellular growth factors produced by neighboring cells. Thus, cell-cycle progression is achieved only in an environment containing mitogenic factors (Sherr, 1994). For neural stem cells (NSC) and their derivative progenitors in the developing neuroepithelium, the secreted factors Wnt and Shh trigger progression through the cell cycle (Fig. 4).

During embryonic development, the neural tube is exposed to a multitude of secreted Wnt canonical ligands. The Wnt canonical pathway is crucial for both patterning and proliferation of neural progenitors and we focus on its mitogenic activity (Wilson and Houart, 2004). The role of Wnt in neural proliferation is supported by the analysis of mice depleted for Wnt ligands or for other effectors of the pathway. Although the *Wnt1*<sup>-/-</sup> or *Wnt3A*<sup>-/-</sup> mice lose both the midbrain and the hippocampal areas, double *Wnt3A/Wnt1* mutant embryos exhibit an additional reduction in the diencephalon, caudal hindbrain, and rostral spinal cord (Lee *et al.*, 2000; McMahon and Bradley, 1990; Thomas and Capecchi, 1990). These results suggest partially redundant functions for *Wnt1* and *Wnt3A* in brain development. Conversely, ectopic expression of *Wnt1* or *Wnt3A* induces enlargement of the neural tube along the DV axis, without altering the cellular identities of neurons (Megason and McMahon, 2002; Panhuysen *et al.*, 2004).

Many Wnt ligands such as *Wnt3*, *Wnt3A*, *Wnt8b*, *Wnt4*, and *Wnt2b* are expressed in the diencephalic alar and roof plates (Fig. 5A). Indeed, the Wnt signaling machinery as well as Wnt target gene expression are enriched in the thalamus, but are absent from the prethalamus of all vertebrates analyzed so far from zebrafish to rhesus monkey (Bluske *et al.*, 2009; Jones and Rubenstein, 2004; Quinlan *et al.*, 2009; Shimogori *et al.*, 2004). However, the early changes in diencephalic patterning upon manipulation of Wnt activity appear to be primarily owing to the altered fate specification rather than to changes in proliferation, indicating that other pathways contribute to diencephalic growth control (Houart *et al.*, 2002; Ishibashi and McMahon, 2002).

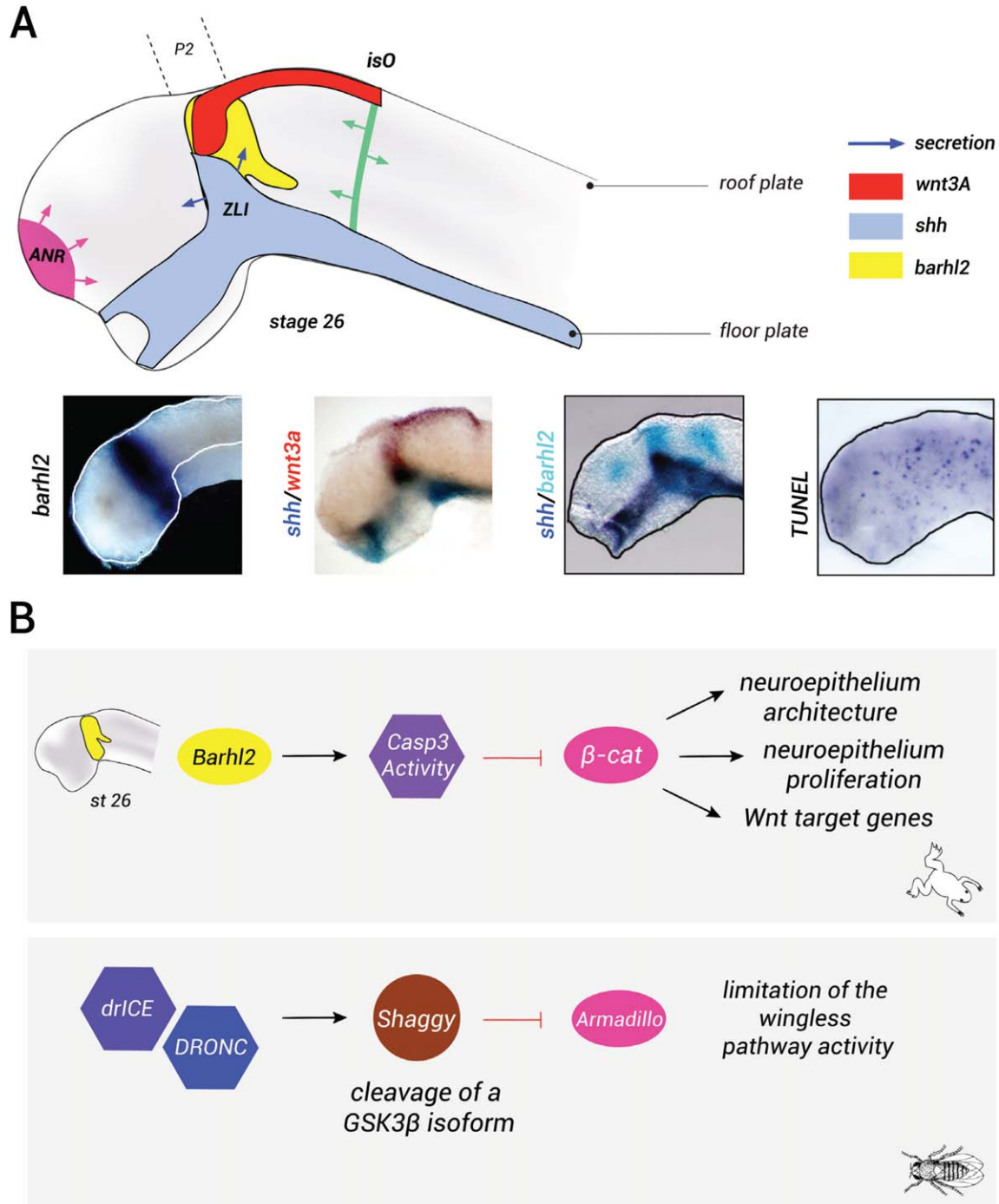
The development of the caudal part of the forebrain is particularly dependent on Shh signaling. This territory receives the combination of Shh signals from the basal plate and from the ZLI. Both signaling structures contribute to the acquisition of a diencephalic regional identity. The analysis of mutant mice for Shh shows a marked reduction in the size of the forebrain and a marked decrease in the size of the diencephalic territory compared to other areas of the brain. This effect partly reflects the role of Shh, which allows the survival

and proliferation of diencephalic progenitor populations (Chiang *et al.*, 1996; Ishibashi and McMahon, 2002), and controls development of the forebrain ventral neuroepithelium (Fuccillo *et al.*, 2006; Gunhaga *et al.*, 2003).

In amphibian, transcripts encoding the *barhl2* gene are detected in the diencephalic primordium (Fig. 5a) (Juraver-Geslin *et al.*, 2014). Fate mapping of the diencephalic histogenic field at the gastrula stage identifies a wide area similar to the expression pattern of *barhl2* in both zebrafish (Staudt and Houart, 2007) and mice (Mo *et al.*, 2004). It has been demonstrated that within the *xenopus* diencephalic territory, *barhl2* acts as a proliferation brake via an unconventional activity of caspase-3 that limits the proliferation of neuroepithelial cells. Indeed, comparative analysis of *Barhl2*-depleted and caspase-3-depleted *Xenopus* embryos reveals a similar dramatic hyperplasia and cellular disorganization of the caudal forebrain neuroepithelium. The measurement of the relative velocity of the cell cycle in *Barhl2* or caspase-3-depleted embryos reveals a similar shortening of cell-cycle length (6 vs. 8 h). The length of the S-phase in these cells remains unchanged (1.5 h). Similarly to what we previously described for caspase-3-depleted embryos, the increase in the speed of the cell cycle observed in *Barhl2*-depleted neural tube has been correlated to  $\beta$ -catenin stabilization and to an increase in the activity of the canonical Wnt pathway. Specifically, both *Barhl2* and caspase-3 depletion generate an increase in cyclin D1 expression. Cyclin D1 controls the length of the G1 phase and it is part of a major cell-cycle checkpoint controlling whether a cell continues to divide or becomes quiescent. It is therefore probable that *Barhl2* and caspase-3 normally act as proliferation brakes by lengthening the G1 phase of the cell cycle. The overexpression of caspase-3 rescues the hyperplasia phenotype in *Barhl2*-depleted embryos. Therefore, in the molecular pathway limiting proliferation and Wnt signaling, caspase-3 acts downstream of *Barhl2* (Fig. 5b) (Juraver-Geslin *et al.*, 2011). It is unknown whether mouse embryos lacking *Barhl2* function exhibit a hyperplasia phenotype of the diencephalic walls.

Taken together, intertwined contributions of the Wnt, the Shh, and the *Barhl2* pathways contribute to diencephalic proliferation. We discuss here how these three pathways interact and control diencephalic primordium growth.

**The Wnt canonical pathway in early brain growth.** Through the control of cell proliferation and cell polarity, the determination of cell identity and axes specification, Wnt functionality covers many aspects of the development (Angers and Moon, 2009; Clevers and Nusse, 2012). Wnt ligands, of which there are 19 in mammals, are secreted glycoproteins modified by the addition of a lipid. The key effector molecule of the canonical Wnt pathway is  $\beta$ -catenin. The mechanisms controlling



**FIG. 5.** Barhl2 acts as a brake on diencephalic progenitor proliferation. **(a)** *barhl2*, *wnt*, and *shh* ligands are coexpressed in the diencephalic primordium. The scheme of a stage 27 neural tube: *barhl2* (yellow) is expressed in the prosomere p2 (p2) within the diencephalon, in a territory giving rise to the ZLI, a secondary organizer that secretes Shh (blue). Shh is also expressed in the anterior neural tube basal plate. Wnt3a (in red) as Wnt3 (data not shown) is expressed in the dorsal (alar plate) part of the prosomere p2, in the midbrain roof plate. The two other major secondary organizers, the ANR (pink) and the isthmus organizer (isO, green), that play roles in patterning the forebrain and the midbrain/hind-brain, respectively, are also shown. The secretion is represented with arrows. Whole mount ISH using *barhl2*, *shh*, or *wnt3a* probes as indicated. Representative dissected neural tube of wild-type embryos is shown, dorsal view anterior left. Whole mount TUNEL analysis: cells undergoing endogenous apoptosis appear in blue. **(b)** Nonapoptotic functions of caspases regulate the levels and activity of  $\beta$ -catenin. In *Xenopus* diencephalic primordium, Barhl2 acts upstream of a nonapoptotic function of caspase-3 that limits both levels and activity of  $\beta$ -catenin and consequently limits the proliferation of neural progenitors, controls the neuroepithelium architecture, and regulates the transcription of Wnt target genes. In *drosophila* SOP cells, the caspases orthologues DRONC and drICE limit the activity of the Wingless pathway by cleaving an isoform of GSK3- $\beta$ , Shaggy, which destabilizes Armadillo ( $\beta$ -catenin orthologue) and consequently limits the Wnt pathway.

the homeostatic balance of Wnt pathway activation are based on the fine regulation of both the expression levels and the activation rate of  $\beta$ -catenin (Fig. 4b).

The Wnt molecular pathway has already been extensively reviewed (Arce *et al.*, 2006; Grigoryan *et al.*, 2008; Hur and Zhou, 2010; Kimelman and Xu, 2006). The key step in the activation or inhibition of the Wnt pathway is the modulation of the activity of GSK3- $\beta$  which targets  $\beta$ -catenin to its degradation by the proteasome. In the absence of Wnt ligands, a protein degradation complex is formed that binds the free  $\beta$ -catenin and leads to its degradation. Cytoplasmic  $\beta$ -catenin levels are low, and thus preventing the activation of the pathway (Fig. 4C). The presence of Wnt ligands stabilizes  $\beta$ -catenin, which is then translocated into the nucleus. In the nucleus,  $\beta$ -catenin displaces Groucho corepressor proteins and in association with LEF and TCF acts as a transcriptional coactivator, allowing the activation of Wnt target genes. Recently, it was demonstrated that Wnt signaling initiates a complex cellular process, leading to GSK3- $\beta$  sequestration in multivesicular bodies into which GSK3- $\beta$  is sequestered and its activity inhibited (Taelman *et al.*, 2010). Finally, as previously mentioned,  $\beta$ -catenin forms a stable complex with the molecules of the cadherin superfamily and it is part of the adherens junctions that regulate cell-cell adhesion (Fig. 4B) (Redies, 2000).

By stimulating  $\beta$ -catenin accumulation, Wnt activation induces the expression of the proto-oncogene *c-myc* (He *et al.*, 1998). *C-myc* encodes a bHLH leucine zipper (bHLHZip) transcription factor that has two distinct roles in G1 progression. On the one hand, it increases the expression of cyclins D1 and D2; on the other hand, it represses p27Kip1 and p21Cip1, and hence promoting cell-cycle progression and enhancing cell proliferation (Fig. 4B) (He *et al.*, 1998). In parallel, Wnt stimulates cell growth through a global increase in protein levels during the G1 phase of the cell cycle. Both steps depend on the key event of the inhibition of GSK3- $\beta$ . The inhibition of GSK3- $\beta$  induces a general increase in protein stability (Taelman *et al.*, 2010) and stimulates growth through the activation of the TOR pathway, which stimulates protein synthesis. Therefore, the Wnt pathway promotes G1 progression and cell growth, whereas the accumulation of cyclin D1 and *c-myc* triggers proliferation (Fig. 4B). This activity of Wnt is crucial for the emergence of groups of cells that will help in creating tissue and eventually a functional structure (Davidson and Niehrs, 2010). Besides its role in G1 progression, Wnt signaling is believed to play a role in progression through mitosis.  $\beta$ -Catenin levels are the highest during the G2/M phase. During the M phase,  $\beta$ -catenin allows centrosome separation at the beginning of mitotic spindle formation, regardless of its role in regulating gene transcription (Bahmanyar *et al.*, 2008; Huang *et al.*, 2007). In addition, the Wnt pathway

allows the establishment of mitotic spindle orientation and therefore affects the distribution of cytoplasmic determinants between the two daughter cells, a key process that determines both the specific identity and the spatial positioning of these cells in a tissue (Siller and Doe, 2009; Walston and Hardin, 2006). Importantly, mechanistic regulation and molecular components of Wnt signaling appear to be conserved during evolution (Davidson *et al.*, 2009).

Indeed, the fundamental activity of Wnt in regulating growth of the neuroepithelium is underscored by the analysis of GOF or LOF mutations introduced at the  $\beta$ -catenin locus in mice. The conditional LOF mutants in the spinal cord show a clear reduction in the number of proliferating neurons, associated with an increase in the number of differentiated cells. The reciprocal GOF induces the opposite effect (Zechner *et al.*, 2003). The conditional LOF mutants for  $\beta$ -catenin in the cortex and in the hippocampus also exhibit decreased neuroepithelial proliferation and disruption associated with the defects in interkinetic nuclear migration and the loss of adherens junctions. Specifically, the nuclei of proliferating cells are abnormally located outside the ventricular zone (Machon *et al.*, 2003). Furthermore, in mice expressing a stabilized form of  $\beta$ -catenin in the cortex, neural progenitors are kept in a mitotic state and do not differentiate. The brains of these mice are enlarged owing to an expansion of the progenitor pool and a horizontal expansion of the ventricular zone with the appearance of convolutions on the surface of the cortex, similar to those observed in higher mammals. The process of apoptosis is also increased in these mice. However, the increase in the number of neuroepithelial progenitors cannot be attributed solely to the change in the cell death rate (Chenn and Walsh, 2002).

In conclusion, through its ability to modulate the activity of GSK3- $\beta$  that promotes a general increase in protein stability, and specifically in  $\beta$ -catenin stability, Wnt signaling promotes concomitantly cell-cycle progression and cell growth.

**Shh is a mitogen for neuroepithelial cells.** - Through its survival and mitogenic activities, Shh contributes to the coherent growth of the neural plate and tube (Jacob *et al.*, 2003; Jessell, 2000; Mehlen *et al.*, 2005; Ribes and Briscoe, 2009). Indeed, Shh regulates both the cell cycle and the apoptosis machinery (Fig. 4a). The two main promoters of G1 progression, cyclin D1 and E, and N-Myc are the targets of the Shh pathway (Katoh and Katoh, 2009; Kenney and Rowitch, 2000; Oliver *et al.*, 2003). In the vertebrate, neuroepithelium electroporation of modulators of Shh activity affects proliferation and apoptosis. In chick's spinal cord, the expression of a dominant negative form for Ptc induces a reduction in size of the neuroepithelium associated with decreased proliferation and induction of apoptosis. Electroporation of Gli3<sup>R</sup> mimics this effect and

causes a cell-cycle arrest associated with a reduction in the expression of cyclin D1. Conversely, overexpression of Gli3<sup>A</sup> in the neuroepithelium induces hyperplasia linked to a decrease in the length of the G1 phase associated with an increase in the expression of cyclin D1, an overexpression of cyclins A, B, and E and late progression in G2. Thus, by regulating the ratio of activating and repressive forms of Gli proteins, Shh promotes G1 and G2 progression in the neuroepithelial cells of the spinal cord (Alvarez-Medina *et al.*, 2009; Cayuso *et al.*, 2006). In the *Xenopus* retina, Shh controls the speed of the neural progenitor cell cycle and stimulates G1/S and G2/M progressions by regulating the expression of the phosphatase Cdc25B, together with late cyclins (B1 and A2) and cyclin D1. Blocking the Shh pathway promotes lengthening of the G1 and G2 phases, whereas Shh overexpression induces premature cell-cycle exit of retinal precursors (Borday *et al.*, 2012). In mouse neuroepithelium, the Shh signal stimulates the progression into mitosis. The entry into mitosis depends on the maintenance of cyclin B/Cdk1 complex in an inactive state by phosphorylation on Thr14 and Tyr15 residues. Cdc25B promotes cycle progression by the activation of the complex (Kristjansdottir and Rudolph, 2004) (Fig. 4A). The inhibition of Shh signaling by cyclopamine decreases Cdc25B expression in the neural tube, whereas overexpression of Gli2<sup>A</sup> by electroporation leads to its accumulation. Thus, Shh promotes progression in G2/M in mice by inducing the expression of Cdc25B in neuroepithelial progenitors. In agreement with these observations, mutant mice for Shh have a delayed and reduced ventral Cdc25B expression, which argues for a conserved role of Shh in regulating the later stages of the cell cycle, G2, and M in mammals (Benazeraf *et al.*, 2006).

In summary, Shh helps control the size of the neural plate and tube by positively regulating progression through the cell cycle, especially during the later stages of the cell cycle.

**Shh and Wnt signals cooperate in patterning and growth along the entire neuraxis.** At the neural plate stage, Wnt and Shh exhibit opposite patterning activities: Shh contributes to the acquisition of a ventral identity, whereas Wnt promotes dorsal neural progenitor fate (Briscoe and Ericson, 2001; Robertson *et al.*, 2004; Zechner *et al.*, 2003). Such antagonism is also observed in the establishment of the spinal cord occurring after neural tube closure, where Shh is expressed in the floor plate and Wnt is expressed in the roof plate. The inhibition of the Shh pathway by Wnt signaling is crucial in the regionalization of the spinal cord. In chicken, the Wnt pathway limits the influence of Shh by inducing the expression of Gli3<sup>R</sup> in the dorsal part of the neural tube. In mice, the expression of a stabilized form of  $\beta$ -catenin dorsalizes ventral neurons and extends the expression of Gli3<sup>R</sup> ventrally. This pheno-

type is partially rescued through Gli3 LOF (Alvarez-Medina *et al.*, 2008; Yu *et al.*, 2008). In the *Xenopus*, postembryonic ciliary marginal zone of the retina, the Shh, and Wnt pathways inhibit each other's expression, and hence allowing them to segregate in mutually exclusive territories. This inhibition partially depends on the transcriptional regulation of SFRP1, on the one hand, and Gli3 on the other hand, which both contribute to this negative crossregulation. Such antagonistic interplay of Wnt and HH signals appears to regulate the extent of neural stem/progenitor cell proliferation in the *Xenopus* retina (Borday *et al.*, 2012). Finally, in the chicken caudal forebrain, these two pathways also antagonize one another. Electroporation of Shh in the developing diencephalon induces a decrease in the expression of Wnt4 and Wnt5A. In vivo Wnt4 expression is inhibited by the Shh signal from the ZLI and Wnt4 expression is progressively restricted to the caudal part of the diencephalic alar plate, a process that contributes to thalamus regionalization (Quinlan *et al.*, 2009). In addition, Wnt8b regulates Gli3 expression within the diencephalon, and hence contributing to *shh* induction and dorsal progression of the ZLI within the caudal forebrain (Martinez-Ferre *et al.*, 2013).

The cooperative action of the two signaling pathways also affects cell-cycle progression (Fig. 4c). Shh mutant mice display a reduction of Wnt activity in the dorsal neural tube that is detected by proliferation defects along the entire DV axis of the spinal cord and a reduction in the expression of *axin2*. This phenotype is compensated in mice lacking both Shh and Gli3 (Litingtung and Chiang, 2000). In the chick spinal cord, the proliferative activity of Wnt is dependent on Shh signaling. The Shh pathway is epistatic to the Wnt-dependent progression in the G1 phase of the cell cycle: Shh permits transcriptional activation of Tcf4 and Tcf3, which then induces the expression of cyclin D1. Electroporation of a mutated form of Ptc inhibits the proliferative effect of Wnt ligands or of a stabilized form of  $\beta$ -catenin. This phenotype is compensated by the coelectroporation of Tcf3 or Tcf4 (Alvarez-Medina *et al.*, 2009). The study of mutant mice for Shh suggests a conservation of these interactions in the diencephalon. Such mice develop a reduced diencephalon with decreased Tcf4 expression, a phenotype consistent with an epistatic role for Shh in Wnt activity in controlling the growth of the diencephalic primordium (Ishibashi and McMahon, 2002).

In conclusion, the integration of Shh and Wnt signals appears strictly necessary for correct DV growth and patterning along the entire neuraxis and specifically in the diencephalon.

**Crosstalk between Barhl2, Wnt, and Shh within the diencephalic primordium.** Little is known about the extracellular signals influencing *barhl2* expression and activity, and how the Barhl2, Wnt, and Shh pathways coordinately control diencephalic



growth. In the caudal forebrain territory, *Barhl2* expression overlaps with that of *Shh*, *Wnt3A*, and *Wnt8b* (Fig. 5A). In chick embryo, a *Wnt8b*-induced *Wnt* signal is a prerequisite for the induction of *Shh* expression and for the emergence of ZLI in the diencephalic primordium (Martinez-Ferre *et al.*, 2013; Zeltser *et al.*, 2001). In zebrafish, *Wnt3* and *Wnt3A* are coexpressed in the ZLI anlage and their early depletion affects its induction (Mattes *et al.*, 2012). Further work is necessary to identify the inductive signals underlying *barhl2* expression in the neural plate and caudal forebrain territory. However, *Wnt* and eventually *Shh* signaling might participate in initiating the expression and/or protein stabilization of *Barhl2* at early development stages. In turn, *Barhl2* would act as a negative regulator of these pathways, initiating a possible *Barhl2*-controlled activity regulatory loop that would limit the growth of the caudal forebrain. At note beside its function in diencephalic growth, *barhl2* plays a key function in the establishment of the ZLI compartment that secretes *Shh* (Juraver-Geslin *et al.*, 2014). The analysis of caudal forebrain proliferation kinetics in chick and mice shows that ZLI cells divide slowly relative to cells in their flanking territories (Baek *et al.*, 2006; Martinez and Puelles, 2000). In addition, the cells in neighboring compartments separate along the boundaries, probably owing to the differences in their adhesive properties (Dahmann *et al.*, 2011).  $\beta$ -catenin acts on thalamic cell adhesiveness and segregation by mediating the interactions between the intracellular cytoskeleton and the cadherins, (Peukert *et al.*, 2011; Puelles, 2007; Redies, 2000). Therefore, the ability of *Barhl2* to limit the rate of cell proliferation and modulate cell adhesion also plays a role in the correct patterning of the caudal forebrain.

The so-called nonapoptotic functions of caspases have been described repeatedly in various developmental contexts including their ability to regulate proliferation and neuronal differentiation. Although the recent studies in *C. elegans* have begun to unveil the molecular basis underlying the ability of caspase-3 to act in multiple ways (Hirose and Horvitz, 2013; Hirose *et al.*, 2010), little is known about such mechanisms in vertebrates (Kuranaga, 2012; Miura, 2011; Yi and Yuan, 2009). Specifically, how *Barhl2* regulates caspase-3 nonapoptotic activity and limits  $\beta$ -catenin levels and stability in the developing diencephalon is unknown. *Barhl2* could control caspase-3 proteolytic activity and specificity by direct interaction. However, the analysis of *Barhl2* and caspase-3 protein sequences and structural motifs do not support such a hypothesis. Alternatively, *Barhl2* could target caspases to specific subcellular compartments, allowing spatial control of their catalytic activities or regulate the transcription of genes that encode for the regulators of caspase-3 activity and/or proteolytic specificity. The current dogma holds that the context-dependent nonapoptotic functions of caspase-3

rely on its ability to exhibit substrate specificity. Although whether the proteolytic activity of caspase-3 is involved in limiting diencephalic proliferation has not been demonstrated, one simple assumption is that caspase-3 directly regulates the activity of an effector protein through proteolytic cleavage. One candidate is GSK3- $\beta$ , the major negative modulator of the canonical *Wnt* pathway. In both *Barhl2*- and caspase-3-depleted embryos, overexpression of GSK3- $\beta$  partially alleviates the hyperplasia phenotype, suggesting that *Barhl2* and caspase-3 may regulate GSK3- $\beta$  activity. The tissue-specific depletion of GSK3 $\alpha$  and GSK3- $\beta$  results in neural hyperplasia owing to increased proliferation and decreased neurogenesis (Kim *et al.*, 2009). Therefore, one hypothesis is that *Barhl2* acts through caspase-3 proteolytic activity and modulates directly or indirectly the activity of intermediaries of the *Wnt* pathway.

The analysis of the literature reveals a very similar mechanism occurring in *Drosophila* neural progenitors. Specifically, in the cells of the sensory organ (SOP), orthologues of caspase-9 (DrONC) and caspase-3 (DrICE) cleave and activate an isoform of GSK3- $\beta$ . This isoform, called Shaggy, subsequently inhibits the activity of the wingless pathway and induces a change in the fate of SOP (Fig. 5B) (Kanuka *et al.*, 2005; Kuranaga *et al.*, 2006). Despite this attractive mechanism, the search for orthologues of this isoform of GSK3- $\beta$  in vertebrates has proven unsuccessful. In addition, the apparent absence of cleavage sites by caspases in GSK3- $\beta$  in vertebrates argues against the conservation of this mechanism and suggests the existence of more complex regulations. In fly, inhibitor-of-apoptosis-proteins (IAP) participate in controlling nonapoptotic functions of caspases. The members of the IAP family, including DIAP in *Drosophila* and its vertebrate orthologue, XIAP (X-linked IAP), interact with caspases through their BIR domain, block their catalytic activities, and activate their degradation (Nakhaei *et al.*, 2012). The levels of DIAP in fly are a critical parameter in determining the level of caspase activity necessary for the execution of nonapoptotic functions (Miura, 2011). The determination of whether a similar mechanism exists in vertebrates has not yet been investigated. However, XIAP is required for *Wnt* signaling in cultured mammalian cells and in *Xenopus* embryos. XIAP is recruited to TCF/Lef where it monoubiquitylates Groucho (Gro)/TLE, decreasing the affinity of Gro/TLE for TCF/Lef and further allowing  $\beta$ -catenin-TCF/Lef assembly and initiation of a *Wnt*-specific transcriptional program (Hanson *et al.*, 2012).

## CONCLUSIONS

In conclusion, genetic and embryological observations in the last 20 years have documented the importance of developmental apoptosis and of unconventional activities of apoptosis effectors, in early neural tube

morphogenesis. Although the effector mechanisms of the cell death program appear conserved, the genetic controls for apoptosis induction are different in neural progenitors and postmitotic neurons. In nematode, the temporal and spatial regulation of developmental PCD is primarily regulated by transcriptional control of the expression of the cell-death execution machinery. Whether this is true in vertebrate is an open question. The observations in *C. elegans*, *Xenopus* embryos, and mice, document that BarH transcription factors act on the apoptotic cascade and on an unconventional function of caspase-3 that limits neuroepithelial cell proliferation. Both activities are strictly dependent on the cellular context. We know little on how these transcription factor activities are implemented and regulated. In vertebrates, Barhl2 could directly or indirectly affect the decision of neural cells to die by transcriptional upregulation of proapoptotic genes or downregulation of antiapoptotic genes. Barhl2 could also control the levels and/or stability of regulators of caspase activity, among them the IAPs. In any case, their involvement in both survival and regulation of an unconventional activity of caspase-3 raises the interesting possibility that the molecular cues controlling the death-survival switch are similar and/or related to those controlling the unconventional activities of apoptosis effectors.

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