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The Multiple Tasks Endured by PI3K during neural tube development

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The multiple tasks endured by PI3K during neural tube development

Las múltiples funciones de la PI3K durante el desarrollo del tubo neural

Memoria presentada por

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“To dare is to lose one's footing for a while. Not to dare is to lose oneself.”

Søren Kierkegaard

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ABSTRACT

Development of the spinal cord involves coordination between exposure to localized extracellular signals and controlled activation of intracellular signaling pathways. This way, neuroepithelial cells firstly proliferate apically to increase the progenitor pool and, later on, initiate neurogenic divisions giving rise to a variety of neuronal cell types. Class IA PI3Ks are heterodimeric enzymes (catalytic+regulatory subunits) activated by receptors tyrosine kinase (RTKs) or G-protein coupled receptors (GPCRs) that, upon extracellular stimuli, modulate diverse target proteins through local production of PtdIns(3,4,5)P3 lipids. Vertebrates express three Class IA catalytic subunits (p110α, p110β, and p110δ), all important for the development of the central nervous system. However, it is unclear to what extent these p110 isoforms have overlapping or distinct biological roles, and what exact functions they hold in neural development.

Analysis of PI3Kα (p110α+regulatory subunit) expression in the embryonic spinal cord revealed abundant mRNA and protein levels in cycling progenitors followed by restriction of PI3Kα exclusively to differentiating neurons. To examine the role of PI3K in progenitors and neurons, we interfered with normal PI3Kα regulation by expressing active mutants or knocking down of p110α in the chicken neural tube. Loss of p110α resulted in high apoptotic rates in both progenitors and neurons, sustaining a role for PI3Kα in neural survival as seen in other studies. Instead, uncontrolled upregulation of PI3Kα activity resulted in severely disrupted neural tubes, with abnormal cell masses in the luminal face of the neuroepithelium and ectopic mitosis. Additionally, we observed alterations in the neural lamination characterized by basement membrane breaches followed by enhanced neural migration and misoriented axonal growth. A thorough analysis of the tissue unveiled loss of polarity as the main cellular mechanism driving the luminal structural aberrations, suggesting a major role of PI3Kα in neuroepithelial apico-basal polarity. Moreover, the rescue of the depolarization phenotype with a dominant-negative form of RhoA proposes local regulation of the Rho family of small GTPases as the molecular mechanism responsible for the PIP3 dependent regulation of adherens junction dynamics. Alternatively, we found the neural overmigration caused by excess of PI3Kα activity explained by increased basal accumulation of PIP3, leading to actin-based membrane protrusions and basement membrane breaches. Coherently, when we assessed the neural positioning after p110α knock-down, we detected neurons inserted in the proliferative layer and reduction of the neuronal cytoskeletal component beta-III tubulin, suggesting that PI3Kα also modulates morphological maturation and apico-basal positioning of differentiating neurons. Interestingly, PIP3-induced overmigration seemed to be carried out through local activation of other two members of the Rho GTPases, Cdc42 and Rac1. These results shed some light upon the PI3Kα/PIP3 specific roles during early neural tube development, stressing out its

function in cell polarity. Furthermore, we propose a mechanism that may partially explain how the PI3Kα/PIP3 signaling is able to control different types of polarity corresponding to different developmental moments. This could help to understand the initial events leading to some neurodevelopmental disorders caused by hyperactivation of PI3K signaling.

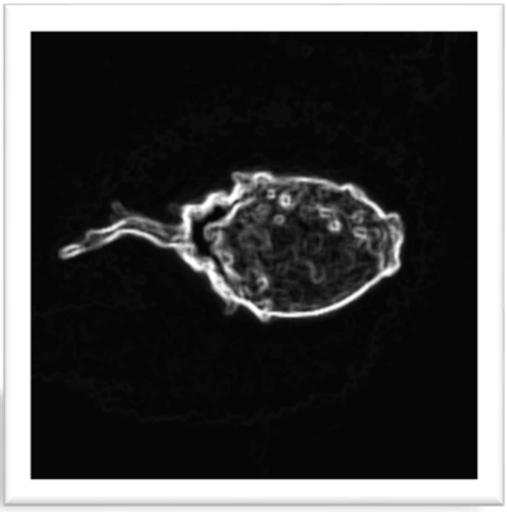
RESUMEN

El desarrollo de la médula espinal requiere una fina coordinación entre señales extracelulares y la activación de vías intracelulares específicas. De este modo se da una primera fase de proliferación de las células neuroepiteliales en la zona apical para aumentar el número de progenitores y una segunda fase de neurogénesis, a partir de la cual se originan diferentes tipos de neuronas. La clase IA de las PI3Ks se encuentra implicada en la transducción de señales a través de receptores tirosina quinasa (RTKs) o receptores acoplados a proteínas G (GPCRs). En respuesta a estímulos extracelulares, controlan la actividad de distintas proteínas diana a través de la producción local de lípidos PtdIns(3,4,5)P3. La clase IA de las PI3Ks, formada por enzimas heterodiméricas, consta de tres tipos de subunidades catalíticas (p110α, p110β, and p110δ). Todas ellas son importantes para el desarrollo del sistema nervioso, sin embargo no están claras las funciones específicas de cada isoforma.

El análisis de la expresión de PI3Kα a nivel de RNAm y proteína en la médula espinal embrionaria reveló una expresión diferencial según el estadío, siendo alta en progenitores antes de la neurogénesis y restringida a neuronas en estadíos más tardíos. Para estudiar su función en progenitores y neuronas, transfectamos formas activas de PI3Kα o suprimimos transitoriamente la p110α en el tubo neural de embriones de pollo. La pérdida de p110α provocó una alta tasa de apoptosis en ambas poblaciones, revelando su importancia en supervivencia. La sobreexpresión de la PI3Kα activa, en cambio, generó disrupciones muy severas del tejido neural caracterizadas por la presencia de masas celulares en la pared ventricular y mitosis ectópicas. En el lado basal, se observaron alteraciones en la laminación neuronal con células atravesando la lámina basal y crecimiento axonal aberrante. Nuestros resultados apuntan hacia la pérdida de polaridad como la principal causa de las aberraciones estructurales apicales, indicando que la PI3Kα tiene una función en la regulación de la polaridad apico-basal. Asimismo, la PI3Kα parece implicada en la maduración del citoesqueleto neural y en el posicionamiento de las neuronas en el eje apico-basal, funciones parcialmente mediadas por miembros de las Rho GTPasas.

INDEX

INTRODUCTION



1. Development of the Central Nervous System

1.1 Formation of the neural tube

During gastrulation, cell movements result in a massive reorganization of the embryo from a simple spherical ball of cells, the blastula, into a multi-layered organism. The primary germ layers (endoderm, mesoderm, and ectoderm) are formed and organized in their proper locations during gastrulation. Endoderm, the most internal germ layer, will form the lining of the gut and other internal organs. Ectoderm, the most exterior germ layer, will give rise to the skin, the nervous system, and other external tissues. Mesoderm, the middle germ layer, will form muscle, the skeletal system and the circulatory system. The vertebrate Central Nervous System (CNS), which is the main topic of this thesis, derives from the neural plate, a region of columnar epithelium induced from the most exterior layer, the ectoderm, on the dorsal surface of the embryo during gastrulation. The process by which the neural plate will give rise to the neural tube, the rudiment of the central nervous system, is called neurulation and an embryo undergoing such changes is called a neurula. There are two major ways of forming a neural tube from the neural plate: primary neurulation, which involves invagination of the neural plate to form a hollow tube and occurs in most of the neural tube in vertebrates, and secondary neurulation, where the neural tube is formed by hollowing out of the interior of a solid precursor in the most caudal part.

During primary neurulation, the original ectoderm divides into three sets of cells: the internally positioned neural plate/tube, which will form the brain and spinal cord; the externally positioned epidermis of the skin; and the neural crest cells, which will migrate to generate the peripheral neurons and glia, the pigment cells of the skin, and several other cell types. Morphologically and in a summarized way, what happens is that shortly after the neural plate has formed, its edges thicken and move upward to form the neural folds, while a U-shaped neural groove appears in the center of the plate, dividing the future right and left sides of the embryo. The neural folds migrate toward the midline of the embryo, eventually fusing to form the neural tube beneath the overlying ectoderm. The cells at the dorsal most portion of the neural tube become the neural crest cells and the neural tube eventually forms a closed cylinder that separates from the surface ectoderm (Figure 1A) (Gilbert, 2000). This separation is thought to be mediated by the expression of different cell adhesion molecules. Although the cells that will become the neural tube originally express E-cadherin, they stop producing this protein as the neural tube forms, and instead synthesize N-cadherin and N-CAM (Derycke and Bracke, 2004). As a result, the surface ectoderm and neural tube tissues no longer adhere to each other.

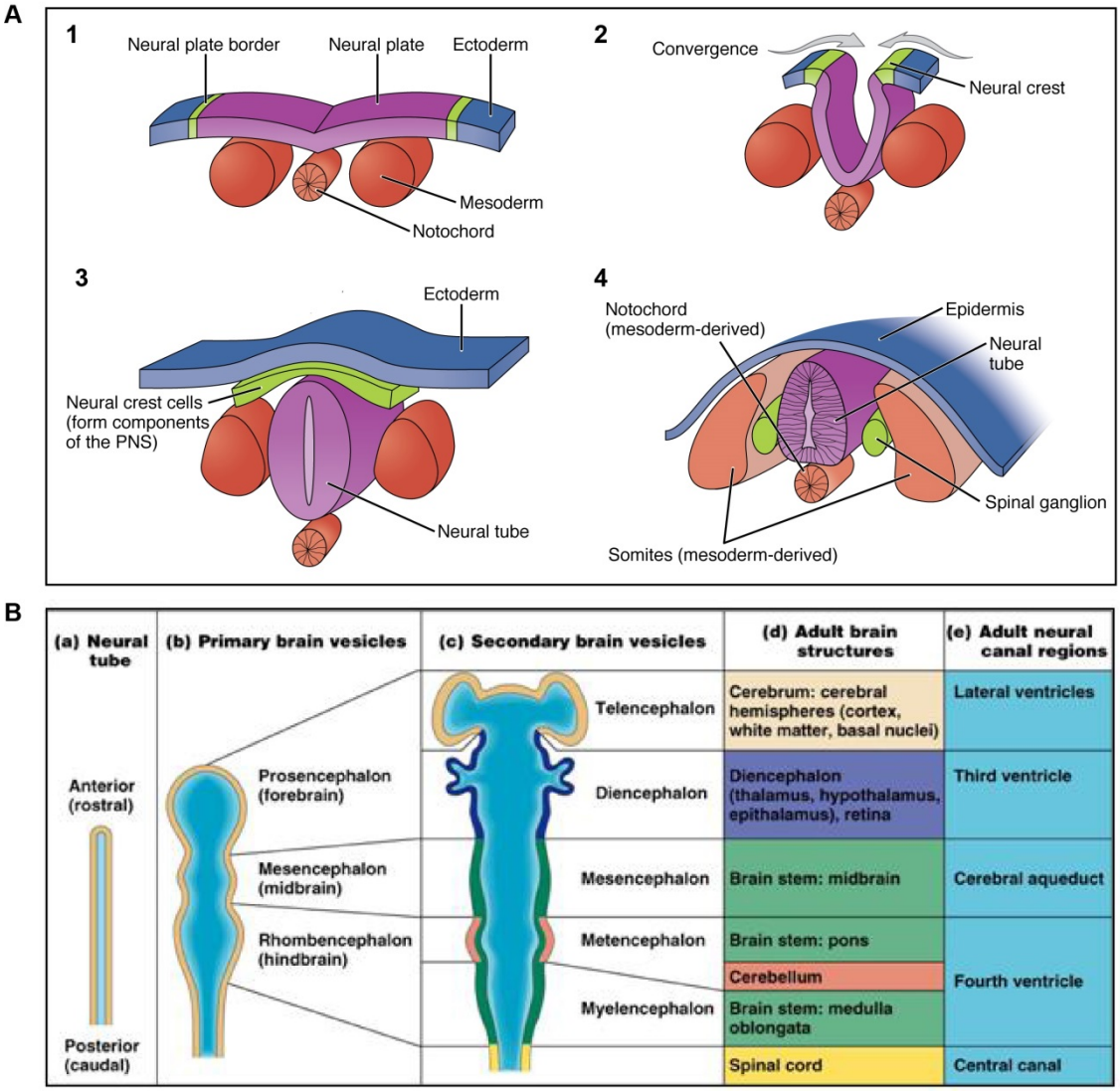


Figure 1. Anatomical development of the neural tube. (A) Transformation of the neural plate into the neural tube (neurulation) displayed in four steps. (1) Differentiation of part of the ectoderm into neuroectoderm (neural plate), separated by the neural plate border that will generate the neural crest cells. The notochord is already positioned centrally in the embryo with respect to both the dorsal-ventral (DV) and left-right (LR) axes. (2) The neural plate bends dorsally forming a groove that will finally close in the dorsal midline. (3) The neural tube, expressing different adhesion molecules to separate from the non-neural ectoderm (epidermis) folds along the dorso-ventral axis and the neural crest cells separate from the tube and migrate to become a variety of different cells. (4) The formed neural tube develops surrounded by epidermal tissue and the somites. Some neural crest cells form the dorsal root ganglia containing the cell bodies of sensory neurons. **(B)** The central nervous system, generated from the neural tube, subdivides in the most anterior part into three primary vesicles that give rise to secondary vesicles and, finally, to adult brain structures making up the brain. The spinal cord will be generated from the posterior neural tube. Images adapted from Anatomy & Physiology, Connexions Web site and 2006 Pearson Education, Inc., publishing as Benjamin Cummings.

Importantly, the closure of the neural tube does not occur simultaneously throughout the ectoderm. In vertebrates as birds and mammals the body axis is elongated prior to

neurulation and it starts earlier in the cephalic (head) region while the caudal (tail) region of the embryo is still undergoing gastrulation. In addition, changes in the shape of the tube will end up in the regionalization of the neural tube along the anterior-posterior (A-P) axis. In the cephalic end (brain and retina), the wall of the tube will be broad and thick and posterior swellings and constrictions will define the several brain compartments. And from the caudal to the head region (spinal cord), the neural tube will remain simpler (Gilbert, 2000) (Figure 1B). A basement membrane, a thin sheet of specialized extracellular material, will separate the neuroepithelium from the surrounding connective tissue along the A-P axis.

1.2 Structure of the neural tube

Previously, it was shortly explained the differentiation of the neural tube into the various regions of the central nervous system on the anatomical level. At the tissue level, the cell populations within the wall of the neural tube rearrange themselves to form the different functional regions of the retina, brain and the spinal cord (Figure 1). Finally, on the cellular level, the neuroepithelial (NEP) cells function as neural stem cells and differentiate, first, into the numerous types of nerve cells (neurons) and, later, into supportive cells (glia) present in the body (Kintner, 2002).

NEP cells, like other epithelial cells, exhibit apical-basal polarity, with their apical plasma membrane lining the lumen of the neural tube while maintaining cell– cell adhesion and the integrity of the VZ, and their basal plasma membrane contacting the extracellular matrix (ECM), which demarcates the outer boundary of the neural tube (Gotz and Huttner, 2005) (Figure 2A). The ECM is a complex tissue specific network made of collagens, proteoglycans, and glycoproteins such as fibronectins and laminins, the latter lengthily implicated in epithelial polarity and morphogenesis. In addition, a large number of ECM-modifying enzymes, ECM binding growth factors, and other ECM-associated proteins interact and cooperate with ECM proteins to assemble and remodel extracellular matrices. These matrices are actively remodeled by cells during development, normal tissue homeostasis, and in several disease processes such as cancer-associated desmoplasia or inflammation. Specialized cell surface-associated ECMs, named basement membranes (BMs), underline epithelial (and neuroepithelial) cells at their basal surfaces (Roignot et al., 2013).

The apico-basal polarity in epithelial cells is achieved by the mutual exclusion of three conserved protein complexes in coordination with the specific tissue geometry described above. These complexes are the apical partitioning defective PAR (Par3, Par3, aPKC) and Crumbs (Crumbs, PALS1, PATJ) complexes, and the basolateral Scribble (Scribble, DLG, LGL) complex (Iden and Collard, 2008). In the neuroepithelium, it is very similar. The apical surfaces of individual cells, known as apical junctional complexes (AJCs), are composed mainly of Ncadherin-based adherens junctions (AJs), but not by

tight junctions as observed in epithelial cells (Gotz & Huttner 2005). These AJCs are composed by three functionally distinct microdomains within the AJ structure. The most apical domain is formed by Par6/aPKC/Cdc42 and the Crumbs complex, followed by Afadin/ZO1 (Zonula Occludens 1) in an intermediate domain and, in the most subapical domain, we can find Ncadherin/β-catenin/α-catenin (AJ) and Par3. In the NEP basolateral membrane, members of the Scribble complex such as LGL have been detected (Afonso and Henrique, 2006; Marthiens and ffrench-Constant, 2009). Additionally, the basal membrane establishes focal adhesions based on integrins with the ECM that also can trigger an outside-in signaling controlling cytoskeletal organization, force generation, differentiation and survival (Hood and Cheresh, 2002; Long et al., 2016). Establishment of AB polarity involves cell-cell and cell-extracellular matrix interactions, polarity complexes and trafficking of membrane components to the apical or basolateral domain. Many protein families are involved in cell polarization, but also lipids like phosphoinositides have been reported as important players since they are regulators of cytoskeletal and membrane dynamics and they participate in membrane trafficking (Gassama-Diagne and Payrastre, 2009).

NEP cells are highly elongated and have a bipolar morphology with a ‘‘pearl-on-a-string’’ shape. In the early developing neural tube, the entire structure is mostly composed of a germinal neuroepithelium consisting of a rapidly dividing cell population with two remarkable interrelated features: pseudostratification and interkinetic nuclear migration (INM) (Taverna and Huttner, 2010). Pseudostratification refers to the fact that although all neuroepithelial cells extend from the luminal (apical) surface of the neuroepithelium to the basal lamina throughout their cell cycle, their nuclei are found at various positions along this apical-basal axis resulting in a multilayer appearance. And this is due to the INM, which refers to the fact that the nuclei of NEP cells migrate to different relative apico-basal positions during the cell cycle. Mitosis (M phase) occurs at the apical surface whereas DNA synthesis (S phase) occurs while the nucleus is at a more basal location with apical-to-basal nuclear migration in G1 and basal-to-apical nuclear migration in G2 (Baye and Link, 2008) (Figure 2A).

Before the onset of neurogenesis (embryonic day 11 in the mouse and around 60hrs post-fecundation in chicken), these NEP cells undergo symmetric proliferative divisions, with one progenitor giving rise to two daughter NEP cells to increase the size of the progenitor pool. As neurogenesis proceeds, they switch to an asymmetric neurogenic mode of division to generate one NEP cell along with one cell committed to a specific neuronal fate. After this neurogenic division, the new-born neuron detaches the apical-cell process from the ventricular surface and then migrates to the lateral (basal) neural tube (Figure 2B). Alternatively, NEP cells can undergo neurogenic symmetric divisions that generate two cells that enter the differentiation pathway and exhaust the stem cell pool (Marthiens and ffrench-Constant, 2009; Saade et al., 2013).

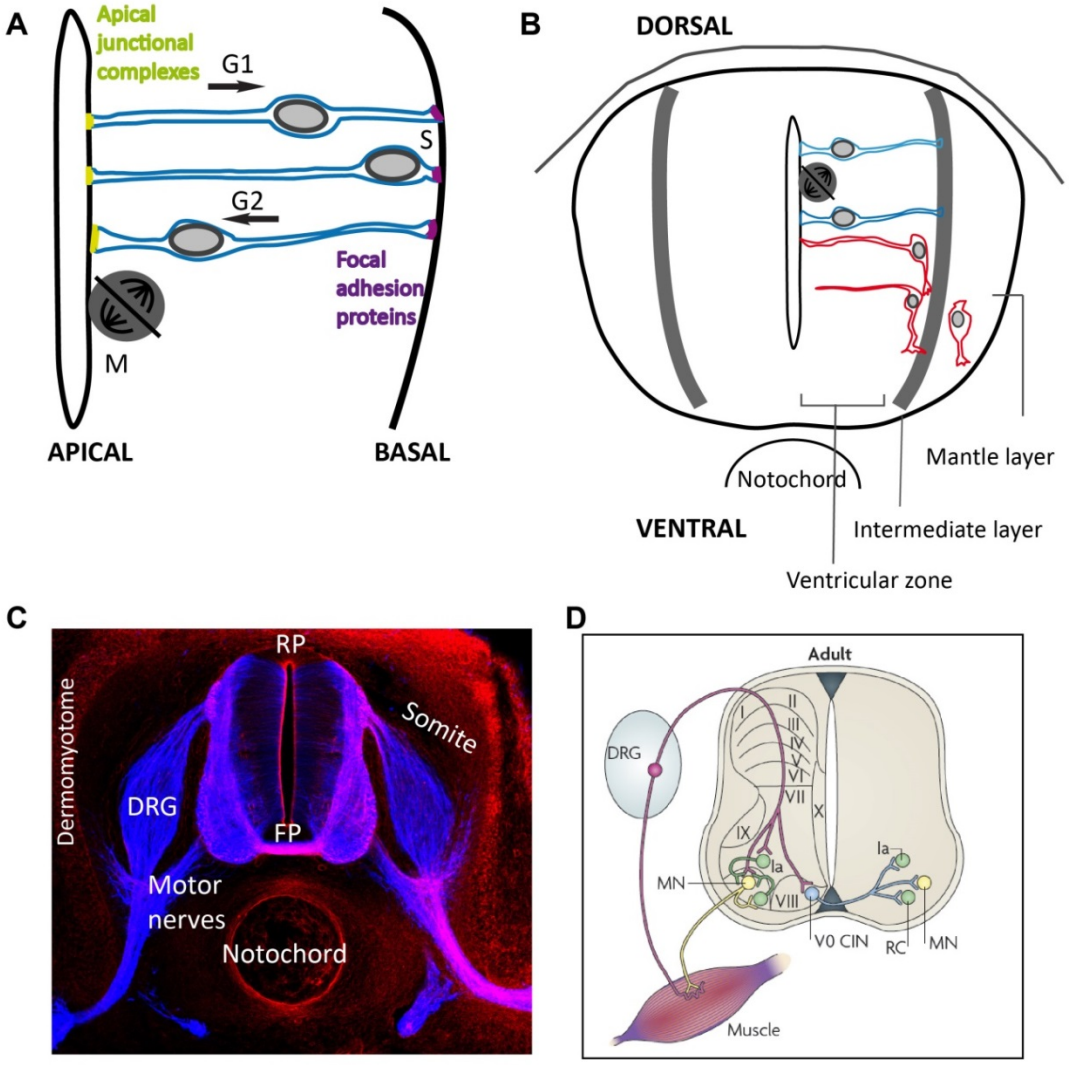


Figure 2. Structure of the neural tube at the cellular level. (A) Neuroepithelial cells exhibit apico-basal polarity with their apical membrane adjoining the lumen, which contains adherent junction proteins and maintains cell–cell adhesion, and the basal process contacting the basement membrane involving integrin-dependent focal adhesions with ECM ligands. Moreover, their nuclei are found at various positions along the apico-basal axis throughout the cell cycle (interkinetic nuclear migration, INM) resulting in a pseudostratified epithelium. **(B)** After a neurogenic division, the post-mitotic neuron releases from the apical side of the tube and migrates laterally forming a basal layer of differentiated neurons. This process is called "lamination" and generates layers within the neural tube: proliferative (ventricular zone), intermediate and neural differentiation (mantle zone) layers. (C) Confocal image of a transverse section of chick embryonic spinal cord after 6 days of incubation showing F-actin (red, cytoskeleton) and beta-III tubulin (blue, early neuronal marker) with labels for the different structures observed. (D) Representation of the neural connections inside the spinal cord showing sensory/afferent neurons (DRG), motor/efferent neurons (MN) and interneurons (Renshaw cells, RC; inhibitory interneurons, Ia; commissural interneurons, CINs). Image adapted from (Goulding, 2009).

It has been recently described that mitotic spindle orientation and the distribution of certain apical markers can regulate the type of division in the neuroepithelium (Das and Storey, 2012; Herrera et al., 2014; Kosodo et al., 2004). For example,

downregulation of N-cadherin or apical aPKC are associated with lateral migration intended to differentiation, as it is loss of apical complex proteins by apical abscission, a process involving actin-myosin–dependent cell constriction of the apical membrane and dismantling of the primary cilium (Das and Storey, 2014; Ghosh et al., 2008). The basal lamina (BL), besides its importance in the structure of the CNS, seems to have also direct functions on neuronal migration possibly by binding growth factor, guidance molecules or BL components themselves such as laminin (ligand of integrins) to cell surface receptors (Franco and Muller, 2011). Hence the tight regulation of the different modes of division as well as cell polarity are essential to balance growth and differentiation during organ development and homeostasis.

The beginning of neurogenesis and localization of neurons to their final destinations will form new layers of cells, in addition to the germinal neuroepithelium (also called, ventricular zone VZ and, later, the ependyma). These basal layers become progressively thicker as more cells are added to it, assuming the name of intermediate layer (IL) where post-mitotic cells localize and, more externally, mantle zone (MZ) where cells differentiate into both neurons and glia and further create a cell-poor marginal zone containing mainly nerve fibers (Figure 2B, C) (Diez del Corral and Storey, 2001; Gilbert, 2000). While in the most cranial part of the neural tube, like in the cerebral cortex, successive waves of migration will establish a laminar structure with different layers containing a characteristic distribution of neuronal subtypes and connections, when the spinal cord matures a further anatomical and functional subdivision appears, although less complex. A longitudinal groove divides it into dorsal (alar) and ventral (basal) halves, receiving inputs from sensory neurons and effecting various motor functions respectively. Interestingly, this mentioned basic three-zone pattern of ventricular, mantle and marginal layers is retained throughout development (Figure 2D). This “less complex” final structure is why the developing spinal cord is an ideal model for studying developmental regulation of growth, polarity and pattern formation in the CNS.

1.3 Patterning of the neural tube

The developmental events recently explained leading up to the formation of the neuroepithelium and, subsequently, to neurons involve inductive events that underlie axis determination. Significantly, NEP cells seem to form from cells that avoid a variety of instructive signals that induce non-neural fates, including the bone morphogenetic proteins (BMPs), which induce epidermal differentiation around the start of gastrulation (Wilson and Edlund, 2001). As a result, NEP cells probably represent a default, ground state, perhaps explaining their ability to generate a variety of cell types (Kintner, 2002). After acquiring the neural identity within the neural tube, a wide array of neurons and glial cells must be produced in an organized temporal and spatial order to form a functional nervous system. This is occurring via inductive interactions that

create organizing centers at the dorsal and ventral poles of the neural tube. The general view is that the acquisition of a specific neural cell fate depends on the initial spatial coordinates of a precursor cell within the neural plate/tube. This initial position defines the exposure of a progenitor cell to specific local environmental signals (morphogenes) released by the induced organizing centers that will restrict its developmental potential in a concentration-dependent manner, so the gradient prefigures the pattern of development (Tabata and Takei, 2004). These local signals direct cell fate by activating or repressing the expression of transcriptional regulators, which, in turn, control the genetic network necessary for the proper specific function of each neural cell (Le Dreau and Marti, 2012).

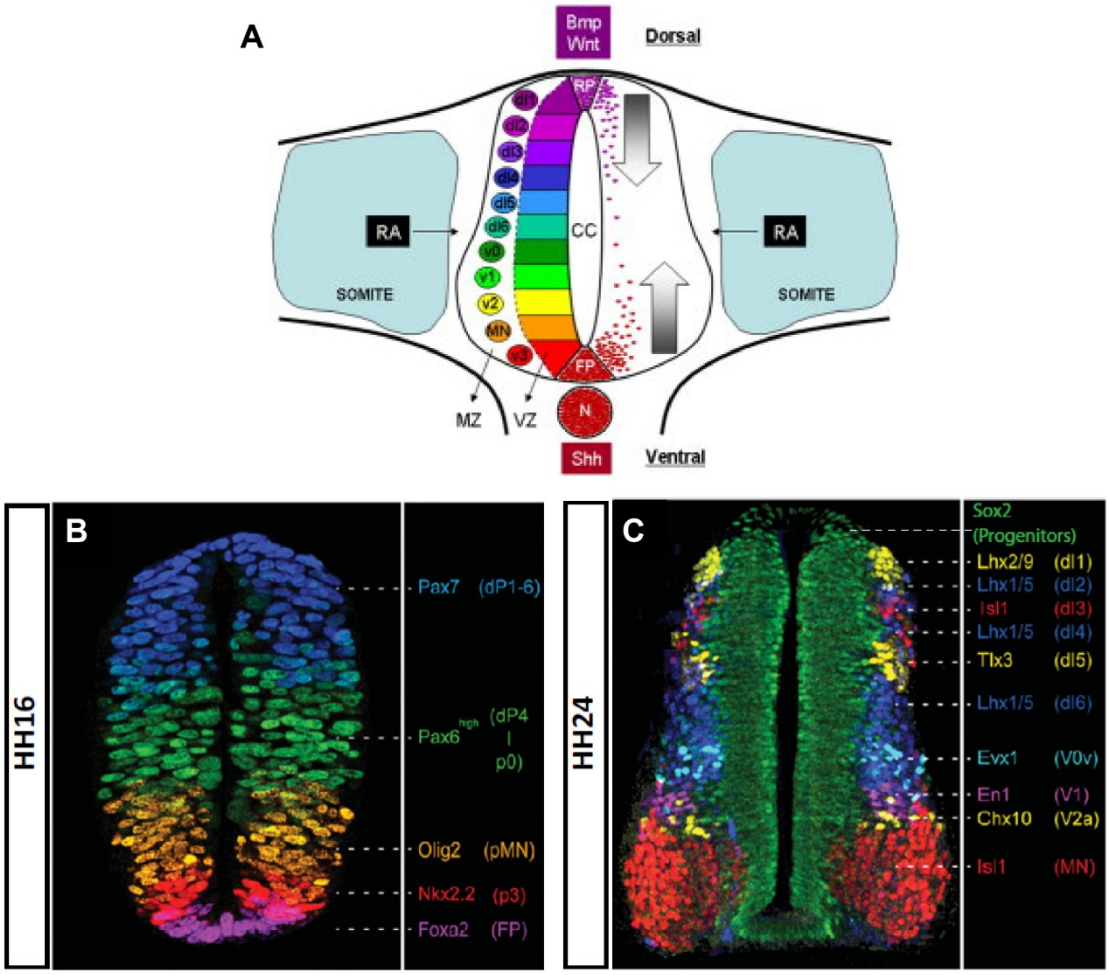


Figure 3. Neural determinants and dorso-ventral (DV) patterning of the neural tube. (A) Scheme of the environmental signals (morphogenes) that control neurogenesis and define the different neural subpopulations along the dorsal-ventral axis (Bmp, Wnt, Shh, RA). **(B,C)** Photographic reconstruction of immunostainings performed on chick neural tube sections at stage Hamburger and Hamilton (HH) 16 and 24 (56hrs and 4 days of development) showing: (B) combinations of progenitor transcription factors that subdivide the neural tube into distinct domains; (C) Sox2+ neural progenitors located in the ventricular zone (VZ, green) and various subpopulations of differentiating neurons generated during the primary neurogenic wave. Images adapted from Ulloa and Martí, 2010 and Le Dreau and Martí, 2012.

The dorso-ventral (D-V) patterning of the neural tube is initiated by two opposed non-neural ectodermal signaling centers within the neural tube: the dorsal roof plate (RP) and ventral floor plate (FP). The dorsal RP produces different Transforming Growth Factor β (TGFβ) secreted factors including the Bone Morphogenetic Proteins (BMPs), such as BMP4 and BMP7 (Le Dreau et al., 2012), and also members of the Wingless (Wnt) family such as Wnt1 and Wnt3a (Muroyama et al., 2002). The RP does not give rise to any neurons itself, but acts as neural organizer of dorsal populations in the neural tube. Selective removal of the RP results in the loss of most dorsal interneurons (dIN). On the other hand, the notochord, located ventrally to the neural tube, is required for the specification of the most ventral population of the neural tube, the FP. Both the notochord and the FP are responsible for ventralizing the neural tube through the release of Sonic Hedgehog (Shh) extracellular signals (Dessaud et al., 2008). There are other two signaling molecules involved in the D-V patterning and generated by the surrounding structures, although they act in the A-P axis. Fibroblast growth factors (FGFs), which are produced by the caudal mesoderm and must be down-regulated before neural differentiation can occur. And Retinoic acid (RA), produced by the paraxial mesoderm (somites), it is an inducer of motor neuron differentiation and responsible for down-regulating FGF (Wilson and Maden, 2005) (Figure 3A).

The patterning genes induced by these signals encode homeodomain transcription factors (TF), able to convert a gradient of extracellular signaling activity into discrete progenitor domains through cross-repressive interactions among members of different homeodomain protein classes (Jessell, 2000). This way, they are thought to specify neuronal subtype identity and, in addition, when and for how long neurons of a particular type will be generated during neurogenesis. This latter function likely depends on interactions between the patterning genes and a family of basic-helix–loop–helix (bHLH) transcription factors referred to collectively as the proneural proteins (Kintner, 2002). The developing spinal cord is thus divided into 11 discrete domains of neural progenitors, with five ventral domains (called p3, pMN, p2-0 from ventral to dorsal) and six dorsal domains (called dP1–6 from dorsal to ventral) which are identified by a particular TF code, and this code determines the neuronal subtype progeny they will produce (Le Dreau and Marti, 2012) (Figure 3B, C).

1.4 The Neural Crest Cells and border control at the spinal cord

Neural crest (NC) cells develop from the dorsal neural tube, originally the border between the neural plate and the adjacent non-neural ectoderm, and migrate out of the neural tube before or during neural tube closure. They are a transient, multipotent, migratory cell population unique to vertebrates that will generate their progeny following multiple intrinsic and extrinsic cues. NC cell migration begins with a complete or partial EMT, which allows NC cells to separate from the neuroepithelium and the ectoderm (Mayor and Theveneau, 2013). The neural tube and all pre-

migratory NC cells express high levels of N-cadherin (2), which is followed by a switch from high to low N-cadherin expression to allow detachment, together with the *de novo* expression of weaker type II cadherins (6/7/11). This is controlled by Snail/Slug, Foxd3 and Sox9/10 in trunk NC cells. In addition, NC cells also express some proteases such as ADAM10 and ADAM13 that are capable of cleaving cadherins further modulating the cell-cell adhesion properties of emigrating NC cells. These changes, together with a change of integrin activity and local remodelling of the extracellular matrix (ECM), trigger NC migration (Mayor and Theveneau, 2013).

NC cells yield pigment cells, neurons and glial cells of the peripheral nervous system (PNS), endocrine cells and, in addition, they originate mesenchymal cells capable of differentiating into connective tissue cells, tendons, cartilage, bone and adipocytes (Le Douarin and Dupin, 2003). These cells are originated along the A-P axis of the neural tube, in the anterior portion of each somite, and they are divided into five domains: cranial, trunk, vagal, sacral and cardiac. Because of its contribution to the nervous system, I will explain mainly the trunk neural crest.

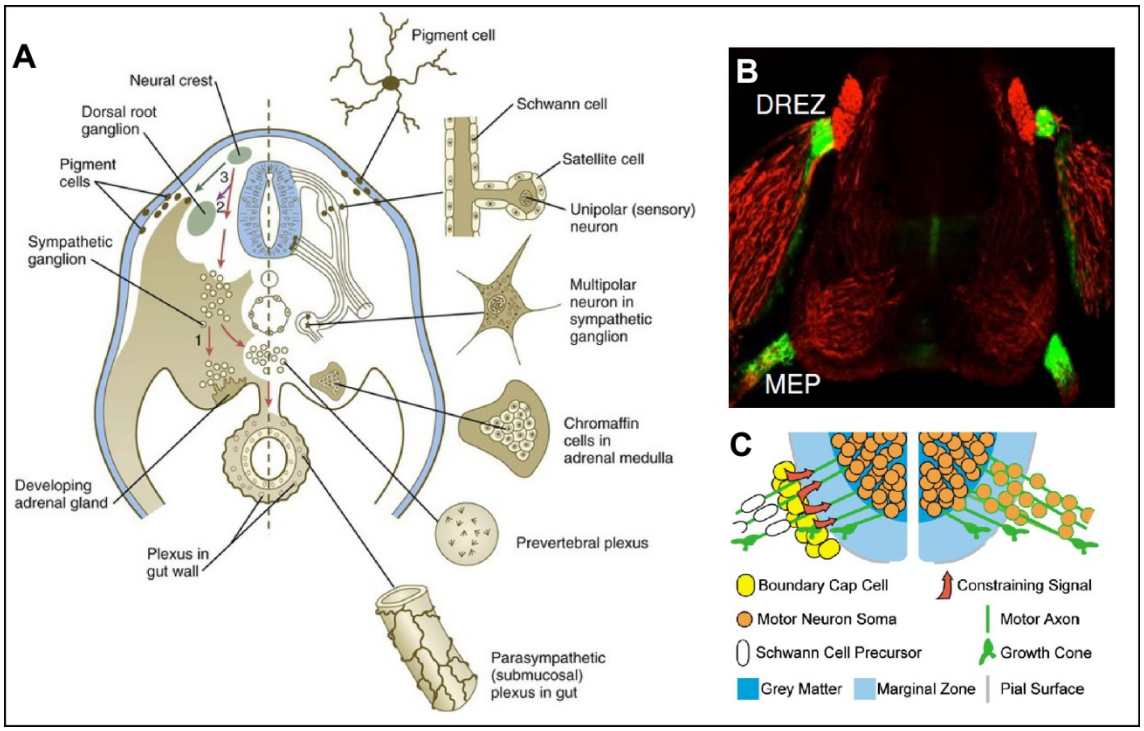


Figure 4. Neural crest cells derivatives. (A) Scheme of migratory pathways and final identity of the migrating neural crest cells in the trunk. Image adapted from the website http://clinicalgate.com/neural-crest/ **(B)** Transverse section from a HH23 chick embryo stained against cad7 (green), expressed by boundary cap cells (BC cells) located in the dorsal root entry zone (DREZ) and motor exit point (MEP), and neurofilament (NF; red). Image adapted from (Bron et al., 2007). **(C)** Schematic diagram illustrating the role of BC cells in the MEP: on the left, clusters of BC cells (yellow) define the PNS side of the CNS:PNS interface; on the right, the absence of BC cells allows motor neuron cell soma to escape through the marginal zone (light blue). Image adapted from (Vermeren et al., 2003).

The trunk neural crest is a transient structure whose cells disperse soon after the neural tube closes. There are three major pathways taken by the migrating trunk neural crest cells. One is the early ventral pathway, where trunk NC cells that go ventro-laterally through the anterior half of each somatic sclerotome will form the dorsal root ganglia (DRG) containing sensory neurons (2, Figure 4A). Others, migrating further ventrally, will become sympathetic neurons, adrenomedullary cells and Schwann cells (1, Figure 4A). And in a third pathway, the dorsolateral, cells will travel between the epidermis and the dermamyotome giving rise to melanocytes (3, Figure 4A) (Mayor and Theveneau, 2013; Mort et al., 2015).

Regarding the spinal cord structure, the recently mentioned NC-derived DRG and the ventrally localized motoneurons (MNs) are unique since their axons enter and exit the CNS respectively along the A-P axis. This occurs through specialized regions called dorsal root entry zone (DREZ) and motor exit points (MEPs), which are breaches in the basement membrane (BM) that covers all the CNS. Boundary cap (BC) cells, which are derived also from the ventral-lateral pathway (as the DRG) of NC cells, are positioned over these breaches from outside of the BM (Figure 4B). The presence of BC cells in the DREZ maintains the integrity between the basal process of NEP cells and the BM while regulates afferent ingrowth of the dorsal root (Golding and Cohen, 1997; Zhu et al., 2015). Meanwhile in the MEPs, BC cells are not required for MN axon exit, but rather restrict MN cell bodies from ectopically migrating out of the CNS (Figure 4C) (Bravo-Ambrosio and Kaprielian, 2011). One of the molecular pathways involved in this control, the semaphorin pathway, belongs to a group of signals aimed to axon guidance and neuronal migration, which link receptor activation to cytoskeletal organization (Mauti et al., 2007).

2. PI3K

The controlled metabolism of phosphatidylinositols (PtdIns), a minor component on the cytosolic side of the cell membrane, is fundamental for signal transduction in eukaryotic cells. They can be phosphorylated in the 3, 4 and/or 5 position resulting in different forms [PtdIns, PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(4,5)P_2, PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3] that play important roles in lipid signaling, cell signaling and membrane trafficking. PtdIns(4,5)P_2 (PIP2) and PtdIns(3,4,5)P_3 (PIP3) represent less than 1% of membrane phospholipids, yet they function in a remarkable number of crucial cellular processes (Czech, 2000; Vanhaesebroeck et al., 2001). PIP3 is the mediator of multiple downstream targets of the phosphoinositide-3-kinase (PI3K) pathway in response to cell stimulation via a variety of membrane receptors and PIP2 can be a substrate of PI3K, or it can serve as a substrate of phospholipase C (PLCγ) leading to the production of the second messengers inositol-(1,4,5)-trisphosphate (IP3) and sn-1,2-diacylglycerol (DAG) (Gassama-Diagne and Payrastre, 2009) (Figure 5).

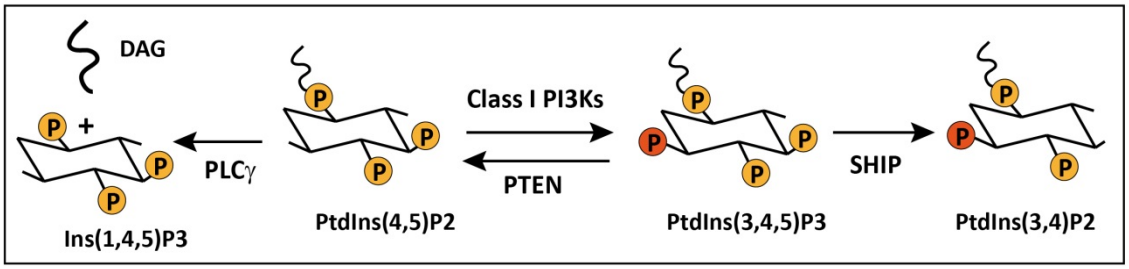


Figure 5. Phosphatidylinositols work as second messengers of signaling pathways. Scheme of the substrates and products of different enzymes that control the presence of specific phosphatidylinositols by phosphorylating or dephosphorylating in positions 3,4 and/or 5 of the inositol ring: PLCɣ (phospholipase C gamma), Class I PI3Ks (phosphoinositide 3-kinase), PTEN (phosphatase and tensin homolog), SHIP (SH-2-containing inositol 5'-phosphatase).

The PI3K family of enzymes is recruited upon receptor activation and produces 3'-phosphoinositide lipids. The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins. These events constitute the start of a complex signaling cascade, which ultimately results in the mediation of diverse cellular activities such as proliferation, differentiation, chemotaxis, survival, trafficking, and glucose homeostasis (Katso et al., 2001). PI3Ks are grouped into three classes (I – III) which differ in regulation, structure, lipid substrate preferences and function (Figure 6). Class I PI3Ks have been the major focus of PI3K studies because they are the isoforms that are generally coupled to extracellular stimuli. In addition, since this thesis has focused on the role of Class IA PI3K in the nervous system, I will explain this family in some more detail.

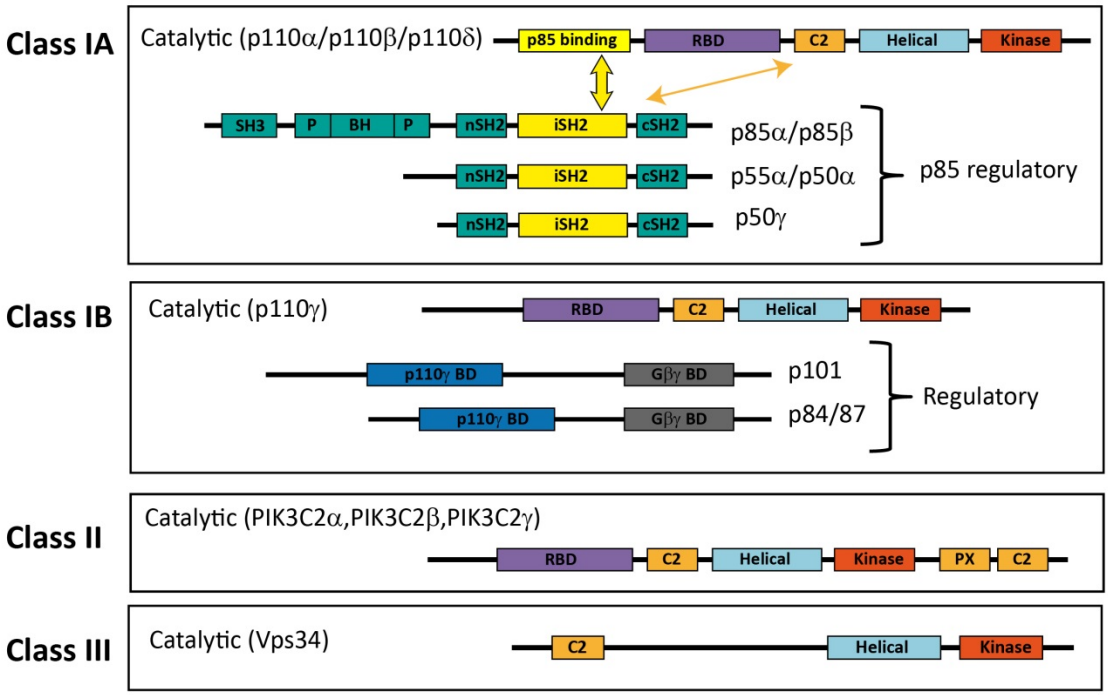


Figure 6. The members of the phosphoinositide 3-kinase (PI3K) family. PI3Ks have been divided into three classes according to their structural characteristics and substrate specificity, although they all act adding a phosphate group into 3 position of the inositol ring. Class I PI3Ks are heterodimers consisting of a p110 catalytic subunit and a p85 regulatory subunit. Class II PI3Ks are monomers with only a single catalytic subunit and a divergent N-terminal domain among genes. Class III PI3K consists of a single catalytic subunit.

2.1 Class I PI3K family members

Class I PI3Ks are heterodimeric enzymes consisting of a catalytic subunit p110 that associates with a regulatory subunit. In vertebrates, the Class I PI3Ks are divided into two subclasses, Class IA and Class IB PI3Ks, based on structural and functional differences (Cantley, 2002) (Figure 6).

The vertebrate genome presents three genes (PIKCA, PIKCB, PIKCD) that code for three highly homologous Class IA catalytic subunits named as p110 (α, β, δ). These are composed of several modular domains, from N to C terminal:

- the NH2-terminal binding domain (ABD) that interacts with the regulatory subunit.
- the Ras-binding domain (RBD) that mediates activation by the small GTPase Ras.
- the C2 domain, involved in phospholipid membrane binding.
- the helical domain, which has been proposed to serve as scaffolding for other domains.

- and the catalytic lipid kinase domain, which exhibits weak, but some homology to protein kinases such as mTOR (mammalian target of rapamycin) or ATM (ataxia telangiectasia mutated), indicating that these proteins may share an ancient evolutionary origin.

All class IA catalytic subunits can associate equally with all of the class IA regulatory subunits. Two genes encode subunits of 85 kDa, termed p85α (PIK3R1) and p85β (PIK3R2), considered as the full-version of the regulatory subunits. They consist of:

- an amino-terminal Src-homology 3 (SH3) domain that binds other proteins via proline-rich sequences [such as FAK (Focal Adhesion Kinase), CAS (Cellular Apoptosis Susceptibility proteins), Apoptin, Ruk, SNX9 (Sorting Nexin 9), Dynamin, Cbl (E3 ubiquitin ligase Casitas B-lineage lymphoma), and BCR-ABL, reviewed in (Mellor et al., 2012)].
- a Bcr homology domain (BH) flanked by two proline-rich regions (P), mediates binding to XB-1 (Tenascin), Rac1, Cdc42, Rab5 or PTEN (reviewed in Mellor et al., 2012).
- an N-terminal SH2 (nSH2) domain that interacts with the helical domain of p110α (Miled et al., 2007) and mediates binding to phosphotyrosine residues.
- an inter-SH2 (iSH2) region containing the binding site for both the ABD and C2 domains of p110α, leading to stabilization and inhibition of p110α respectively (Elis et al., 2006; Huang et al., 2007).
- and a C-terminal SH2 (cSH2) domain, which mediates binding to phosphotyrosine residues as the nSH2.

The PIK3R1 gene also produces two major alternative transcripts encoding the smaller proteins p55α and p50α. The N-terminal domains of these PIK3R1 derived isoforms, consisting of 34 and 6 amino acids respectively, are distinct and might allow additional signaling input and output, but their specific roles are not well known. A third gene (PIKR3) encodes p55γ (or p55PIK), a protein with similar structure to p55α (Brachmann et al., 2005). Notably, the regulatory subunit p85α seems the most prevalent.

It has been established that Class IA PI3Ks occur as obligatory dimers in the cell since no evidence for free p85 or p110 subunits could be obtained (Geering et al., 2007) and overexpression of monomeric p110α had very little activity when compared to co-expression with the regulatory subunit (Yu et al., 1998). The Class IA regulatory subunits would play three key roles: stabilizing the p110 subunit, inhibiting p110 basal lipid kinase activity, and facilitating activation downstream of phosphorylated tyrosine motifs through interaction with their nSH2 and cSH2 domains (Burke and Williams, 2015; Hofmann and Jucker, 2012). However, there are also evidences pointing to the existence of a small pool of free p85 tightly controlled by nuclear sequestration (Chiu et al., 2014) or FBXL-2 (F-box/LRR-repeat protein 2) mediated proteasomal degradation (Kuchay et al., 2013) suggesting additional signaling roles for p85 subunits.

The class IB only presents one gene encoding a catalytic subunit p110ɣ (PIK3CG) and it can associate with either a p84 (also referred to as p87, PIK3R6) or a p101 regulatory subunit (PIK3R5) (Shymanets et al., 2013).

2.2 Class I PI3K activation

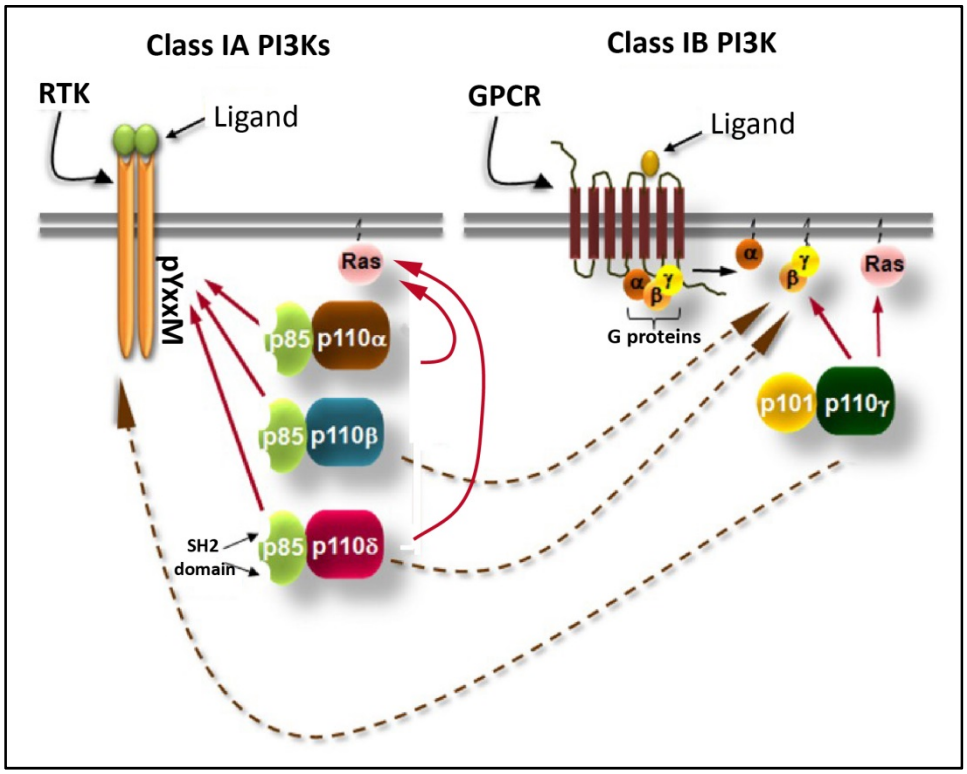


Figure 7. Differential activation of Class I PI3K catalytic subunits. Class I PI3Ks can be activated by receptors tyrosine kinase (RTK) or by G protein-coupled receptors (GPCRs). P110α and δ are mainly associated to RTK signaling, while p110ɣ activation relies on GPCRs and p110β activation can be mediated by both types of receptors. Image adapted from (Tzenaki and Papakonstanti, 2013).

Activation of Class IA PI3Ks generally starts with binding of a ligand to receptor tyrosine kinases (RTKs), which dimerize and autophosphorylate at tyrosine residues, and it can go downstream through three independent pathways. The first way consists of the direct binding of the regulatory subunit to phospho-YXXM motifs within the RTK. The second depends on adaptor proteins like the insulin receptor substrate 1 (IRS1) or the GRB2-associated binder (GAB). And the third way is via the Ras superfamily of small GTPases independently of the regulatory subunit through the binding of GBR2 to the receptor and activation of Sos followed by Ras, which directly binds the catalytic subunit (Yang et al., 2012). Alternatively, the p110β catalytic subunit seems unable to interact with Ras and it can be additionally activated by G protein–coupled receptors (GPCR) or by the Rho subfamily of small G proteins, specifically by Cdc42 and Rac1 (Burke and Williams, 2015; Castellano and Downward, 2011; Fritsch et al., 2013).

Regarding the p110δ isoform, several studies have linked it also to GPCRs. On the other hand, Class IB PI3Ks are mainly activated by GPCRs (specifically by the Gβγ subunits of heterotrimeric G-proteins), but it seems that they also could interact with Ras and tyrosine kinases (Kurig et al., 2009; Tzenaki and Papakonstanti, 2013) (Figure 7).

PIP3 signaling is mainly counteracted by two phosphatases: the phosphatase and tensin homologue (PTEN) and the SH2 domain containing inositol 5'-phosphatases (SHIP1 and 2), which convert this lipid back to PIP2 [PtdIns(4,5)P_2] or to PtdIns(3,4)P_2 respectively (Figure 5). PTEN discovery was a major breakthrough in the context of PI3Ks and disease because it is one of the most frequently inactivated tumour suppressors in many cancers, stressing a high frequency of mutations in endometrial carcinoma, glioblastoma, prostate cancer, breast, colon, and lung tumors. And PTEN inactivation leads to constitutive activation of the class I PI3K pathway (Li et al., 1997; Salmena et al., 2008). Instead, members of the SHIP-5 phosphatases seem to have inhibitory roles in the immune system and, possibly, in insulin signaling (Hori et al., 2002; Huber et al., 1998).

2.3 Structural effects of oncogenic PI3Kα mutations

P110α is practically the only PI3K catalytic subunit mutated in a broad variety of cancers. Analysis of these mutations has allowed a better comprehension of the regulation of its enzymatic activity. Notably, the majority of mutations are activating and they cluster in so-called ''hot-spot'' regions in the kinase domain and the adjacent helical domain (Figure 8). The molecular mechanisms by which these mutations activate p110α are being unraveled, but the main models refer to two mechanisms. One mechanism could be the facilitation of p110α binding to membranes in the case of the H1047R mutation affecting the kinase domain resulting in increased enzymatic activity with respect to the wild type. Another mechanism promoted by mutations affecting the helical domain, such as E542K and E545K, would go through weakening of the inhibitory interaction exerted by the regulatory subunit mainly through its nSH2 domain, while preserving the stabilizing interaction between p85 iSH2 and the adapter-binding domain of p110α. Likewise, low frequency mutations or deletions in the regulatory subunits encoded by the PIK3R1 gene have been also reported in tumor samples, most of them localized in the SH2 or iSH2 domains. It was found that these mutants also retained their ability to stabilize p110α but lost their inhibitory activity, similarly to the second mechanism mentioned (Berenjeno and Vanhaesebroeck, 2009; Gabelli et al., 2010). In this work, we increase PI3K activity to study its function in the neural tube based on this knowledge.

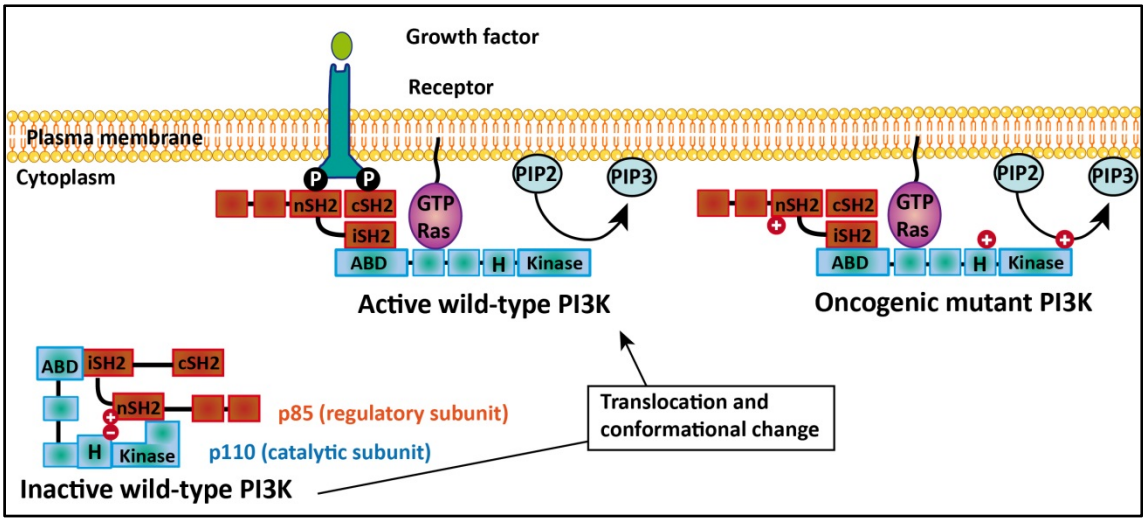


Figure 8. Class IA PI3Ks can be activated by growth factor receptors or oncogenic mutations. Different mutations in PIK3CA or PIK3R1 can induce gain-of-function of p110α through distinct molecular mechanisms, such as altering the inhibitory loop or increasing the positive surface charge of p110α and therefore enhancing its recruitment to cellular membranes.

2.4 Class I PI3K effectors

Once the receptor is activated, Class IA p110 catalytic subunit colocalizes proximal to its substrate PtdIns(4,5)P_2 (PIP2) and produce PtdIns(3,4,5)P_3 (PIP3), a key lipid second messenger that controls a wide range of cellular responses via recruitment to the plasma membrane of downstream effector proteins with PIP3-binding domains (such as the pleckstrin homology domain, PH) including adaptor proteins, protein kinases and nucleotide-exchange factors (GEFs) or GTPase-activating proteins (GAPs) for GTPases of the Ras, Rho and ADP-ribosylation factor (Arf) families (Burke and Williams, 2015; Vanhaesebroeck et al., 2012).

One of the best-studied PIP3 effector proteins is the Ser/Thr protein kinase Akt (also known as protein kinase B, PKB), a member of the AGC family of protein kinases. The Akt kinase family is comprised of three highly homologous isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). Phenotypic analyses of Akt isoform knockout mice have documented Akt isoform specific functions in the regulation of cellular growth, glucose homeostasis and neuronal development respectively, although they also have overlapping functions (Gonzalez and McGraw, 2009).
Recruitment of Akt to the membrane through binding of its PH domain to PIP3, leads to its phosphorylation and activation by phosphoinositide-dependent kinase (PDK1) in Thr308 and mammalian target of rapamycin complex 2 (mTORC2) in Ser473, although a number of other kinases may also function in specific settings. Once fully phosphorylated, Akt is locked in a catalytically-competent conformation, loses the PIP3 requirement and translocates to a variety of intracellular locations including the cytoplasm, mitochondria and nucleus, where it phosphorylates specific substrates that

modulate numerous processes including gene expression, protein synthesis, cell cycle, cytoskeleton and metabolism. Some of the downstream effectors phosphorylated by Akt are GSK3β (glycogen synthase kinase 3), p70S6K (ribosomal protein S6 kinase), BAD (Bcl-2-associated death promoter), IkB kinase, endothelial nitric oxide synthase, mTOR (mammalian target of rapamycin), 4E-BP (eukaryotic translation initiation factor 4E binding protein), forkhead transcriptional factors (FOXp), Caspase 9, PRAS40 (proline-rich Akt substrate) or p27 (cyclin-dependent kinase inhibitor 1B).Therefore, activation of Akt can impact in diverse secondary pathways controlling cellular proliferation, evasion from apoptosis, invasive migration, angiogenesis and metabolic reprogramming among others (Figure 9) (Fayard et al., 2010; Rajala, 2010; Toker, 2012; Vasudevan and Garraway, 2010).

Besides Akt, another PI3K downstream protein, PDK1, also turned out to be very important for cell signaling. PDK1 serves as the master regulator of the AGC-family of kinases, most of which require an additional phosphorylation event for complete activation through a parallel-acting pathway. PDK1 localization at the plasma membrane is also dependent upon a functional PH domain. Once in the membrane, PDK-1 phosphorylates numerous kinases, such as the mentioned Akt or PKCs (protein kinase C enzymes), that have been implicated in cell growth, metabolic control, proliferation, survival and polarity (Arimura and Kaibuchi, 2007; Bayascas, 2010; Mora et al., 2004).

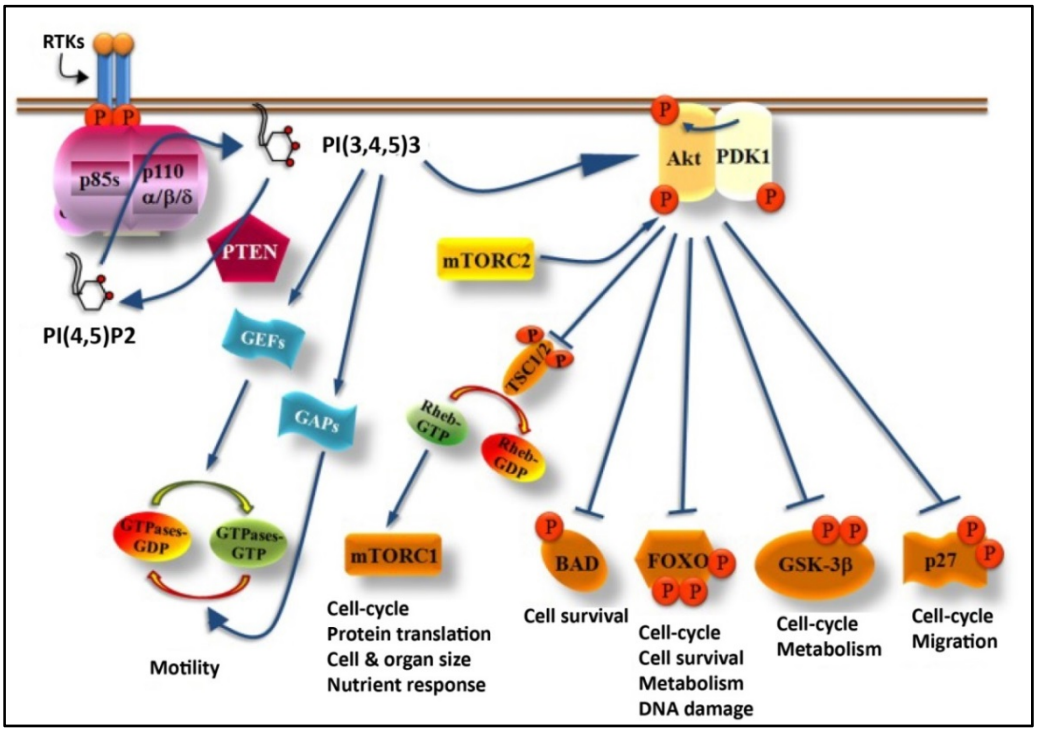


Figure 9. Class IA PI3Ks participate in multiple cellular functions. Upon PI3K activation, membrane PIP3 recruits proteins with pleckstrin homology (PH) domains such as Akt, which becomes phosphorylated and active, or GEFs and GAPs that regulate the activity of small GTPases. Akt activation results in phosphorylation of a variety of effector proteins including TSC1/2, BAD, FOXO, GSK3β or p27, which

control several cellular processes. Modulation of small GTPases can derive in cytoskeletal changes. Image adapted from Tzenaki and Papakonstanti, 2013.

Another family of proteins regulated by the presence of PI3K and PIP3 at the plasma membrane is the superfamily of small GTP-binding proteins (G proteins), including the Ras and Rho families. These proteins act as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) forms controlled by regulators. Guanine nucleotide exchange factors (GEFs) activate Rho proteins by catalyzing the exchange of GDP for GTP. GTPase-activating proteins (GAPs) enhance the GTPase activity to hydrolyze GTP to GDP, so that returning to the inactive conformation. And guanine-nucleotide dissociation inhibitors (GDIs) form a large complex with the Rho protein, helping to prevent diffusion within the membrane and into the cytosol and thus acting as an anchor and allowing tight spatial control of Rho activation. These and other post-transcriptional and post-translational regulatory mechanisms precise the control of individual Rho GTPases, which determine the spatial and temporal context of specific cell functions (Takai et al., 2001). As explained previously, Ras can bind the p110 subunit leading to p110 stabilization in the membrane and PIP3 generation and, simultaneously, interaction of Ras with p110 can activate downstream factors of Ras in a PI3K-dependent manner. The Ras family has been largely associated with signaling pathways contributing to normal and aberrant cell growth, differentiation or apoptosis through control of MAPK kinases and transcription factors (Castellano and Downward, 2011). Regarding the Rho family, its activation/inhibition by PIP3 occurs because there are some GEFs and GAPs having a PH domain therefore allowing interaction with phospholipids of the cell membrane and recruitment of Rho GTPases. Interaction of GEFs and GAPs with Rho GTPases modulates the GDP–GTP exchange activity of the latter in specific locations of the plasma membrane. Rho-related protein function is to integrate extracellular signals with specific targets regulating cell morphology, cell aggregation, tissue polarity, cell motility, cytokinesis and transcriptional control (Hanna and El-Sibai, 2013; Teramoto et al., 2003). To increase the level of complexity, the PI3Ks, Ras and Rho family of small GTPases can influence each other's activities by feed-back processes needed for regulating certain cellular processes, for example, maintaining cell polarity while regulating cell migration (Yang et al., 2012).

2.5 Class I PI3K actions (where and what)

Multiple signaling pathways can activate Class I PI3Ks. Each isoform has a different ability to integrate signals from these inputs, and many of the isoform-specific roles of PI3Ks are dependent on their ability to successfully synergize inputs from multiple pathways with a high level of complexity (Vanhaesebroeck et al., 2010). For example, mammals have five distinct Class IA regulatory isoforms, potentially giving rise to fifteen distinct p85–p110 combinations. When co-expressed, they all can bind each

p110 isoform, although it is not clear whether endogenous regulatory isoforms show preference for specific p110 isoforms.

At present, clear specific roles of the distinct regulatory isoforms are unknown, but there are more and more studies addressing distinct cellular functions dependent on specific regulatory isoforms (Hiepen et al., 2014; Hill et al., 2001; Mellor et al., 2012; Pensa et al., 2014; Vanhaesebroeck et al., 2005). Further knowledge of expression levels in health and disease are contributing to a better understanding of the PI3K pathway. Regarding their expression, members of the regulatory subunit family have distinct, but overlapping, tissue distributions. p85α and p85β are ubiquitously expressed, while p55γ is expressed mainly in brain and testis. p50α and/or p55α are present in insulin-sensitive tissues including fat, muscle, liver, and brain (Chen et al., 2004; Geering et al., 2007). Class IB regulatory subunits have been found to be mainly expressed in the immune system (Burke and Williams, 2015).

With reference to the expression and functions of the catalytic isoforms, p110α and p110β isoforms seem to have broad tissue distribution while the p110δ and p110ɣ are primarily expressed in hematopoietic cells and leukocytes (Burke and Williams, 2015; Geering et al., 2007; Vanhaesebroeck et al., 2005). Interestingly, it was recently unveiled high expression of p110δ also in the nervous system while p110ɣ was found additionally in the heart, pancreas, liver and skeletal muscles (Eickholt et al., 2007; Liu et al., 2009). Selective genetic inactivation of different p110 isoforms in mice and the use of p110 isoform-specific inhibitors have been valuable in delineating their distinct functional roles. For example, it has been reported that traditional knockout (KO) of p110α in mice leads to early embryonic lethality around embryonic days 9.5–10.5 while KO of p110β leads to even earlier embryonic lethality at E3.5 associated with defective cell proliferation. Notably, normal development and cell proliferation seem to depend more on the kinase activity of p110α, but p110β must have a still unknown non-redundant essential function during early development that explains this phenotype (Bi et al., 2002; Jia et al., 2009). Another parameter to take into account when deciphering isoform-specific functions and KO phenotypes is their lipid kinase activity, which is significantly higher for p110α compared to the other Class IA catalytic isoforms (Meier et al., 2004). So far, p110α has emerged as the main isoform regulating growth factor signaling through RTKs (IGF, EGF, PDGF…), while p110β action seems more linked to GPCRs and/or Gβɣ subunits activation, although it also can respond to RTK activation and compensate for the lack of other p110 subunits (Foukas et al., 2010). Recent studies have demonstrated that both isoforms might have redundant functions, such as contributing to insulin action in the liver (Brachmann et al., 2005), but they also play distinct roles in cellular signaling. For example, p110β is differentially recruited by the small GTPase Rab5 in the endocytic pathway, or they function differently in growth or in oncogenic transformation (Christoforidis et al., 1999; Samuels and Velculescu, 2004; Vanhaesebroeck et al., 2005). Related to their

role in tumorigenesis, there is also a striking difference since the gene encoding p110α (PIK3CA) is the only PI3K catalytic subunit gene found to be mutated in cancer, which results in its overactivation, while loss of the tumour suppressor PTEN increases PI3K activity mainly through enhanced p110β signaling (Wee et al., 2008).

3. Class IA PI3K in the nervous system

3.1 Expression

All class IA PI3K subunits are expressed in both the developing and mature mammalian nervous system. In the rat embryo, the p110α catalytic subunit was found throughout the entire CNS, particularly enriched in neurons at developmental stages that correspond to extension and guidance of neuronal processes, with gradual decrease in expression during postnatal development to lower expression in the adult. In the adult brain, p110α seems expressed at relatively high levels in the olfactory bulb, hippocampus, and cerebellum, while in all other regions, p110α is expressed at low levels in neurons (Ito et al., 1995; Waite and Eickholt, 2010). In adult murine tissue analysis, p110β was found highly expressed in brain, far exceeding p110α levels (Geering et al., 2007). As mentioned before, p110δ PI3K appeared to be particularly enriched in the nervous system, especially in the spinal cord, dorsal root ganglia (DRG), cranial sensory ganglia and peripheral nerves during embryonic development (Eickholt et al., 2007).

Moreover, changes in PI3K levels or activity have been observed in neuronal disease or damage with upregulation following axonal crush in hypoglossal motoneurons and after transient ischemia in spinal motoneurons or global ischemia in brain. Conversely, there are reductions of PI3K in Alzheimer's disease brain and in spinal cord tissue from pmn/pmn mice, a murine model of motoneuron disease. The high levels of PI3K expression during normal nervous system development and observed changes in expression in neurodegenerative conditions support the contribution of PI3K to processes that underlie cell growth and differentiation in the nervous system (Rodgers and Theibert, 2002).

3.2 Activation and functions

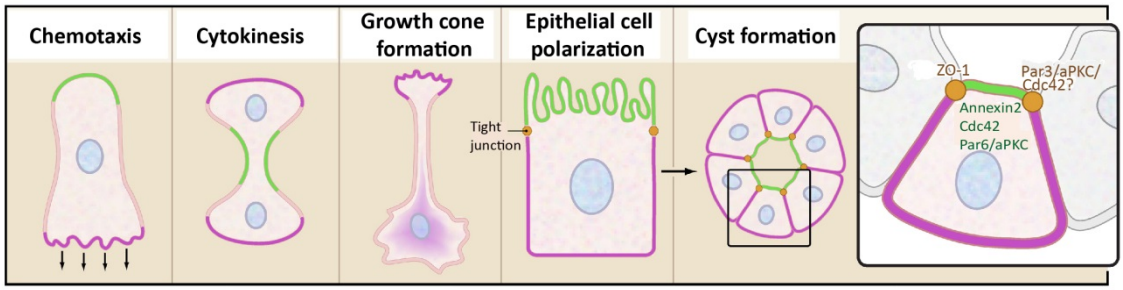


Figure 10. Spatial restriction of phosphoinositides as key regulator of cellular polarity. The cellular distribution of PI(3,4,5)P3 (purple) and PI(4,5)P2 (green) during chemotaxis, cytokinesis, growth cone formation, epithelial cell polarization, and cyst formation is shown. The segregation of PI(3,4,5)P3 and PI(4,5)P2 creates subcellular domains, attracting specific signaling components involved in maintaining polarized responses, as represented in the cyst formation. Image adapted from (Comer and Parent, 2007).

Cellular polarity is an important feature of many eukaryotic cells that respond to spatial cues. Its proper regulation requires the orchestration of cell-cell and cell-substratum interactions with intracellular signal transduction events. In multiple cell types spatial restriction of phosphatidylinositol signaling components drive polarization in different contexts as mediators between the external input and the internal response, like chemotaxis in motile cells, cytokinesis during cell division, growth cone formation in neurons, epithelial polarization or cyst formation (Figure 10) (Comer and Parent, 2007; Martin-Belmonte and Mostov, 2007).

In neuronal cell types, highly polarized, phosphatidylinositol distribution thus participates in several processes in response to diverse extracellular signals. Upstream, PI3K activity in neural cells can be increased by neurothrophic factors, secreted proteins that stimulate survival and differentiation of neural stem cells and neurons including NGF, GDNF and BDNF; by growth factors such as IGF-1; through activation of the Wnt pathway; via axon guidance cues such as netrin or semaphorins; through extracellular matrix proteins such as laminin or integrins; via chemokines such as fractalkine; and through neurotransmitters (Rodgers and Theibert, 2002; Tahirovic and Bradke, 2009; Waite and Eickholt, 2010; Wolf et al., 2008).

Dowstream, genetic manipulation and pharmacological approaches are unveiling the cellular events regulated by the PI3K in neural cells:

A) Survival

One of the first PI3K-mediated functions discovered in neurons was survival, principally mediated through localization and activation of the serine/threonine kinase Akt (Dudek et al., 1997). Dominant negative Akt blocks neuronal survival in the presence of trophic factors and expression of activated Akt is sufficient to support neuronal survival even in the absence of trophic factors. PI3K/Akt signaling seems to be necessary and sufficient for neuronal survival in many neuronal cell types and it has been proposed to be a convergence point for regulating diverse survival signals, including regulation of pro-apoptotic proteins (Bad, Caspase-9), protein kinases (GSK3β) and transcription factors such as the FoxO family, very important in the insulin pathway (Brunet et al., 2001; Hannenhalli and Kaestner, 2009; Rodgers and Theibert, 2002). Another downstream effector of PI3K crucial in neural survival is activation of the 70kDa ribosomal S6 kinase (p70S6K), a key step for upregulating the protein synthesis pathway and needed for insulin stimulated retinal cell survival (Rajala, 2010).

B) Mitosis

Previous evidences suggest a role for PI3Ks in cell cycle progression in several non-neuronal cell types through modulation of its activity at different cell cycle phases. For example, Class IA PI3Ks need to be attenuated to enter the M phase, but they are

required to correctly progress throughout mitosis. Activation of p110α seems to be important for progression through prometaphase and enrichment of PIP3 at the midcortex participates in the recruitment of the dynein/dynactin complex that anchors the mitotic spindle to the cell membrane. Therefore, misregulation of Class I PI3K activity has been associated to spindle orientation defects (Silio et al., 2012). During late mitosis, so anaphase and telophase, the PI3K signaling pathway must be attenuated again and, during cytokinesis, PTEN actively controls PIP3 levels in order to smoothen the cell membrane avoiding F-actin based ruffles and regulate the formation of the actomyosin ring underlying the cleavage furrow (Campa et al., 2015; Janetopoulos et al., 2005). Regarding neuroepithelial cells, little is known about PI3K controlling cell division, but given that mitotic processes share common mechanisms among cell types and PI3K seems involved in AB polarity in the neuroepithelium (essential for mitotic localization) it is likely that PI3K also participates in any of the mentioned steps (Ghosh et al., 2008; Tsuda et al., 2010). Indeed, it would be very interesting to study.

C) Neural migration

PI3K signaling is also involved in neural migration. As previously explained, after a neurogenic division the newly born neuron migrates basally to its final destination while begins to differentiate and to form neurites through major changes in the neuronal cytoskeleton. Neuronal migration depends on PI3K signaling for some neuronal types. For example, the extracellular matrix protein Reelin – secreted by Cajal–Retzius cells in the marginal zone of the cortex – binds to VLDLR and ApoER2 receptors, induces tyrosine phosphorylation of the adaptor protein Dab1 and it can activate PI3K downstream and transmit the signal. This way, Reelin controls the radial migration of cortical neurons and directs the organization of neurons in the cortical plate in a PI3K-dependent fashion (Valiente and Marin, 2010). Interestingly, it has been also reported as modulator of spinal cord neuron position in the rodent spinal cord (Kubasak et al., 2004; Vaswani and Blaess, 2016). Additionally, classical neurotrophins such as BDNF also control the movement of neurons via PI3K, in this case affecting tangential movements of neurons from the medial ganglionic eminence (MGE) into the developing cortex during cortical development (Polleux et al., 2002).

Although neurons may migrate by distinct modes or paths, there appears to be a general basic model of neuronal movement requiring exquisite regulation of three repetitive steps. First, the cell extends a leading process that extends and contracts as it explores the microenvironment. Next, it is followed by the translocation of the nucleus into the leading neurite. Finally, there is the retraction of the trailing process, an aspect of migration that is very poorly understood. PI3K activation underlies some of the molecular events needed to perform the structural rearrangements required for cell migration, such as phosphorylation of Akt and GSK3β, mTORC2 activation or

spatiotemporal activation of Rho GTPases, although the role of PI3K in migration has been mainly studied in migrating fibroblasts and epithelial cells or in cells during chemotaxis, not in neurons (Ayala et al., 2007; D'Arcangelo, 2014; Kolsch et al., 2008; Kurokawa et al., 2005).

D) Neuronal morphology and polarity

D.1) Local enrichment of PIP3 promotes neural asymmetry

Neurons are among the most polarized cell types in the body and are compartmentalized into two molecularly and functionally distinct domains: the axon and the dendrites. Neurons typically form a single axon and multiple dendrites, which underlie the directional flow of information transfer in the central nervous system. Dendrites integrate synaptic inputs which propagate along the axon, making presynaptic contacts onto target cells. There are still important questions revolving around the relationship between the neural progenitor polarity in the neuroepithelium previously presented and post-mitotic neuron polarity in vertebrates. Dividing neuroepithelial progenitors present a strong apico-basal polarity where the apical and baso-lateral membranes, with different molecular compositions, are separated by cadherin-based adherens junctions (AJ). Likewise, the centrosome or microtubule organizing center (MTOC) is associated to the apical domain of NEP cells. It is not so clear for several neural subpopulations, like the spinal cord neurons, whether and/or how any of the proteins or organelles associated to the AJC of progenitors are inherited and arranged by post mitotic neurons upon cell-cycle exit. It is logical to think that there might be a functional relationship between the Par polarity complex or the centrosome position on the axon initiation site during the transition to the final neural morphology, since both elements have been connected to neural polarity and axonal growth (de Anda et al., 2010; Parker et al., 2013; Vyas et al., 2013).

The PI3K pathway has been also proposed as a crucial mechanism for the morphological changes explained above, although most of the studies have been performed only *in vitro*. Interestingly, there are cues pointing to a role of PI3K in apico-basal polarity prior to neural differentiation (Ghosh et al., 2008). And with regard to neural differentiation, accumulating evidence indicates that local enrichment of PIP3 is one of the first mechanisms that regulates the breaking of symmetry to implement “polarization” and, likewise, persistent accumulation at the tip of a neurite is required for coordination of cytoskeletal dynamics and axon specification. This importance of PI3K and PIP3 in neural polarization is based on the fact that pharmacologic inhibition of PI3K activity using LY294002 or Wortmannin prevents axon formation and, conversely, overexpression of a constitutively active catalytic p110α leads to the formation of multiple axons. Coherently, overexpression of the antagonist PTEN disrupts the development of polarity. Additionally, using the PH domain of AKT fused to GFP (PH-AKT-GFP) as a biosensor for PIP3 formation, it has been shown that PIP3

accumulates selectively within a single neurite following local application of laminin, signal that enhance axonal growth, in immature hippocampal neurons (Arimura and Kaibuchi, 2007; Menager et al., 2004). There are two independent mechanisms reported that would maintain PIP3 at the tips of axons and, therefore, the axonal identity. Firstly, the local production of PIP3 by PI3K at the axon tip promoted by Shootin, a PI3K-interacting protein that overexpressed led to aberrant accumulation of PI3K in minor neurites and to the generation of supernumerary axons. Consistently, Shootin knockdown inhibited axon formation, suggesting a critical role for Shootin and PI3K in establishing axonal identity (Toriyama et al., 2006). The second proposed mechanism is the local transport of PIP3 containing vesicles to the prospective axon by the guanylate kinase-associated kinesin (GAKIN), since studies with mutant forms of GAKIN generated similar effects to those described for other PIP3 modulating proteins (Horiguchi et al., 2006).

D.2) Local enrichment of PIP3 activates several signaling pathways controlling cytoskeleton

There are several molecular cascades triggered by PI3K activation contributing to these neural maturation processes. Some of the final targets involve intermediate Akt/PDK1 activation, but others require alternative activation of members of the Rho family of small GTPases or the Ras/MAPK pathway, which also controls gene expression.

An important signaling cascade required for neural polarization that can be activated by PI3K/PDK1 in specific regions of the plasma membrane involves the PAR protein complex Par3–Par6–aPKC (atypical protein kinase C), which triggers the recruitment of some signaling molecules while restringing other polarity protein complexes that establish the different membrane identities (Iden and Collard, 2008; Suzuki and Ohno, 2006). Before neurogenesis, as previously explained, the NEP cells of the neural tube display an apico-basal polarity and their membranes have different protein complexes. For example, the PAR3–PAR6–aPKC complex determines the apical side of the tube. However, after a neurogenic division, these same polarity markers have to rearrange themselves and participate in the final neural morphology. In both morphologies, the Par complex localization is, at least, partially controlled by membrane phosphoinosites through localized PDK1/Cdc42 dependent aPKC activation (Figure 11) (Balendran et al., 2000; Barnes and Polleux, 2009; Ghosh et al., 2008).

Concerning activation of the PI3K/Akt signaling pathway in neural polarity, another relevant role to describe is the inactivation of the constitutively active GSK3β, which regulates microtubule dynamics (among other molecules) through regulation of collapsin response mediator protein 2 (CRMP2), APC, Tau, and microtubule-associated proteins (MAPs). These proteins, free from GSK3β inhibition, promote microtubule stability and subsequent axon formation (Figure 11) (Hur and Zhou, 2010).

Neuronal polarization also requires major reorganization of the growth cone cytoskeleton, a highly motile cellular compartment at the tips of growing axons that senses environmental clues including components of the ECM, axon guidance molecules and growth factors that have been shown to alter axonal growth behavior through changes in PI3K signaling. The axonal growth cone is composed of a central region filled with organelles and microtubules and a peripheral, highly dynamic, actin-rich region containing lamellipodia (sheet-like protrusions) and filopodia (hairlike protrusions). It has been proposed that a loose actin network at the tip of the future axon enables microtubules to selectively engorge into the axonal growth cone, forming the platform for subsequent axon elongation (Witte et al., 2008). Therefore, during axonal growth, the growth cone undergoes net elongation and retraction driven by the rapid polymerization and depolymerization of the actin filaments. Among many other molecules, Rho GTPases have been reported as important regulators of axon specification by activating specific effector molecules to change cell morphology or motility though modulation of actin dynamics (Arimura and Kaibuchi, 2007). Importantly, some Rho GTPases are controlled by PI3K dependent lipid signaling as explained in the previous section. The most extensively studied members of the Rho family of GTPases are RhoA (Ras homologous member A), Rac1 (ras related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42), and they have well established roles in regulation of the actin cytoskeleton (Figure 11) (Azzarelli et al., 2014; Hanna and El-Sibai, 2013). For example, it has been reported that activation of Cdc42 or Rac1 enhances neurite elongation through assembly of dynamic actin-based protrusions via PI3K activation, whereas RhoA activation has been associated with inhibition of neurite formation involving PTEN upstream and myosin II downstream (Gallo, 2006; Gallo, 2008; Polleux and Snider, 2010; Waite and Eickholt, 2010).

Finally, PI3K signaling has been revealed important to develop a dendritic branching pattern and the establishment of dendritic spines, essential structures for synapse development (Kumar et al., 2005).

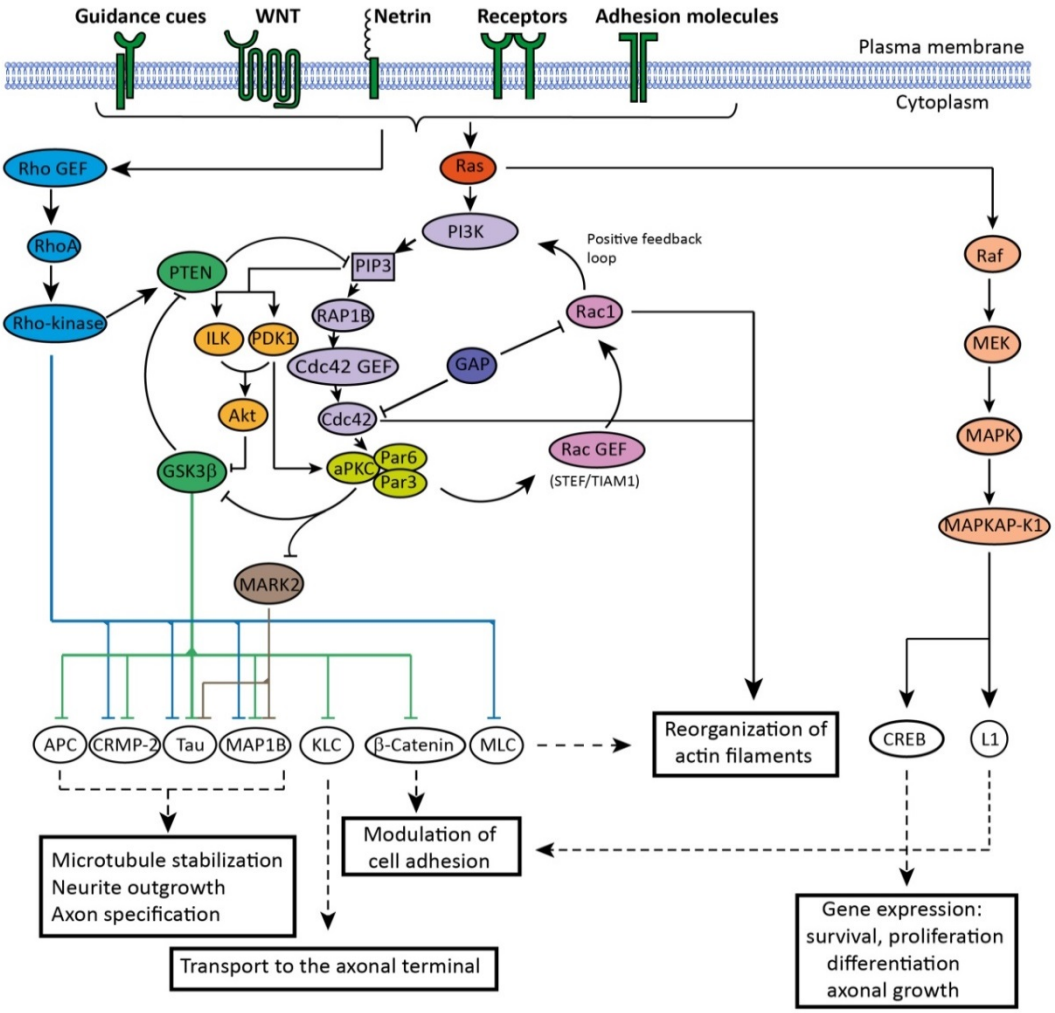


Figure 11. Pathways regulated by PI3K activation in neuronal morphology. In response to the activation of cell surface receptors by extracellular ligands or adhesion molecules, PI3K and/or Ras become activated. Subsequent production of PIP3 recruits and activates Ras-related protein 1b (RAP1B) and integrin-linked kinase (ILK), or phosphoinositide-dependent kinase 1 (PDK1). RAP1B activates cell division cycle 42 (Cdc42). Partitioning defect 6 (PAR6) specifically associates with the GTP-bound form of Cdc42 activating the PAR3–PAR6–aPKC (atypical protein kinase C) complex of polarity. PAR3 can then interact with, and activate, the Rac guanine nucleotide exchange factors (GEFs) T-lymphoma and metastasis 1 protein (TIAM1) and SIF and TIAM1-like exchange factor (STEF), and thereby activate Rac1. Active Rac1 and Cdc42 interact with effector molecules that regulate filamentous actin reorganization. Additionally, Rac1 can activate PI3K forming a positive feedback loop. Specific GTPase-activating proteins (GAPs) for Rac1 and Cdc42 will inactivate both GTPases respectively and break this feedback loop. Activated ILK or PDK1 phosphorylates Akt (also known as protein kinase B) which, in turn, phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β). Microtubule affinity-regulating kinase 2 (MARK2) is inhibited by aPKC. As GSK3β or MARK2 activity promotes microtubule destabilization, inactivation of either of these kinases has a positive effect on microtubule formation and neurite outgrowth, and also effect on directional transport and cell adhesion. Inactive GSK3β or RhoA can activate phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which decreases the levels of PIP3, inhibiting the signaling. Activation of Ras has mainly effects on gene expression through the cyclic AMP-response element binding protein (CREB). APC, adenomatosis polyposis coli; CRMP2, collapsin response mediator protein 2; KLC, kinesin light chain; MAP1B, microtubule-associated protein 1B; MAPKAP-K1, mitogen-activated protein kinase-activated protein kinase 1; MEK, mitogen-activated protein kinase kinase.

4. Class I PI3K in the nervous system in disease

So far, we have gone through the wide range of steps where the PI3K works as an instructive signal leading to the develoment of the CNS. That includes the mediation of extrinsic signals (growth factors, axon guidance cues, extracellular matrix components) and regulation of intrinsic effectors that originate a varied arrangement of morphological and expression changes mainly controlling survival, migration or neural morphogenesis. Therefore, it is not surprising that subtle aberrations of this pathway can lead to neurodevelopmental disorders.

4.1 PI3K in brain disorders

The importance of Class IA PI3K activity in the nervous system led to functional and genetic studies analyzing the link between dysregulation or mutations in specific catalytic subunits and distinct brain disorders (Table 1).

- **p110α**

The alteration of p110α was found associated with megalencephalies and hemimegalencephalies (MEG/HMEG; Lee et al., 2012), characterized by overgrowth of either one of the two cerebral hemispheres, and focal cortical dysplasia (FCD), which includes cortical dyslamination, cytomegaly and dysmorphic neurons (Jansen et al., 2015). These brain malformations lead to increased brain growth, developmental delay and seem to be the origin of the majority of intractable localization-related epilepsy in childhood (Iffland and Crino, 2016).

- **p110β**

The p110β catalytic subunit is the predominant subunit associated with GPCRs (Burke and Williams, 2015). Notably, p110β activity has been linked to activation of group I of metabotropic Gq-coupled glutamate receptors (mGluRs), a family of receptors involved in glutamate dependent excitatory signaling in the CNS and overactivated in human Fragile X syndrome mental retardation (FXS) (Gross et al., 2010; Valenti et al., 2002). FXS is the most common form of inherited intellectual disability and monogenic cause of autism. It is caused by loss of function of FMRP, an mRNA binding protein involved in the negative regulation of mRNA translation at synapses. Apparently, p110β protein levels and activity are increased in FXS patients and mouse models, contributing to neural dysfunctions and defects in cognitive function by affecting protein synthesis (Gross and Bassell, 2014).

- **110δ**

The catalytic subunit p110δ is essential for axonal outgrowth during development and in regenerating neurons. Increased expression and/or dysregulation of this isoform has been observed in patients with schizophrenia (Law et al., 2012). Schizophrenia is a severe neuropsychiatric disorder with a complex genetic etiology, but high risk for schizophrenia has been associated to neuregulin 1 (NRG1)-receptor tyrosine kinase ErbB4, a pathway very important in synaptic maturation and plasticity. It seems that aberrant p110δ signaling could be one of the downstream mediators of ErbB4 CYT-1, the isoform identified from genetic studies as a risk factor for this disease.

- **p110ɣ**

The Class IB catalytic subunit, p110ɣ, also seems to have some roles in the CNS, although its main studied functions take place in the immune system. P110ɣ function in the brain has been involved in N-methly D-aspartate (NMDA)-type glutamate receptors signaling and excitotoxicity when there is an excessive release of glutamate in stroke, trauma, and neurodegenerative disorders (Gross and Bassell, 2014; Kim et al., 2011). Additionally, it seems to be a genetic link between p110ɣ disfunction and autism, but the mechanisms remain to be elucidated.

	PI3K subunit *(gene symbol)*	Neuronal signaling pathway	Physiological function in the brain	Neurological disease	Transgenic mouse models	Antagonists
Class IA	**p110α** *(PIK3CA)*	*Insulin receptor*	*Insulin-dependent plasticity/LTD*	megalencephaly, hemimegalencephaly Riviere et al. (2012) Epilepsy *Alzheimer's disease*	→knockout (not viable) Bi et al. (2002) →transgenes with cancer mutations Koren and Bentires-Alj (2013)	INK1117[a] BYL719[a] A66
	p110β *(PIK3CB)*	mGlu1/5 S6, protein synthesis *Rac, Rab5*	protein synthesis Gross and Bassell (2012)	FXS Gross et al. (2010) Autism Cusco et al. (2009) *Alzheimer's disease*	→knockout (not viable) Bi et al. (2002) →conditional knockout (liver) Jia et al. (2008)	TGX-221[b] GSK2636771[a] AZD-6482[a] AZD8186[a]
	p110δ *(PIK3CD)*	Nrg1/ErbB4 RhoA	axon outgrowth and regeneration in sensory neurons Eickholt et al. (2007)	Schizophrenia Law et al. (2012)	→knockout Jou et al. (2002) →kinase-negative transgene[b] Okkenhaug et al. (2002)	CAL-101[a] IC87114[b] TGR 1202[a] AMG319[a] PIK-294
Class IB	**p110γ** *(PIK3CG)*	NMDA Rap1, p38 *PDE3B*	NMDA-LTD, behavioral flexibility Kim et al. (2011)	Autism Serajee et al. (2003) *Excitotoxicity/Brain ischemia/Epilepsy*	→knockout[b] Sasaki et al. (2000) →kinase-negative transgene Patrucco et al. (2004)	AS-605240[b] CZC24832

Table 1. Summary of the current knowledge about neuron-specific signaling and function of class I PI3K catalytic subunits including a list of available tools for their future study (transgenic mouse models and drugs). Adapted from Gross and Bassell, 2014.

4.2 Gestational diabetes

Another specific context where PI3K signaling can be altered and leading to neural abnormalities like those previously mentioned is uncontrolled gestational diabetes. Diabetes during pregnancy causes high blood-sugar levels in the mother that may go into the child bloodstream, affecting his development. It is a common complication that occurs in approximately 7% of all pregnancies. It is not dangerous per se when there is control of maternal glycemic levels. However, there are several risks for the intrauterine and postnatal growth in the case of uncontrolled diabetic mothers. High levels of glucose and insulin in the blood flow could lead to hyperactivation of the PI3K pathway in the fetus. In fact, there are several studies linking the effects of maternal glucose intolerance on the long term growth and development of the offspring, reporting correlation between levels of exposure to maternal hyperglycemia and neurobehavioural abnormalities, attention span and motor functions among other consequences (Krakowiak et al., 2012; Ornoy, 2005).

4.3 Cancer

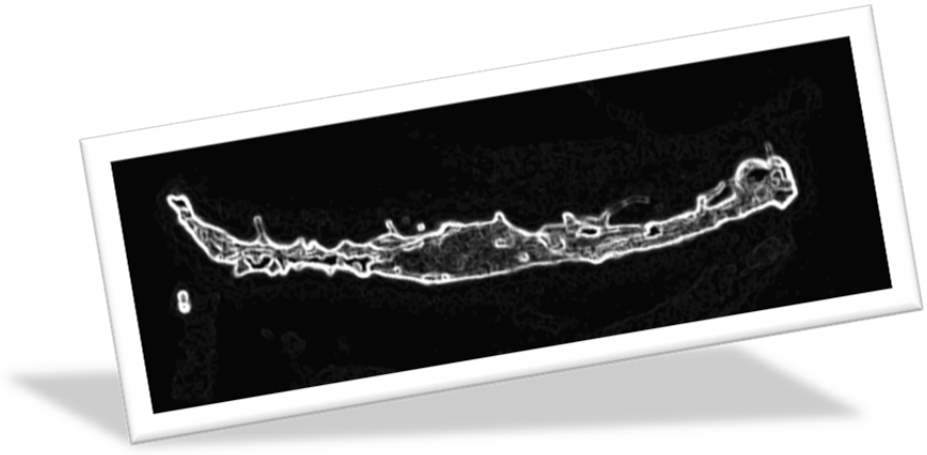
Regarding its role in cancer, activation of the PI3K signaling pathway is frequently found in common human cancers, brought about by oncogenic receptor tyrosine kinases (RTKs) acting upstream, PTEN loss, or activating mutations of PI3K itself (Yuan and Cantley, 2008). These mutations affect mainly the PIK3CA gene leading to constitutive activation of the p110α protein, and concentrate in two hotspots, the most common being E542K and E545K in the helical domain, and H1047R in the kinase domain. There are also somatic mutations affecting the regulatory subunits, although less frequent (Thorpe et al., 2015). In the CNS, mutations in this pathway are often present in the Glioblastoma Multiforme (GBM), the most malignant type of glioma and, in addition, the most abundant malignant cancer of the adult human brain. Unfortunately, survival rates of patients suffering GBM are approximately 15 months and the highly infiltrating growth of glioblastoma cells into the brain tissue complicates surgery or ionizing radiation. By analyzing gene members of several affected pathways, it was found that there are critical alterations mainly in three pathways: RB1 pathway (RB1, CDK4, and CDKN2A; altered in 68% of GBMs), TP53 pathway (TP53, MDM2, and MDM4; altered in 64%), and the PI3K/PTEN pathway (PIK3CA, PIK3R1, PTEN, and IRS1; altered in 50%) (Klinz et al., 2011; Parsons et al., 2008).

Concerning the other tumour types with alterations in the PI3K/PTEN pathway (endometrial, breast, ovarian, colorectal, bladder, lung, GBM…), it is worth noting that they are solid malignancies often originated from epithelial cells (Jokinen and Koivunen, 2015; Wymann and Marone, 2005). As we have seen, PI3K/PTEN pathway plays important roles in multiple cellular functions such as cell metabolism, proliferation, cell-cycle progression, survival or angiogenesis and promotes tumor

progression and metastasis when hyperactivated in a wide number of tissues. In this introduction, I have highlighted the role of PI3K in another process, cell polarity, which is required for the proper tissue integrity and influences all the previously mentioned cell functions. Evidences in epithelial cells also support the loss of cell polarity as a causal mechanism for tumor initiation or progression and it is also considered a hallmark for cancer (Ellenbroek et al., 2012; Lee and Vasioukhin, 2008).Therefore, the role of PI3K in polarity might also be crucial for the tumor progression in some of the mentioned cases. That is why it is really important to study the molecular pathways behind cell polarity.

The results from the present thesis might help to understand the mechanisms behind PI3K hyperactivation leading to disruptions in the tissue architecture of the neuroepithelium, which are on the base of several brain disorders and, possibly, contributing to carcinogenesis in polarized tissues.

OBJECTIVES



Objectives

The aim of this thesis is to decipher the specific functions of Class IA PI3Ks during embryonic development of the spinal cord in vertebrates expecting to better understand the specific signaling events affected by aberrant PI3K signaling in the nervous system in disease.

My main objectives are:

1. Studying the expression of different Class IA PI3K catalytic and regulatory isoforms in the developing chick spinal cord.

2. Determining the Class IA PI3K dependent survival of neuroepithelial cells and neurons.

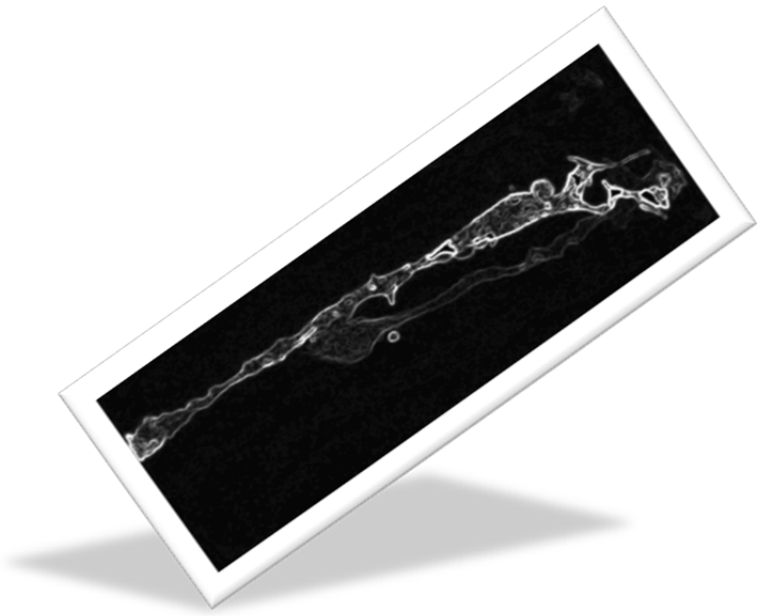
3. Deciphering the Class IA PI3K role in the establishment and maintenance of apico-basal polarity in neuroepithelial cells.

4. Studying the role of Class IA PI3K in regulating cell positioning and tissue structure of neuroepithelial cells and neurons.

5. Uncovering the role of Class IA PI3K during neural differentiation and axonal growth.

6. Understanding the consequences of Class IA PI3K hyperactivation during embryonic development of the neural tube.

MATERIALS AND METHODS



Materials and Methods

Chick embryo as model system

Chicken embryos have some advantages as model of study for developmental biology. Firstly, there is an easy access to the embryos along the whole development and, after the manipulation, they can keep growing. Secondly, there are several techniques to manipulate gene expression in the chick neural tube such as *in ovo* electroporation, retroviral infections or pharmacological inhibitors. And finally, it is possible to decide the number of embryos for each experiment.

Chick *in ovo* electroporation

In ovo electroporation can be used in gain-of-function experiments by overexpressing wild-type or active forms of genes and in loss-of-function experiments by transfecting dominant negative forms or interference RNAs.

Eggs from White-Leghorn chickens were incubated at 37.5ºC in an atmosphere of 45% humidity. Embryos were staged according to Hamburguer and Hamilton stages (HH) (Hamburger and Hamilton, 1992). Chick embryos were electroporated with Affinity purified DNA at 50ng-3ug/ul in H_2O with 50ng/ml Fast Green. Briefly, plasmid DNA was injected into the lumen of HH stage 10-14 neural tubes (although it is possible to inject in other stages), the electrodes were placed on either side of the neural tube and electroporation carried out using an Intracell Dual Pulse (TSS10) electroporator delivering five 50 millisecond square pulses of 20-25V. Since DNA migrates towards the positive pole, only the cells in one of the sides of the neural tube become transfected. Thus, one of the sides is wild-type while the other is a mosaic, containing both transfected and non-transfected cells.

Transfected embryos were allowed to develop to the specific stages indicated in each experiment, then dissected, fixed and processed for immunohistochemistry or in situ hybridization. Alternatively, after dissection they could also be lysed for protein extraction and quantification of luciferase levels or western blot.

Cell line cultures

NIH-3T3 mouse fibroblast cell line was cultured in DMEM (Thermo Fisher) formulated with 10% FBS (Foetal Bovine Serum) and 50 mg/L of Gentamicin in an atmosphere of 5% CO_2 and 37°C. To maintain the cell line, it was necessary to split the plate each 3 days. To sub-culture, we first poured off media from flask and replace it with sterile PBS to get rid of any FBS from the residual culture media. Next, we added enough trypsin EDTA to cover the cells at the bottom of the flask and we rolled it gently to ensure trypsin contact with all cells. The flask was placed back in the 37°C incubator for

a few minutes. As soon as cells detached (the flask may require a few gentle taps), we added some culture media to the flask (the FBS inactivates the trypsin) and pipetted the required volume of cells into new flasks.

Transient cell transfections were performed by electroporation with the Microporator MP-100 (Digital Bio, Seul, Korea), applying two pulses of 1000V and 20ms. Cells were kept in culture for 24hrs until reaching about 70-80% confluency.

For the Akt phosphorylation analysis, cells were transfected and kept in normal media for 24hrs. Four hours before being collected for western blot, we poured off the media, washed them with sterile PBS and added serum-free DMEM.

DNA constructs

We used different DNA constructs in order to modify the PI3K pathway in transfected cells.

Backbones

pCIG: Chicken β-actin promoter and CMV-IE enhancer with a multiple cloning site (MCS) upstream of an internal ribosomal entry site (IRES) and three nuclear localization sequences tagged to Enhanced Green Fluorescent Protein (EGFP) in pCAGGS expression vector. This construct allowed us to independently express our protein and EGFP and this way monitor the transfected cells.
pCIEGO: similar to pCAGGS.
pCDNA3.0: CMV promoter and without GFP.
pRK5: CMV promoter and without GFP.
pCMV6: CMV promoter and without GFP.
pSHIN: modification of pSUPER vector for expression of shRNAs and co-expressing EGFP.
px330-Cas9-SV40-EGFP: kindly donated by Eduard Batlle, for CRISPR/Cas9 expression and simultaneous EGFP reporter.
pCSCMV:tdTomato: CMV promoter expressing a red fluorescent protein (tdTomato).
pEGFP-N1: CMV promoter. Vector for fusing EGFP to the C-terminus of a partner protein.

Constructs

- Class IA PI3K: bovine p110α was subcloned from pBluescript (Hiles et al., 1992) into pCIG and pCIEGO (for experiments with PIP2 and PIP3 sensors) and p110β was obtained from Addgene (#34891, Peter Vogt) and inserted in pCIG. All regulatory isoforms (p85α, p55α, p50α and p55ɣ) were in pCAGGS (Inukai et al., 1997). Active mutants p110α H1047R, p85α W333R and p55α W63R were created by site-directed mutagenesis.

- PTEN: human PTEN fused to EGFP was kindly donated by Ignacio Torres-Alemán (Fernandez et al., 2014) and subcloned into pCIG vector with a C-terminal HA-tag (YPYDVPDYA).

- Akts: original mouse wild-type Akt1 cloned in pCMV6 was kindly donated by Thomas F. Franke and from it we obtained Akt S473F, Akt T308I and Akt K179M (kinase defective) by PCR and site-directed mutagenesis. A C-terminal HA-tag (YPYDVPDYA) was added to all Akt constructs by PCR and an N-terminal myristoylation motif (MGSSKSKPK) for membrane localization was inserted in Akt WT, Akt S473F, Akt T308I and Akt K179M.

- Rho GTPases: 5xmyc tagged Cdc42 WT, Cdc42 T17N (dominant negative) and Cdc42 G12V (active mutant) cloned in pCDNA3.0 were kindly donated by Bao-Liang Song (Xie et al., 2011). Myc-tagged dominant negative mutants inserted in pRK5 Rac1 T17N (#15904, Alan Hall) and RhoA T19N (#15901, Alan Hall) as well as active mutants Rac1 Q61L (#12983, Gary Bokoch) and RhoA Q63L (#12964, Gary Bokoch) were obtained from Addgene. Myc-tagged wild-type Rac1 and RhoA were obtained by site-directed mutagenesis and inserted in pRK5.

- Shp110α: to knock down p110α gene through RNA interference. 19bp target sequences were designed with *Oligoengine 2.0* and cloned into pSHIN vector. The two sequences used are:

shp110α1: CGATAAGCAGAAGTACACC
shp110α3: AGGGAGCATTGCAGTTCAA

- CRISPRp110α: to knock out p110α gene through DNA editing we designed three guide RNA sequences against the *Pik3ca* gene using the MIT website (crispr.mit.edu) and cloned them into the px330-Cas9-SV40-EGFP vector. The sequences are:

gRNA1	F: GACTCTAGAATGCCTCCGTG	R: CACGGAGGCATTCTAGAGTC
gRNA2	F: GACACTCCACAAGGATCCTCG	R: CGAGGATCCTTGTGGAGTGTC
gRNA3	F: GTCACCAGATGATGGTCGGGG	R: CCCCGACCATCATCTGGTGAC

- PIP2&PIP3 sensors: PH-Akt and PHD inserted in pEGFP-N1 were kindly donated by F. Martín-Belmonte (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007).

Immunostaining

Embryos were fixed during 4hrs or overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. For chick embryo immunohistochemistry, embryos were rinsed, sunk in blocks made of 5% agarose/10% sucrose dissolved in PBS and sectioned in vibratome (VT 1000S, Leica) to obtain sections of 40-70um thickness. Immunostaining of free-floating sections were perfomed following standard procedures: a first step of permeabilization with PBT (Triton 0.1% or 1%) for 10min followed by a second step of blocking with PBS-BSA (PBS 1X, 0.1% TX-100, 0.05% Azide, 0.5% BSA) for 15-30min. Afterwards, sections were incubated in the primary antibody diluted in PBS-BSA overnight at 4°C, washed three times for 10min with PBS-BSA and incubated in secondary antibody in the same buffer for 2hrs at RT in the dark. Afterwards, if Rhodamine Phalloidin or Hoescht staining were required, the sections were washed once from the secondary antibody and incubated with the mentioned dye for 15min at RT in the dark. Sections were washed three times in PBS-BSA for 10min before mounting on a glass slice using mowiol and cover it with a thin glass coverslip. For immunocytochemistry of cultured cells, cells were fixed for 15 min with 4% PFA and permeabilization was carried out using Methanol (100%) for 10min, but the rest of the protocol was similar to the one just described.

Microscopy images were obtained using confocal microscopes SPE, SP2, SP5 (Laser-spectral confocal microscope Leica) or Spectral Confocal Microscope Zeiss LSM780 and, later on, processed using Adobe Photoshop CS5 or ImageJ/Fiji software.

Antibodies and reagents:

ANTIGEN	SUPPLIER	REFERENCE	SPECIES
Bromodeoxyuridine	DSHB	G3G4	Mouse
β-Tubulin III/Tuj1	Covance	MMS435P	Mouse
HuC/D	Molecular Probes	A21271	Mouse
GAPDH	Enzo	ADI-CSA-335-E	Mouse
ZO-1	Invitrogen	339100	Mouse
aPKCζ (H-1)	Santa Cruz	sc-1778	Mouse
Lhx3	DSHB	67.4-E12	Mouse
HNK-1	Sigma	C6680	Mouse
Myc	Home made		Mouse
Islet1	DSHB	40.2D6	Mouse
Lhx1/5	DSHB	4F2	Mouse
Flag-M2	Scientif Imaging SysteMouse	discontinued	Mouse
Ncadherin	Invitrogen	13-2100	Rat
Olig2	Millipore	AB9610	Rabbit
Laminin	Sigma	L9393	Rabbit
Islet2	McMahon		Rabbit
Sox2	Invitrogen	48-1400	Rabbit

p110α	Santa Cruz	sc-196	Rabbit
Cleaved caspase3	Millipore	AB3623	Rabbit
HA	Zymed	71-5500	Rabbit
Akt	Cell Signaling	9272	Rabbit
P-Akt (Thr308)	Cell Signaling	13038	Rabbit
RFP	Home made		Rabbit
GFP	Home made		Rabbit
p85-NSH2	Home made		Rabbit
Flag	Home made		Rabbit
Rhodamine-Phalloidin	Invitrogen	R415	
Hoescht	Molecular Probes		

Alexa Fluorescent conjugated secondary antibodies were obtained from Life Technologies.

BrdU incorporation assay

To perform proliferation assays in chicken embryos, they were given a 4h pulse of BrdU (0.5ug/ul) and then fixated for 4hrs in 4% PFA in PBS. Embryos were sectioned in vibratome as previously explained (immunostaining) and the free-floating sections were permeabilized in PBT (1%) for 10min and then in Methanol (100%) for 10min, washed twice in PBT (0.1%) for 5min and incubated in HCl 2N for 30min. Afterwards, we stopped the treatment incubating with NaBorate (0.1M, pH8.5) twice for 5min and then sections were washed again in PBT (0.1%) for 5min before starting the standard immunostaining procedure using anti-BrdU antibody (Molecular probes-Invitrogen, mouse, *A21301MP*).

In situ hybridization

Embryos were fixed 4hrs or overnight at 4ºC in 4% PFA in PBS, rinsed and processed for whole mount RNA in situ hybridization following standard procedures using probes for chicken PI3K isoforms p110α, p110β, p110δ, pan85α (recognizes the three isoforms from the gen *Pik3r1*: p85α, p55α, p50α), p85α, p55α, p50α, p85β and p55ɣ. They were designed from mRNA differential sequences for each gene obtained from the *Gallus gallus* genome (NCBI) and they were around 250bp. Hybridization was revealed by alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim). Hybridized embryos were rinsed in PBT (1%), post-fixed in 4% PFA, vibratome sectioned and photographed on a Leica DMR Microscope.

p110α

CACCTGAGCTACCCAAACACATATACAATAAACTAGATAAAGGGCAAATAATAGTGGTGATATGGGTAAT
AGTTTCACCTAACAACGATAAGCAGAAGTACACCTTAAAAATCAATCATGACTGTGTGCCTGAGCAAGTT
ATTGCTGAAGCAATTAGGAAGAAAACACGAAGTATGTTGCTGTCATCTGAACAACTGAAGCTTTGCGTGT
TGGAGTACCAGGGCAAGTATATTCTGAAAGTGTGTGGCTGTG

p110β

CACACTTACCCACCAGAACAGGAACCCGTTGTCCCAGAGAACTTCCAAGATAAGCTTTATAGTGGAAATC
TTGTAGTGGCTATTCATTTTGATAACTGCCAGGATGTATTTAGTTTTCAAGTGTCTCCTAACATGAATCC
CATCAAGTTGAATGAACTGGCAATCCGAAAACGTTTGACTATCCATGGAAAAGAAGATGAGGAAATTGAT
CCTGCTGACTATGTGCTGCAAGTTAGTGGGAGGCTAGAATATGT

p110γ

GCCTTGGAACTGGACTCTTGCACACACCCATCAAGAACATTTTTGTCAACGTCAAGTTTCAGTCTGGCGG
GGAGAGCTTCACTTTCCAGATCTCCCCAAATGAATTCCCTATCACATTAATGAGCTATGCTGTCAAGAAG
CAGGCTACTGTCTTCCGCCATGAGACAATGGAGAACCCAGAGGACTACACACTGCAGGTGAATGGGAAGT
ACGAATATCTCTATGGGAACTACCCCCTGTACCAGTTCCAGTACATTCGC

Pan85α

TTGAAGAGCAGTGCCAGACACAAGAGAGATACAGTAAGGAATACATTGAAAAATTTAAACGAGAAGGAAA
TGAAAAAGAAATACAAAGAATTATGCACAACTATGAAAAACTGAAGTCTCGAATAAGTGAAATTGTGGAC
AGCAGGCGTAGATTAGAAGAGGACTTAAAGAAGCAAGCAGCTGAATATAGAGAGATAGACAAGCGAATGA
ACAGCATTAAGCCAGATCTCATACAGCTGAGGAAGACAAGAGACC

p85α

CATTGACTTGCACTTAGGAGATATATTAACTGTGAATAAAGGTACCTTACTAGCACTTGGATTCAACGAA
GGGGAAGAAGCAAAGCCTGAGGAAATTGGTTGGTTAAACGGCTTTAATGAAACCACAGGGGAGAGGGGGG
ATTTTCCAGGAACTTACGTAGAATACATAGGAAGAAAAAAAATATCCCCCCCAACCCCGAAGCCTCGTCC
TCCTCGGCCCCTTCCTGTAGCACCAAGTCCGTCAAAAGCTG

p55α

ATGATGAAACAGGAGTGGTCTGGTGACATTATTTTGACATGATTGGCTGAGGATTATGATGTAATAGGTT
ACAGTGCAGCCCCTTATAGGTTTTAAAATGAATTCCAAGACACCATTACAAAGAGAGCCTGACTCTTTCC
TTGTATCTGAGCTTACTCAGTGAAACTCATACAAATGTACAATACTGTTTGGAATATGGAAGAACTGGAT
ATAGAATATCCTAAGACAGATATAAACTGTGGCACAGACTTG

p50α

TGTAAGCAGTTGAGAAGAGGCAAAAGGCTGGGATTTTCTCATTGCACATTGCAGAGATTTTGGTGGAACA
GTGGTTGGAAAGCAAAGTCTTCTTTTTCAGAATTATCTTTTACTTCAGAGAATTTTCCTCAGAGAAGTTC
TATGTTTCGCCTTTTTTTTTTAAGATTTTTATGAGAGGAAGCCAATTTTTCAGGTTGTTGGATTACCTTT
TCAGGCTGTATTACAAGGGCAAGCATCTG

p85β

ATCTCTGTCCCTTCTCACCGGCCCCGAGTGCAGAGACCCCTGCCTGCAACACCAGGAGCCAGTGGCTGCC
GACTGCAGGAGGCTCTTGAGCAAGGGTCTCCTCTCCCTGACCTGCAGGAGCAGTTCAGCCCTCCAGAGGT
TGCGCCTCCGCTGCTGGTGAAGCTGGTGGAAGCCATCGAGAAGAAAGGGTTAGACAGCGAGATGTTATAC
AGGACATCTGGCTCCGAACTGAGGCAAGCTCTAAGAACCGATTGGAGCCT

p55γ

ACTGGAGGGAGGTGATGATGCCCTATTCCACTGAGCTCATCTTTTACATTGAGATGGACCCTCCAGCTCT
TCCTCCAAAGCCCTCCAAACCAATGACTCCAGTGAACACAAATGGAATAAAGGACAATTCCAGCTTCTCT
CTGCAGGAAGCAGAATGGTACTGGGGGGACATCTCAAGGGAAGAAGTAAATGACAAATTACGAGACATGC
CAGATGGAACATTCCTGGTGCGTGATGCATCGACCAAGATGCAGGGAGACTAC

Western Blot

Electroporated cells were washed once in PBS before harvesting in 1X SDS Laemmli Buffer (50mM TrisHCl, 10% Glicerol, 2% SDS, 100mM DTT or 5% β-mercaptoethanol, pH 6.8) and sonicating. Cell lysates were heated at 100°C for 5min and spin out before loading them into 10% SDS-PAGE gels, followed by transference to nitrocellulose membranes. Membranes were blocked in 1X TTBS (150 mM NaCl, 0.05% Tween-20 and 20 mM Tris-HCl [pH7.4]) with 5% w/v nonfat dry milk for 1hr and incubated in primary

antibodies with gentle agitation overnight at 4°C. They were washed 3 times in TTBS before adding Alexa-labeled secondary antibodies for 2hrs at room temperature. After washing 3 times in TTBS, protein detection was carried out using an Odyssey Infrared Imaging System from LI-COR to detect fluorescence. The molecular weight was calculated using Bio-Rad Precision Molecular Weight Markers.

Luciferase reporter assay

The reporter constructs NeuroD-Luciferasa (Huang et al., 2000) and beta-III-Tubulin-Luciferase (βtub3 enhancer/promoter from Bergsland et al., 2011) was cloned into pGL3 backbone) were co-electroporated with the renilla-luciferase reporter construct carrying the CMV immediate early enhancer promoter (Promega) for normalization and 4 to 6 times the experimental conditions (transfected DNA). Embryos (n=10) were dissected after 24, 40 or 48hpe and GFP+ neural tubes were homogenized with a syringe in Passive Lysis Buffer and centrifuged 2min at 13000rpm before the reading. Luciferase and renilla activities were measured using the Dual-Luciferase Reporter Assay System (Promega), the Sirius Luminometer (Berthold) and the FB12 Sirius Software.

RT-qPCR

Neural tubes were electroporated on either sides with shRNA or the control vector (pSHIN) at HH12 and GFP+ tubes were dissected 24hrs later. RNA was extracted and purified using the High Pure RNA Isolation Kit (Roche) and the total RNA was analyzed using NanoDrop (NanoDrop ND-1000 Spectophotometer, ThermoScientific). Afterwards, similar amounts of RNA were retro-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the qPCR was performed in a LightCycler480 System (Roche) using SYBR Green (Bio-Rad). Oligonucleotides specific for chick glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for normalization.

Sequence of the primers:

PIK3CA (1)	F: ttggtgactgtgtgggactc	R: cgacacagtagccagcacaa
PIK3CA (2)	F: acttgcgtcaagacatgctg	R: ctgcaatgctccctttaagc
GAPDH	F: cctctctggcaaagtccaag	R: catctgcccatttgatgttg

Expression quantification with Image J

The expression levels of beta-III tubulin were quantified in confocal pictures of HH-12 chicken neural tube slices electroporated with different constructs and stained with antibodies against beta-III tubulin (Tuj1) and F-actin (Phalloidin). The area of the neuroepithelium was manually delimitated in both non electroporated (NE) and

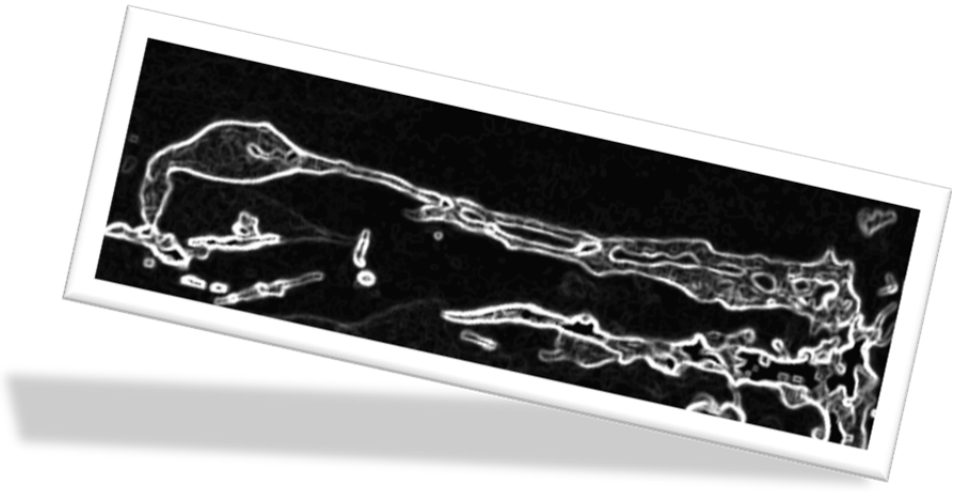
electroporated (E) sides of the neural tube. The mean pixel intensity value of the encircled areas was calculated for beta-III-Tubulin and F-actin staining. For each picture, the ratio between the values obtained in NE and E sides was calculated. Finally the beta-III-Tubulin ratios were divided by the corresponding F-actin ratios.

$$\text{Actin pondered beta-III tubulin mean pixel intensity} = Tuj1\ \left(\frac{E}{NE}\right) / Phall\ \left(\frac{E}{NE}\right)$$

Statistical analysis

For the statistical analysis it was used the GraphPad Prism software (version 4.0). Quantitative data are presented as mean ± SD from at least three independent experiments. One-way ANOVA followed by the Tukey's test or Student's t-test were used to determine significance using 95% of confidence interval.

RESULTS



1. Class IA PI3K subunits are expressed in the chick neural tube

1.1 Class IA PI3K subunits are differentially expressed along time

Since the function for Class IA PI3Ks in the developing spinal cord can be postulated based on expression patterns, it was essential to study the expression of the catalytic subunits (p110α, p110β and p110δ) and the regulatory subunits (p85α, p55α, p50α, p55ɣ and p85β). Therefore, we designed in situ hybridization (ISH) probes marked with digoxigenin (DIG) based on predicted mRNA sequences from the *Gallus gallus* genome to analyze whether these isoforms were expressed at two different embryonic stages in the chicken neural tube and their specific location. The chosen stages corresponded to 50-56 hr post fertilization (hpf; Hamburger-Hamilton [HH] stage 14-16), when most of mitosis are self-expanding proliferative divisions; and 96 hpf (HH stage 22), when neurogenesis has already began (Hamburger and Hamilton, 1992) (Figure 12A-B).

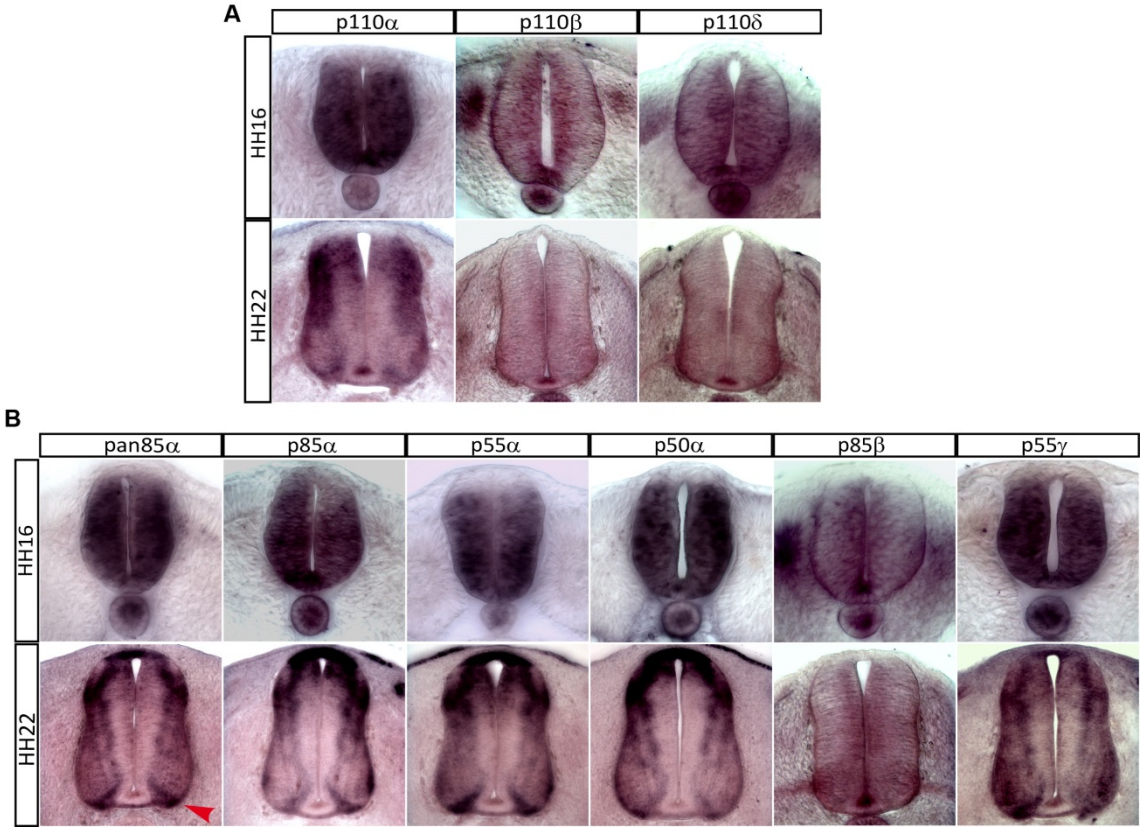


Figure 12. Class IA PI3K isoforms are differentially expressed at two different stages of the chick embryonic spinal cord. (A, B) These images show transverse sections at caudal regions of neural tubes from chicken embryos stained by in situ hybridization against the indicated genes at HH stages 14-16 (50-56hrs) and 22 (96hrs)**.(A)** Expression pattern of the Class IA PI3K catalytic isoforms p110α, p110β and p110δ. **(B)** Expression pattern of the Class IA PI3K regulatory isoforms panp85α (recognizes the three splice variants expressed by the *Pik3r1* gene), p85α, p55α, p50α, p55ɣ and p85β. The red arrowhead in the HH22 pan85α condition points to the V3 interneuron domain.

Specific and strong signal was detected for all isoforms except for p110β, p110δ and p85β, which showed weak expression in the ventricular zone (VZ) at HH14-16 and almost no expression at the later stage. Instead, p110α, p85α, p55α, p50α and p55ɣ were highly expressed at HH14-16 in the whole neural tube excluding the most dorsal part, which corresponds to the Roof Plate (RP). This pattern seemed to change with the onset of neurogenesis, restricting their expression at HH22 to the intermediate layer (IL) where post-mitotic cells can be identified, to the mantle zone (MZ) where neural differentiation occurs and to the RP while excluding their expression from the VZ occupied by neural precursors. It is worth mentioning the more intense expression of the regulatory subunits at HH stage 22 spatially localized in the V3 interneuron domain, a major group of excitatory commissural interneurons right below the motor neuron domain and essential for producing a stable and robust locomotor rhythm (Borowska et al., 2013; Zhuang and Sockanathan, 2006).

1.2 Class IA PI3K is stabilized in differentiated neurons after the onset of neurogenesis

In order to confirm the expression of Class IA PI3Ks at the protein level and due to the lack of antibodies against the Class IA PI3K subunits for chicken, we designed an experiment to indirectly detect endogenous expression of regulatory isoforms based on the fact that free p110α subunit is rapidly degraded unless it is bound to a regulatory subunit. There are several reports supporting that there are no free monomers of catalytic or regulatory subunits, but they only exist as heterodimers where the regulatory subunit acts stabilizing the overall conformation of the p110 through binding to its N-terminal domain (Geering et al., 2007; Yu et al., 1998). Performing chick embryo *in ovo* electroration at HH stage 12, we transiently expressed low concentrations of a bovine p110α alone at two different HH stages (10 and 14) and analyzed bovine p110α expression at 24, 40 and 48hrs post-electroporation (hpe) assuming that the overexpressed p110α would avoid degradation only in the presence of endogenous regulatory isoforms (Figure 13A, C). It is worth noting that the first stage analyzed in this experiment corresponds to the earliest stage shown in the ISH (HH14-16) and the HH stage 14 analyzed 48hrs later to HH22. Our results showed a differential stabilization of overexpressed p110α dependent on the developmental stage and the cell type, validating our method (Figure 13C). As control, we performed the same experiment in parallel co-electroporating bovine p110α with a C-terminal Flag-tagged p85α expecting to have similar p110α expression at all stages as it resulted (Figure 13C'). Besides, immunostaining against Flag revealed a perfect co-localization between the two subunits when co-expressed as expected (Figure 13B).

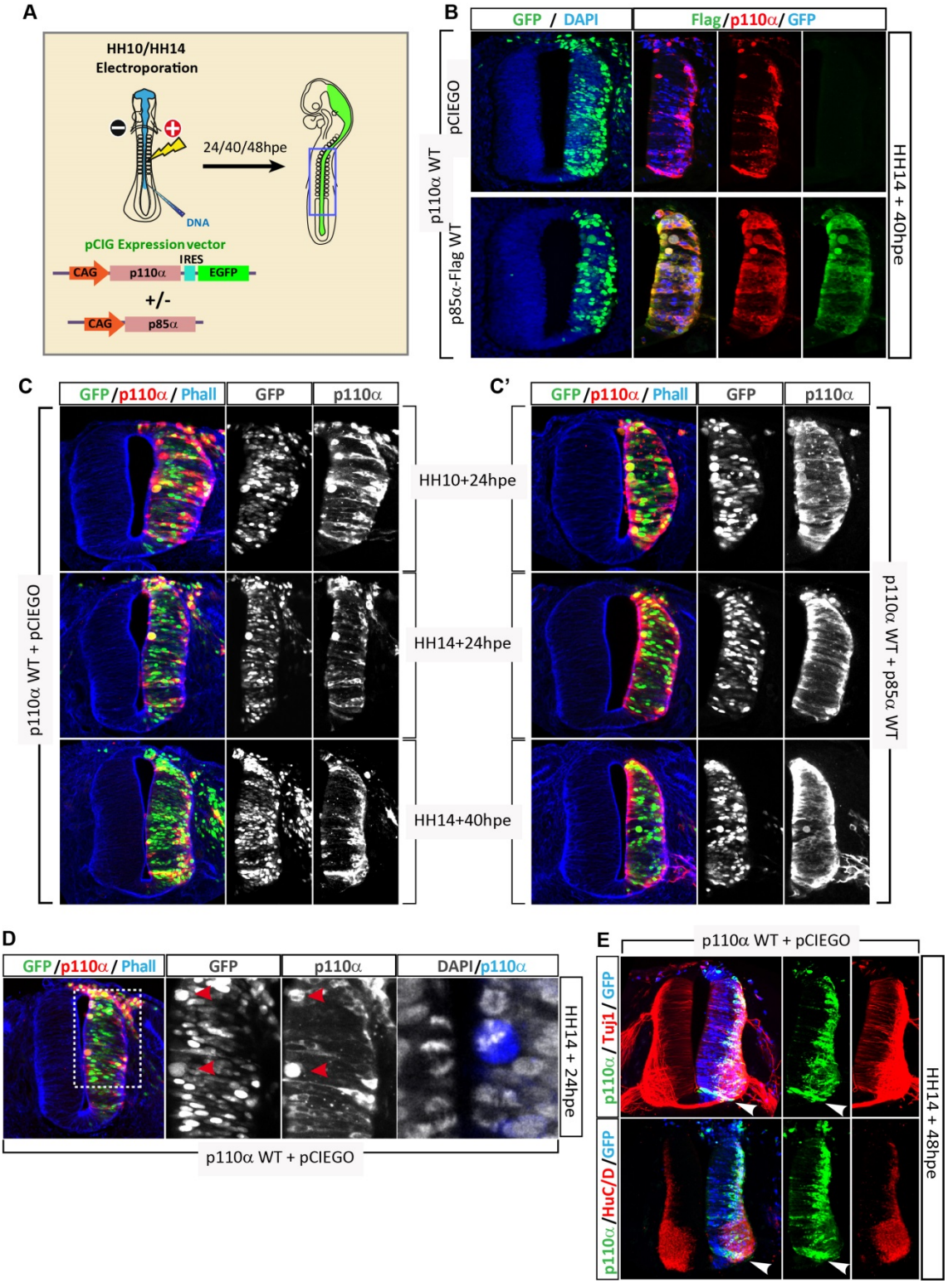


Figure 13. Overexpressed p110α stabilizes only in cells expressing endogenous regulatory class IA PI3K.

(A) HH-10 and 14 chicken neural tubes were electroporated with a wild-type form of bovine p110α in the presence and absence of Flag-tagged p85α at the level of spinal cord and analysed 24, 40 and 48hpe. **(B)** Neural tubes electroporated at HH14 and analysed 40h later were stained with antibodies against GFP (green, transfection) and DAPI (blue) at the left of the panel and against Flag (green) , p110α (red) and GFP (blue, transfection) at the right of the panel. **(C)** Transverse sections were stained with

antibodies against GFP (green transfection), p110α (red) and phalloidin (blue, labels F-actin). Between the two panels, it is indicated the HH stage of transfection and the hours post-electroporation. **(D)** Neural tubes electroporated at HH14 and analysed 24h later were stained with antibodies against GFP (green transfection), p110α (red) and phalloidin (blue). The middle panels show amplifications of the electroporated area and arrowheads point to two electroporated cells in the VZ stabilizing high levels of p110α. One enlarged image at the right of the panel shows staining with DAPI (grey) and p110α (blue) of the lowest pointed cell undergoing mitosis. **(E)** Neural tubes electroporated at HH14 and analysed 48h later were stained with antibodies against p110α (green), GFP (blue, transfection) and two neuronal markers in red: on the first panel, it was used the early neuronal marker Tuj1 (beta-III tubulin) and on the second panel, the later neuronal marker HuC/D. The white arrowheads point to the V3 interneuron domain.

When analyzing this experiment more in detail, p110α revealed high expression in most of the transfected cells of the earliest stage (essentially progenitors) while, at later stages, p110α expression appeared restricted to some progenitors undergoing M phase (Figure 13D) and differentiated neurons (Figure 13E). Interestingly, the early neuronal marker beta-III tubulin (Tuj1) was highly coincident with cells stabilizing p110α. Another compelling observation concerning the figure 2E is the high stabilization of p110α in where it should be the V3 interneuron domain, coincidental with the more intense mRNA expression of the regulatory subunits shown in the previous figure.

These experiments confirm the expression pattern of class IA PI3K subunits previously obtained by ISH, they reveal an accurate regulation of PI3Kα protein expression along time in the neural tube and suggest that PI3K might be performing different functions at different developmental stages.

2. PI3Kα is necessary for the survival of neuroepithelial cells and neurons in the embryonic spinal cord

During central nervous system development, balance between neuronal survival and apoptosis plays an important role in the regulation of the final numbers of each cell type (Raff et al., 1993). The election between the two possible outcomes depends on the reception of the right amount of growth factors, such as insulin or insulin-like growth factor-1 (IGF-1), and neurothrophic factors like BDNF, NGF or Neurothrofins, which would suppress an intrinsic suicide program while stimulating the neurons to survive. These factors activate several intracellular pathways, including the PI3K pathway, which has been reported to be necessary and sufficient for survival in several neuronal types mainly through activation of the serine/threonine kinase Akt (Brunet et al., 2001; Hetman et al., 1999; Rodgers and Theibert, 2002). Moreover, Class IA PI3K catalytic subunits have unique roles in mediating distinct forms of neuronal functions such as protein synthesis, axonal outgrowth or synapse development, so specific p110 dysregulation could lead to neuronal dysfunctions and, ultimately, to apoptosis (Gross and Bassell, 2014).

2.1 Lack of PI3Kα induces apoptosis in the neural tube

Therefore, in the first place we wanted to investigate whether p110α was needed for neural survival in this specific model. For that, we cloned in pSHIN (a vector specialized in strong expression of short hairpins [shRNA]) two shRNAs targeting specifically the chick p110α isoform (Figure 14A). Firstly, we checked the effect of both shp110α combined through electroporation of HH stage 12 neural tubes and posterior analysis 24hrs later of p110α mRNA levels by RT-qPCR comparing with the empty vector. We obtained a consistent reduction between 15-20% of p110α mRNA levels after shRNA electroporation (Figure 14B). To confirm the result, we repeated the experiment with a different set of primers with similar outcome (data not shown). It is worth noting that these experiments were performed using manually dissected neural tubes, where the electroporation efficiency never reaches the 100% of the population and hence the real expression drop is diluted due to the presence of non-electroporated cells.

After validating our tool, we performed knock-down experiments by electroporating the shp110α in HH stage 12 embryos and analyzing the levels of apoptosis 24, 48, 72 and 98hrs later through staining against cleaved Caspase3. Quantification of electroporated cells expressing active Caspase3 at 24hpe revealed a 3 times increase of apoptosis when compared with embryos electroporated with the empty vector (Figure 14C,D). At later times of exposure, the electroporated side of the tube looked smaller and there were many cells undergoing apoptosis. Interestingly, not only

neuroepithelial (NEP) cells seemed very affected, but also neurons as seen in embryos after 72 and 98hrs where apoptotic cells localized in the mantle zone (Figure 14E).

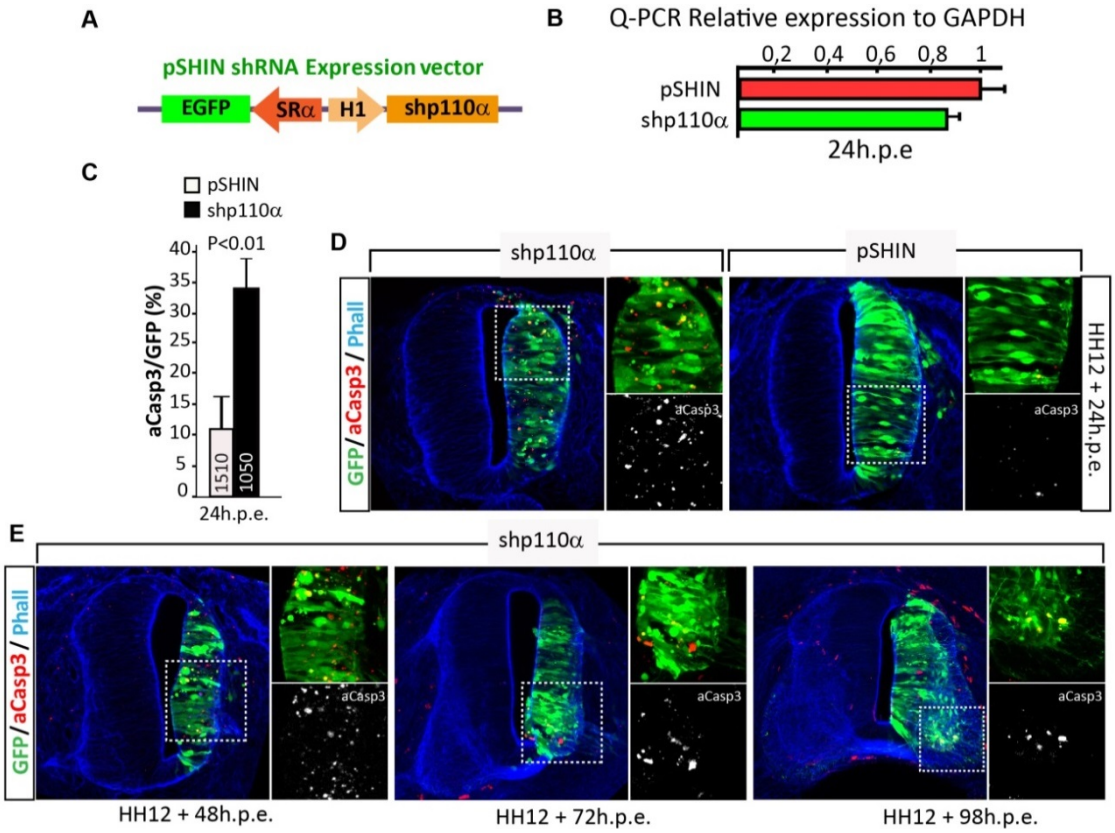


Figure 14. p110α PI3K is needed for survival of progenitors and neurons of the chick neural tube.
(A) Scheme of the DNA construct used in the figure for knocking-down p110α expression coupled to an EGFP reporter of transfection. **(B)** The transcriptional strength of the shp110α was assessed by Q-PCR in electroporated HH12 chicken embryos after 24hrs. **(C)** Cell quantification of chicken neural tubes 24h after transfection with the control (pSHIN) or shp110α. The percentage of aCasp3+ transfected cells was evaluated comparing the electroporated sides of the control and the p110α knock-down of at least 4 embryos. The total number of GFP+ cells counted at each data point is shown within each bar. Significant differences were tested by Student's t-Test. **(D)** Representative images of the experiment (C) that were stained against GFP (green, transfection), active Caspase3 (red, marker of apoptosis) and phalloidin (blue). The right panel shows enlarged images of the area within the white box. **(E)** Representative images of chicken neural tubes 48, 72 and 98hrs after transfection at HH stage 12 with the control vector (pSHIN) or shp110α that were stained against GFP (green, transfection), active Caspase3 (red, marker of apoptosis) and phalloidin (blue). The right panel shows enlarged images of the area within the white box.

2.2 After the onset of neurogenesis, neurons become more dependent on PI3Kα activity than progenitors

Afterwards, since we had previously seen that PI3Kα expression changed along development and concentrated in neurons at later stages, we assessed if both

progenitors and neurons were differentially affected by the reduction of p110α after 40 and 72hrs of p110α suppression. We counted the number of progenitors (Sox2) and neurons (HuC/D) of the electroporated side and compared them with the control side (Figure 15A, B). Notably, in both time points there was a similar result with 30 to 40% of reduction in progenitors and around 50% of reduction in neurons, revealing that both cycling and differentiated populations were affected, but the neuronal population was importantly more affected. These results are consistent with previous reports, confirming the importance of PI3Kα for progenitor and neural survival also in the chick embryonic spinal cord and unveiling a higher dependency on p110α of neurons compared to progenitors at later stages, which might be related to its expression pattern at later stages.

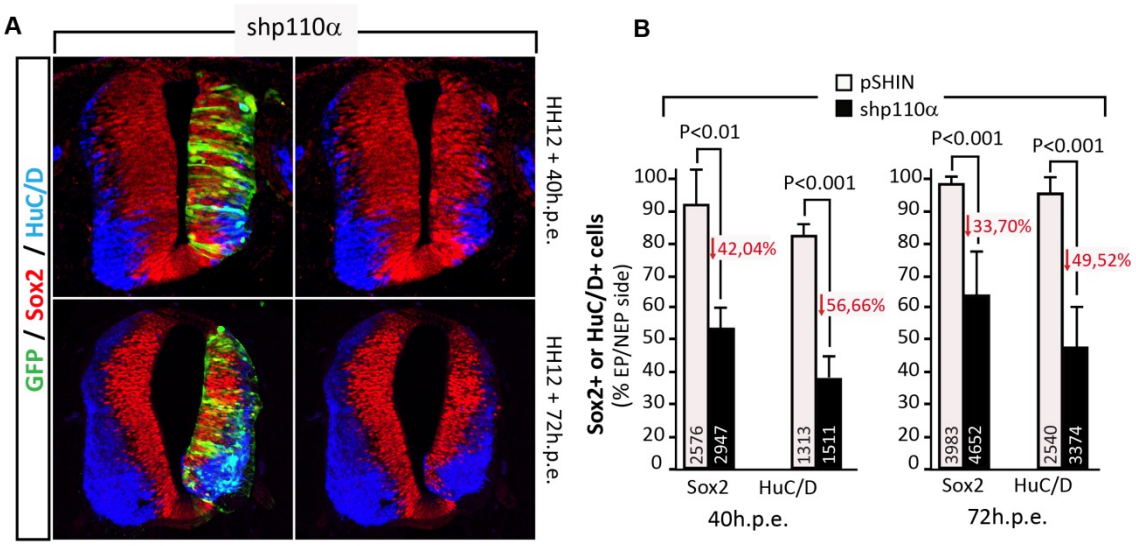


Figure 15. p110α PI3K is needed for neural survival at later stages. (A) Representative images of chicken neural tubes 40 and 72hrs after transfection at HH stage 12 with shp110α that were stained against GFP (green, transfection), Sox2 (red, progenitors) and HuC/D (blue, neurons). **(B)** Cell quantification of experiment (A) to analyze the proportion of remaining progenitors and neurons compared the non-electroporated side. The number of Sox2 and HuC/D positive cells in the electroporated side (EP) was compared in each case to the non-electroporated side (NEP) in at least 4 embryos. The total number of cells counted in the control side for each data point is shown within each bar. Significant differences were tested by Student's t-Test. The differences for each population between transfection with the empty vector and the shp110α are indicated in red.

3. PI3Kα misregulation alters cell localization within the neuroepithelium

As it was mentioned in the introduction, the neural tube is characterized by an apico-basal (AB) polarity. Neuroepithelial (NEP) cells extend from the luminal (apical) surface to the extracellular matrix (or basement membrane) and their membranes contain different apical, lateral and basal protein complexes as well as different lipid domains. PI3K pathway has been reported as an important player in AB cell polarization and neuronal morphology (Comer and Parent, 2007; Menager et al., 2004), hence we decided to study whether PI3Kα was participating in the establishment and/or maintenance of AB polarity in the chick NEP cells.

3.1 Constitutive PI3Kα activation by oncogenic mutations on the regulatory and catalytic subunit

To assess AB polarity, we performed gain and loss-of-function experiments and analyzed distribution of polarity markers. In order to transiently increase PI3Kα activity in the neural tube, we generated constructs with two types of mutation: (a) a hotspot active mutation affecting the kinase domain of p110α and highly mutated in a broad range of tumor types (Pal and Mandal, 2012; Samuels and Velculescu, 2004); (b) a mutation designed by us in the first amino acid of the N-terminal (nSH2) domain of the p85α (W333R) or p55α (W63R, same mutation, but in a shorter isoform). It has been reported that the nSH2 domain of p85α mediates inhibition of p110α through its interaction with the helical domain of p110α (Yu et al., 1998) and cancer-derived mutations affecting the iSH2 and nSH2 domains showed gain of function (Jaiswal et al., 2009; Sun et al., 2010). To date, W333R has only been detected as somatic mutation in two samples from colorectal adenocarcinoma (COSMIC: http://cancer.sanger.ac.uk/cosmic). This regulatory mutant would also allow us to perform further gain of function studies on p110β.

To verify the activating effect of these mutants, we performed *in vitro* transfections in the NIH3T3 mouse embryo fibroblast cell line, which is responsive to insulin stimulation through the PI3K pathway. Firstly, Flag-tagged p85αW333R and p55αW63R were co-transfected with p110α and low concentrations of Akt1 in serum-deprived conditions to use Akt1 phosphorylation as a downstream read-out of PI3K activation in absence of external stimuli. Flag-tagged wild-type p85α, p55α, p50α and p55ɣ regulatory isoforms were also co-transfected as negative controls while an N-terminal myristoylated form of p110α targeted to membrane was used as positive control (Zhao et al., 2005) (Figure 16A). As it was expected, the W333R and W63R active mutants induced constitutive and growth-factor independent activation of Akt signaling as well

as myr-p110α (Figure 16B). None of the other conditions induced phosphorylation of Akt in absence of serum, but all isoforms had similar expression levels, except for the less expressed p55ɣ. Secondly, we confirmed the enhanced activity of oncogenic p110αH1047R by performing a similar experiment. As positive controls, we chose p85αW333R with wild-type p110α and a dominant active Akt form (myr-Akt-HA) with PIP3-independent enhanced localization at the plasma membrane (Aoki et al., 1998) (Figure 16C).

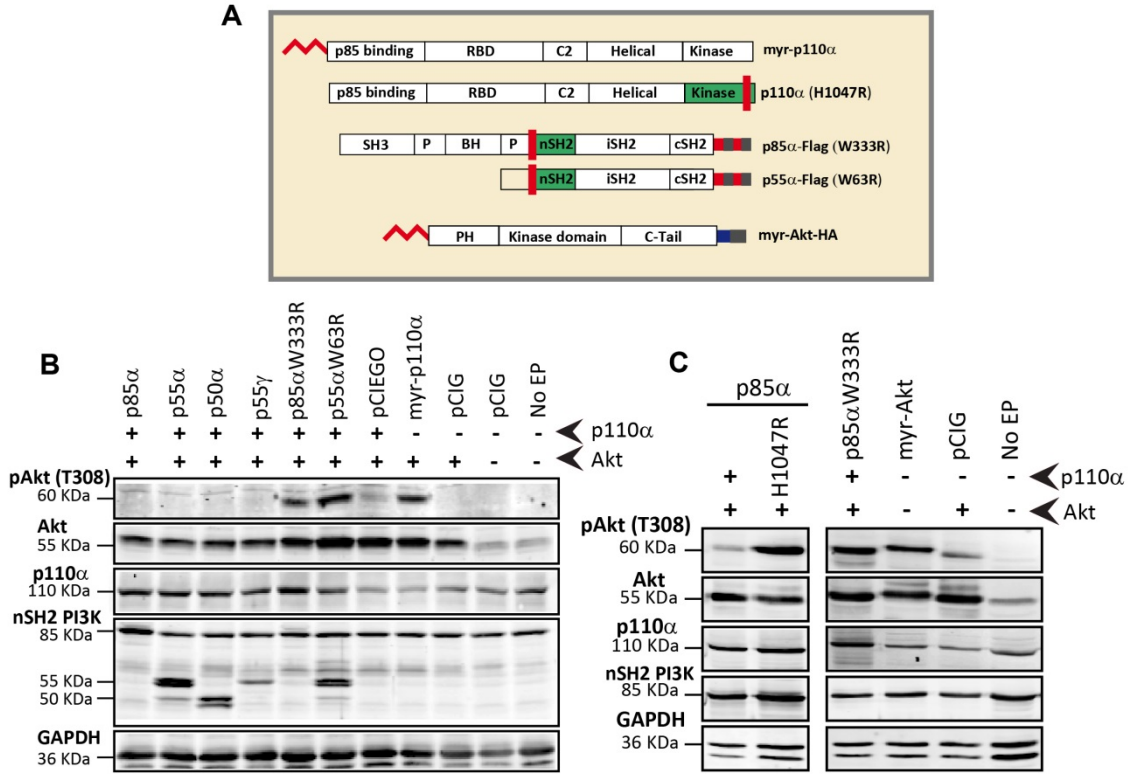


Figure 16. Mutant PI3Kα is constituvely active and growth-factor independent. (A) Representations of the different mutants used in the figures (B,C). **(B,C)** NIH3T3 cells were transfected for 24hrs with the constructs indicated on the top of the panel and analyzed in western blots with the antibodies indicated on the left to see the levels of Akt phosphorylation at Thr308 in free-serum conditions. Staining for total levels of Akt, p110α (overexpressed and endogenous) and the nSH2 domain of the regulatory subunits (overexpressed and endogenous) are shown. GAPDH staining was used as loading control.

3.2 PI3Kα activity is required for AB polarity

After validating our tools, we assessed the distribution of apical markers in embryos electroporated at HH stage 12 with aPI3Kα or shp110α. It should be mentioned that transfection with PI3Kα wild-type isoforms had very mild effects on NEP polarity and that it is why we decided to use active mutants (data not shown). We found no differences on the phenotypes using the p85α and p55α active forms combined with a wild-type p110α or a wild-type regulatory isoform with the oncogenic p110αH1047R,

so it has not been specified in the figures and they are all referred as active PI3Kα (aPI3Kα). Nevertheless, most of the experiments were carried out with the first combination.

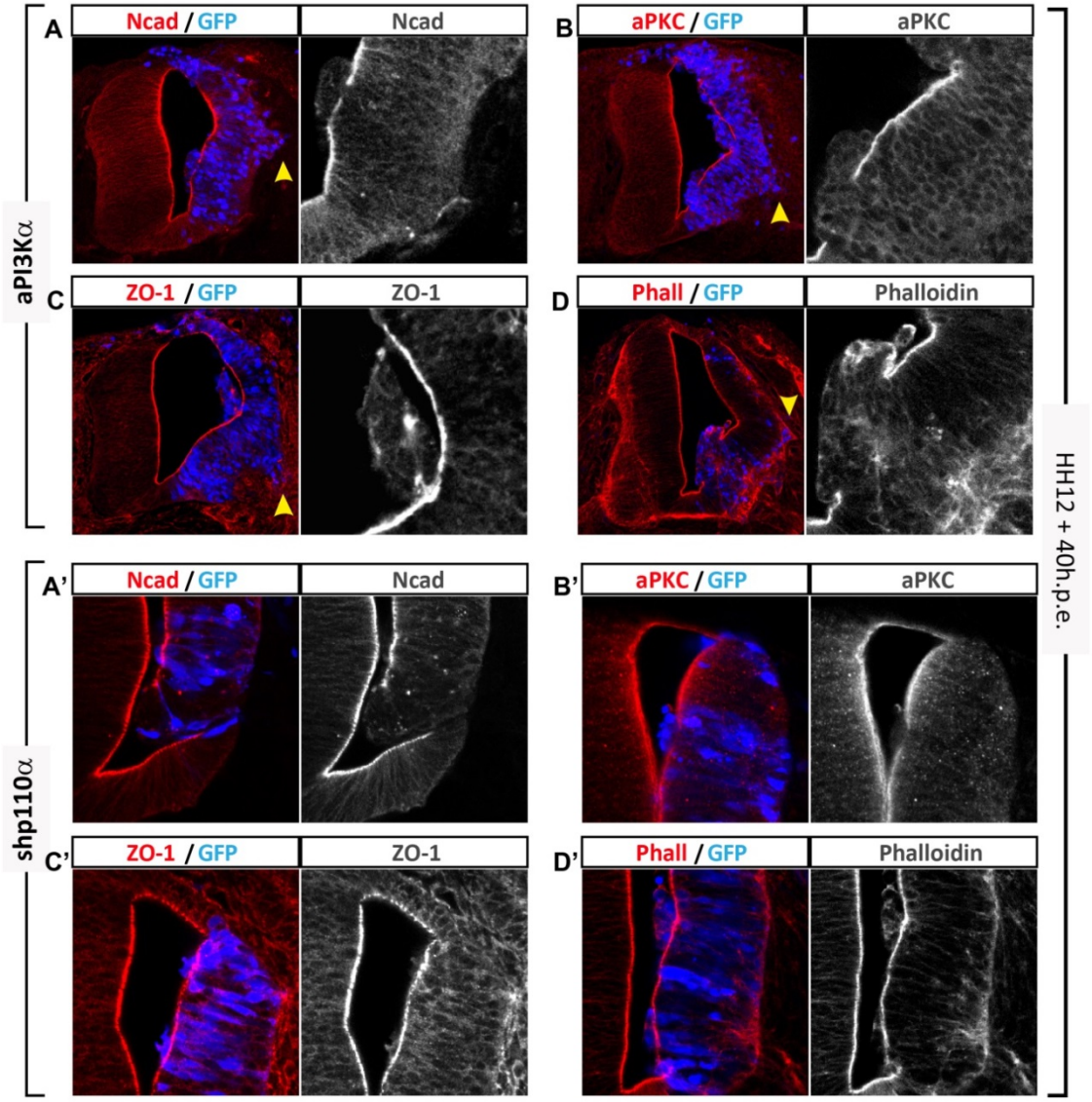


Figure 17. Misregulation of PI3Kα activity alters NEP cell polarity. Representative images of chicken neural tubes electroporated at HH12 and analyzed 40hrs later, stained against GFP (blue, transfection) and different polarity markers (red): Ncadherin (A,A'), aPKC (B,B'), ZO-1 (C,C') and phalloidin (D,D'). Enlarged images showing areas of transfected cells with polarity deficiencies are shown at the right of the panel for each staining. **(A-D)** Neural tubes electroporated with an active PI3Kα form. **(A'-D')** Neural tubes electroporated with shp110α to reduce PI3Kα activity.

Analyzing the aPI3Kα phenotype at different time points, we found that the first malformations in the NEP were evident already at 17hpe and more clearly visible at 24 and 40hpe. Transfected cells were forming ectopic cell masses inside the ventricle and showed an altered distribution of apical markers (Ncad, aPKC, ZO1) or phalloidin (F-actin staining), whose accumulation at the AJC was no longer maintained (Figure 17A-

D). Interestingly, there was also distortion of the basement membrane easily observed in the phalloidin staining (Figure 17D), but more remarkably detected by the presence of transfected cells beyond the basal limits of the NEP (arrowheads in Figure 17A-D). In line with the PI3Kα gain-of-function phenotype, p110α knock-down generated loss of apical polarity events shown with the same markers (Figure 17A'-D'), although they start to appear after 40hpe. Interestingly, lack of p110α did not alter the basement membrane of the tube.

3.3 PI3Kα-dependent apical depolarizations cause structural rearrangements of the NEP organization and ectopic mitosis

In addition to the disruption of NEP polarity, we observed transfected and non-transfected cells undergoing mitosis (PH3+) in ectopic places like inside the luminal space or in more basal locations contrasting with the non-electroporated half of the neural tube, where mitosis always occur close to the lumen (Figure 18A,A').

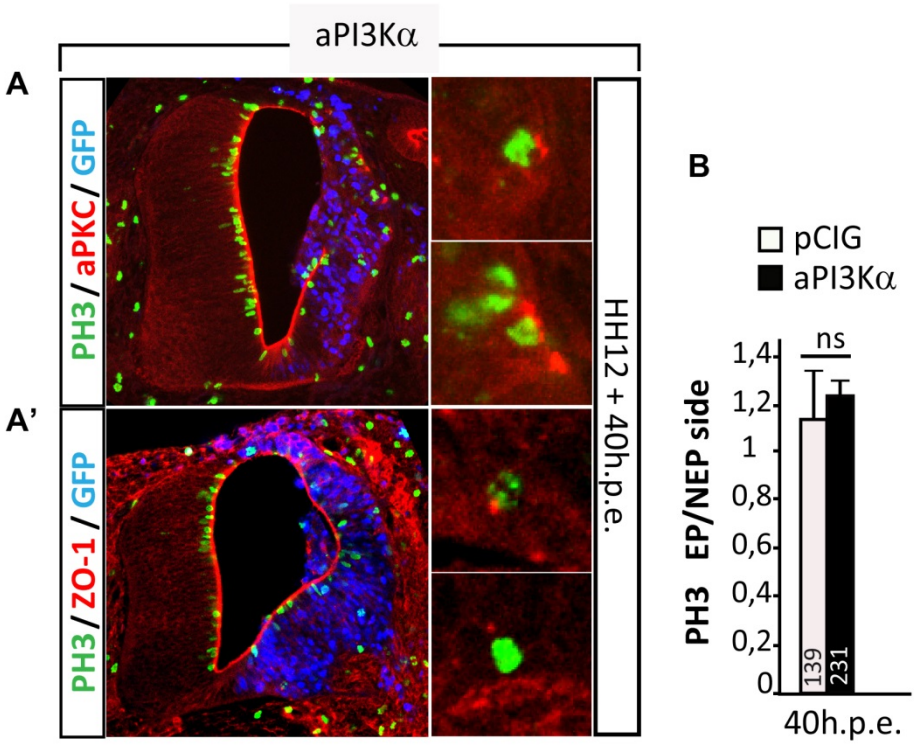


Figure 18. PI3Kα activity regulates localization of the AC. (A-B) HH-12 chicken neural tubes were analyzed 40hrs after electroporation with aPI3Kα. **(A, A')** Representative images of chicken neural tubes stained with antibodies against GFP (blue, transfection), aPKC on the top or ZO-1 on the bottom of the panel (red) and PH3 (blue, marker of M-phase). Enlarged images showing mitotic cells localized ectopically are shown at the right of the panel for each staining. **(B)** Cell quantification of experiment (A) to measure the number of mitotic cells. The graph shows the mean ±s.d. of the ratio obtained by dividing the number of PH3+ cells in the electroporated side (EP) by the non-electroporated one (NEP). The number of PH3+ cells counted (n) is displayed within each bar. Differences were tested by the Student's t-Test.

The presence of discrete clusters of apical proteins such as aPKC o ZO-1 accumulated next to mislocalized cells in M phase suggested that formation of ectopic apical polarity complexes could be the cause of cells entering division at ectopic positions, as it has been reported to occur after high Par3 or active aPKC overexpression in the chick neural tube (Afonso and Henrique, 2006; Herrera et al., 2014). However, aPI3Kα apparently did not caused cell cycle arrest since the number of PH3+ cells remained unaffected compared to the control side (Figure 18B). This result will be discuss later since we recently obtained some results supporting a role of PI3Kα during NEP cell mitosis, so the interpretation of this result should be done carefully.

Moreover, we found that severe NEP aberrations generated by misexpression of aPI3Kα could lead to an extra-lumen formation originated from the luminal face of the tube through bending of the apical side. Notably, we noticed that the general structure of the tissue was maintained because most of mitotic cells were still found around the ventricle and differentiated neurons migrated away of the new ventricle (Figure 19A, B). In addition, there was no ectopic induction of apoptosis unless the apical depolarizations were too severe (data not shown). These results suggest that PI3Kα-induced epithelial malformations could be mainly due to disruptions in cell polarity and not direct changes in cell death, proliferation or differentiation.

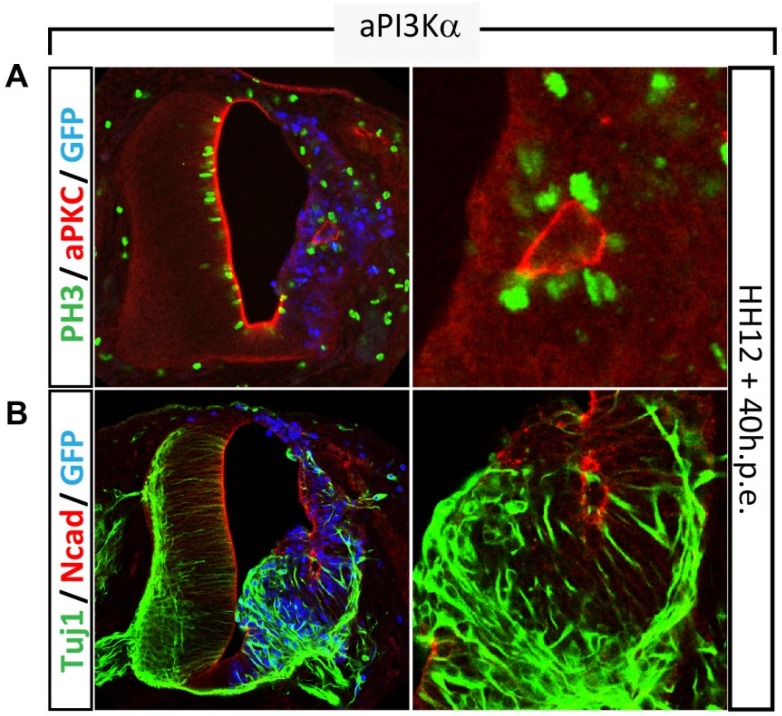


Figure 19. High PI3Kα activity can induce new lumen formations, but NEP cells still proliferate and differentiate. (A,B) Representative images of chicken neural tubes electroporated with aPI3Kα at HH12 were analyzed 40hrs later. Enlarged images showing areas of transfected cells surrounding new lumens are shown at the right of the panel for each staining. **(A)** Transfected sections were stained against GFP (blue, transfection), aPKC (red) and PH3 (green). **(B)** Transfected sections were stained against GFP (blue, transfection), Ncadherin (red) and Tuj1 (beta-III tubulin, green).

3.4 High PI3Kα activity induces basal migrations throughout the basement membrane

As it was previously mentioned in this section and visualized in figure 17A-D, non-regulated high PI3Kα activity generated epithelial disruptions affecting not only the apical, but also the basal side of the neural tube. Both membrane protrusions and cell bodies of electroporated cells were seen slipping out of the neural tube and sometimes fusing the NT with the Dermomyotome, a mesorderm-derived structure close to the NT. Notably, high expression of Ncadherin maintained migrating cells attached to each other and in contact with the NT. When we analyzed the continuity of the basement membrane through staining against laminin, one of its main components, breaches in the basement membrane were detected coincident with the presence of displaced transfected cells (Figure 20A). Next, we confirmed that epithelial malformations and basal breaches were occurring at all levels along the anterior-posterior embryonic spinal cord (Figure 20B), although we never checked if the effect was more severe towards the cranial or the caudal NT, which have different maturation stages at the same HH developmental stage. Nevertheless, we did test if the PI3Kα gain-of-function phenotype was stage-dependent by electroporating aPI3Kα 12h and 24h later than in previous experiments, corresponding approximately to HH16 and HH18 (Figure 20C, C'). Intriguingly, we found that apical depolarization events were still occurring, but there was a highly significant "dorsalization" of the basal exits compared to earlier overexpression, meaning that there were no basal migration events at the ventral half of the tube after transfecting at HH18. Finally, we checked whether the PI3Kα gain-of-function phenotype was specifically generated by an uncontrolled increase of membrane PIP3 and discard aberrant protein-protein interactions generated by the overexpression. Hence, we co-transfected HH stage 12 embryos with aPI3Kα and its antagonist, PTEN, and measure the intensity of the phenotype by quantifying the number of confocal images showing apical depolarizations, basal exits or both (Figure 20D). Co-expression with wild-type PTEN significantly reduced the severity of the described phenotype, confirming overproduction of PIP3 as the main cause of the gain-of-function phenotype. It should be noted that wtPTEN overexpression alone induces high levels of apoptosis, which were abrogated when co-transfected with aPI3Kα.

These results advocate that tight regulation of PI3Kα activity is very important for AB polarity and disruptions on PI3K/PTEN pathway can lead to alterations of the NEP structure highly severe at early stages.

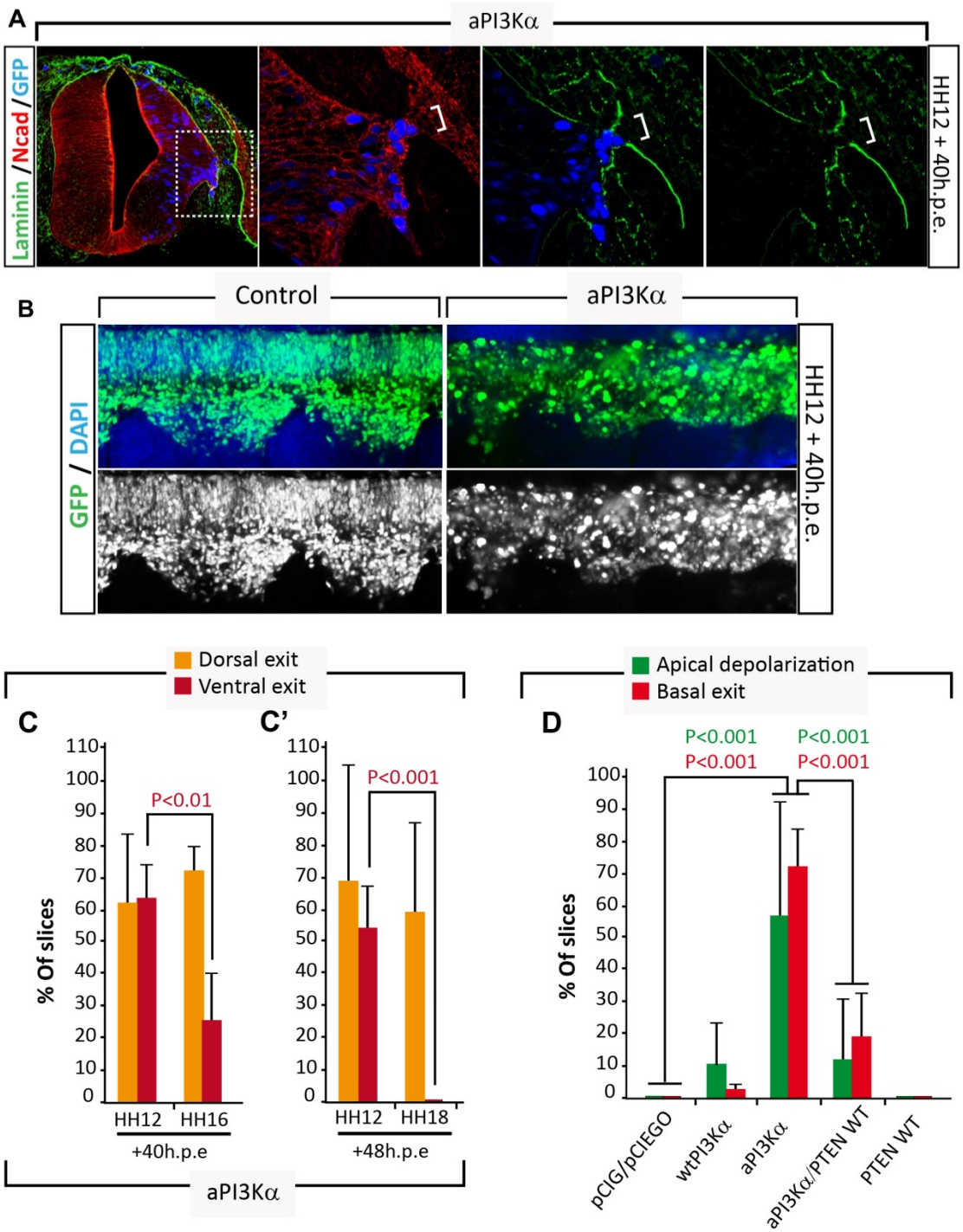


Figure 20. High PI3Kα activity induces stage-dependent basal migrations and invasion of the surrounding mesoderm. (A) Representative images of chicken neural tubes electroporated with aPI3Kα at HH12 and analyzed 40hrs later which were stained against GFP (blue, transfection), Ncadherin (red) and laminin (green). The three panels on the right show enlarged images of the area within the white box and the white square bracket points to one of the breaches in the basement membrane. **(B)** Embryos electroporated at HH stage 12 with the DNAs indicated were analyzed 40hpe for the general NT structure in a whole-mount preparation. Lateral view shows GFP+ cells (green) of the NT and Neural Crest streams across the somites (blue, DAPI). **(C,C')** The bar graph shows the percentage of sections from chicken embryos electroporated with aPI3Kα at the stages indicated on the bottom of the graph that presented basal migrations comparing the dorsal (orange) and ventral (red) NT between stages. The total number of sections (n) studied in each condition was HH12+40hpe=43; HH16+40hpe=29;

HH12+48hpe=39; HH18+48hpe=39. **(D)** The bar graph shows the percentage of sections from chicken embryos electroporated with the indicated DNAs that presented loss of apical polarity (green) and/or basal exits (red) for each treatment. The number of sections studied was pCIG/pCIEGO=54, wtPI3Kα=56, aPI3Kα=83, aPI3Kα/PTEN=47, PTEN=35. Significant differences in (C, D) were tested by one-way ANOVA followed by the Tukey's test.

4. PI3Kα regulates apico-basal positioning

In previous results, we have described that alterations in PI3Kα activity generate disruptions in the AB polarity of the NEP and high PI3Kα activity induces breaches in the basement membrane caused by membrane protrusions and cell body migrations. So far, we have hypothesized that this phenotype was merely structural and it was not originated from changes in proliferation, differentiation or cell fate, but we have not proved it yet.

4.1 PI3Kα does not regulate NEP cell proliferation

In the first place, we verified that proliferation rates were normal since we had previously seen ectopic mitosis, but the number of cells in M phase was not affected. For that, we transfected aPI3Kα and shp110α in HH stage 12 embryos and analyzed cell proliferation by BrdU incorporation assay at 24 and 40hrs later (Figure 21A,A'). As it was proposed, cell proliferation at 24hpe was not compromised when increasing PI3Kα activity, but at 40hpe we observed several proliferating cells in the basal region, which should normally contain only post-mitotic neurons (Figure 21B). This result is similar to the one shown in figure 18 with ectopic PH3+ cells. Regarding the loss-of-function experiment, there was a small but significant reduction in cell proliferation which could be due to the higher apoptosis shown in this condition.

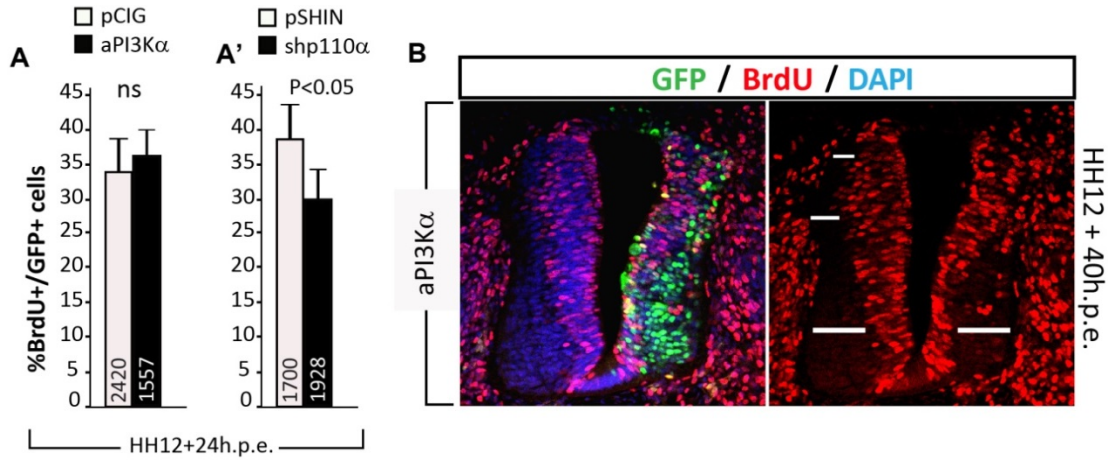


Figure 21. High PI3Kα activity does not regulate cell proliferation of NEP cells. (A,A') Cell quantification of BrdU incorporation in chicken neural tubes electroporated with aPI3Kα (A) and shp110α (A') and analyzed 24hpe. The graph shows the mean ±s.d. of the ratio obtained by dividing the number of BrdU+ cells by the total number of transfected cells. The number of GFP+ cells counted (n) is displayed within each bar. Differences were tested by the Student's t-Test. **(B)** Representative images of the experiment (A), but analyzed 40hpe, stained against GFP (green, transfection), BrdU (red) and DAPI (blue). The white bars delimit the non-proliferative area, which is no longer preserved in the electroporated side.

4.2 PI3Kα activity does not promote neural differentiation

Afterwards, we assessed whether PI3Kα activity was inducing neural differentiation and what was the identity of those cells migrating through the basement membrane. We addressed the first question setting out two different approaches. On the one side, we carried out promoter-dependent luciferase expression assays for the gain-of-function at 24 and 48hpe using the NeuroD promoter, which is only active in the neuronal population (Figure 22C). And on the other side, we performed aPI3Kα and shp110α electroporation in HH12 embryos and counted the number of progenitors (Sox2+) and neurons (HuC/D) after 40hrs (Figure 22A,B). Interestingly, the luciferase changes were not significant and the counted proportion of both populations was preserved (Figure 22A), but AB localization was not, so the initially separated ventricular and mantle layers were merged (Figure 22B): in the gain-of-function, progenitors were seen more basally located than neurons and abandoning the tube, but this often occurred involving also "migrating" neurons (Figure 22D); in the loss-of-function, besides the increased apoptosis previously presented, depolarized neurons stayed in the ventricular zone or closer compared to neurons in the control side.

This experiment shed some light on the second question: upon transfection with active PI3Kα, both progenitors and neurons had the ability to migrate out beyond the mantle zone. Another possible interpretation of this result is that only NEP cells can move out of the NT and then they differentiate in their new position. It is not so easy to distinguish among both hypothesis, but we have certainly seen immature neurons beta-III tubulin positive with their cell bodies inside the NT, but extending a membrane protrusion out through the basal lamina (Figure 24). Hence we believe that NEP cells and, at least, immature neurons have this invasive capacity since they also perform migrations in wild-type conditions to reach their final position in the NT (see introduction). We have also evidences supporting that, once opened a hole in the basement membrane, any non-transfected neuron could potentially slip out.

In any case, these findings are very interesting since progenitors basally located maintained their proliferative state in spite of not being in contact with the luminal side anymore and, afterwards, they were able to differentiate "normally" embedded in the mesoderm. These findings are also intriguing because alteration of PI3Kα activity is not only causing changes in cell polarity, but it seems to be specifically regulating cell positioning in the AB axis, which is important for the spinal cord structure based on layers and for determining the limits of the central nervous system.

4.3 PI3Kα activity controls AB positioning of NEP cells and neurons

Later on, in order to confirm this role of PI3Kα in regulating AB positioning in the embryonic chick neural tube, we performed similar gain and loss-of-function

experiments, but analyzed them later (72hpe) because neurogenesis would be more advanced (Figure 22E, F).

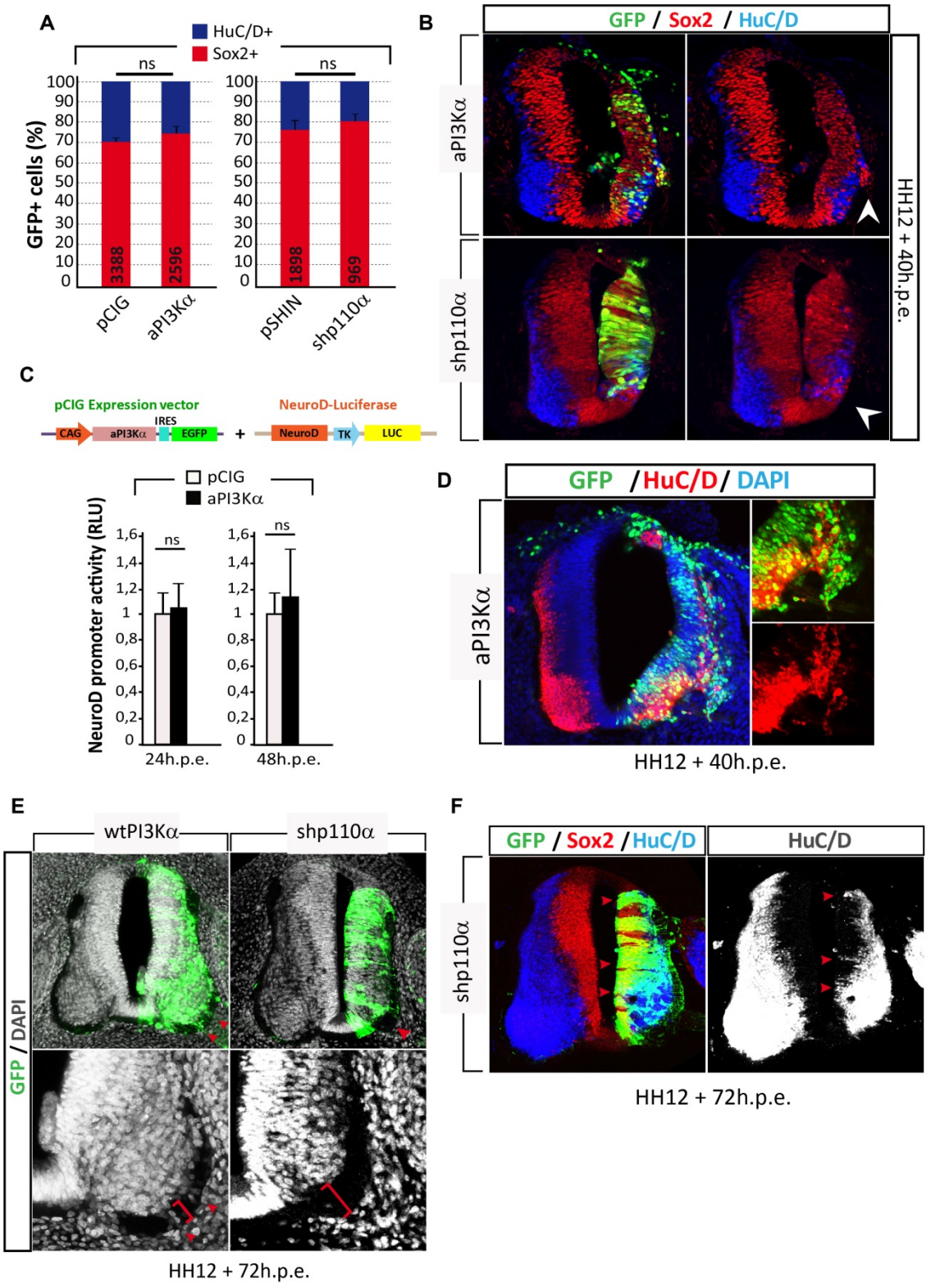


Figure 22. PI3Kα activity promotes apico-to-basal migration without affecting neural differentiation. **(A)** Cell quantification of progenitors (Sox2+) and neurons (HuC/D) in chick neural tubes tubes electroporated at HH12 with aPI3Kα (left) and shp110α (right) and analyzed 40hpe. The graph shows the mean ±s.d. of the ratio obtained by dividing the number of Sox2+ or HuC/D+ cells by the total number of

transfected cells. The number of GFP+ cells counted (n) is displayed within each bar. Differences were tested by one-way ANOVA followed by the Tukey's test. **(B)** Representative images of the experiment (A) stained against GFP (green, transfection), Sox2 (red) and HuC/D (blue). **(C)** aPI3Kα or the control vector (pCIG) was electroporated at HH12 along with reporter vectors expressing luciferase driven by the NeuroD promoter. Luciferase activity was assayed at 24 or 48 hpe. Significant differences were tested by the Student's t-Test. **(D)** Representative images of chicken neural tubes electroporated with aPI3Kα at HH12 and analyzed 40hrs later stained against GFP (green, transfection), HuC/D (red) and DAPI (blue). The two panels on the right show enlarged images of migrating cells. **(E)** Representative images of chicken neural tubes electroporated at HH12 with wtPI3Kα (left) or shp110α (right) and analyzed 72hrs later which were stained against GFP (green, transfection) and DAPI (grey). The lower panels show amplifications of the motor neuron domain and arrowheads point to the marginal zone. **(F)** Representative images of the experiment (E, shp110α) which were stained against GFP (green, transfection), Sox2 (red) and HuC/D (blue). Arrowheads point to neurons in the ventricular layer.

Regarding the gain-of-function experiment, this time we used a wild-type PI3Kα instead of the active form since we wanted to observe more subtle effects on the tube. Notably, we detected cells bodies on the motor neuron domain moving basally through the marginal layer, which should only contain axonal fibers (red arrowheads on Figure 22E, left). On the contrary, reduction of p110α left a significant gap between MNs and the edge of the neural tube on the same area, possibly caused by neurons which partially migrated but died afterwards (red arrowheads on Figure 22E, right). In addition, we found out mature neurons apically located, inside the ventricular zone (red arrowheads, Figure 22F), suggesting that PI3Kα is a needed signal for neurons to correctly migrate towards the basal side.

These results support the previous hypothesis, proposing PI3Kα as an important signal not only for AB polarity, but also as a basal signal needed for the neurons to correctly locate to their final position.

5. PI3Kα is not involved in cell fate

In the previous sections, we have demonstrated that PI3Kα activity is required for maintenance of cell polarity and AB positioning of NEP cells and neurons. We have also seen that unregulated high PI3Kα activity induces the exit of cell bodies throughout the basement membrane towards the surrounding mesoderm ignoring the physical and chemical borders of the central nervous system (CNS). Now, we want to assess if this is caused by alterations in cell fate.

5.1 High PI3Kα does not induce Neural Crest Cell fates

Since these "migratory" and "invasive" features of the PI3Kα gain-of-function experiments were highly coincident with epithelial-to-mesenchymal transition processes (EMT) like those performed by neural crest cells (NCCs) in the most dorsal part of the tube (Ferronha et al., 2013), we decided to test whether high PI3Kα activity was promoting a NCC phenotype in cells from the NT. To achieve this, we looked at NCC specific markers such as Sox9, Sox10 or Slug using in situ hybridization to measure mRNA levels (Figure 23B), and the HNK-1 marker by immunohistochemistry (Giovannone et al., 2015) (Figure 23C). Interestingly, we did not find coincidence between these markers and transfected cells along the NT and therefore the NCC induction hypothesis was dismissed.

5.2 High PI3Kα does not induce Motor Neuron fates

So far, we have not discarded the possibility of being affecting the cell fate of NEP cells from the NT. Therefore, we studied different neural populations of the NT by immunohistochemistry after electroporation of HH stage 12 embryos with the aPI3Kα mutant form. In the first place, we checked the MN population, highly interesting because in wild-type conditions they are the only neurons able to project their axons out of the CNS. In addition, MNs are the main neural population being generated in the neural tube at the stages we are analyzing (Saade et al., 2013).

There are several mechanisms that dictate positioning of the cell bodies of MNs and it has been unveiled an intrinsic capacity of these cells to migrate (Bron et al., 2007; Chauvet and Rougon, 2008; Hu et al., 2007). Consequently, if we were inducing ectopic MNs they might open new exit points and migrate out avoiding the endogenous regulation. Surprisingly, we did not find any mislocalised induction of MNs after aPI3Kα electroporation that could explain the basement membrane breaches along the dorso-ventral axis (Figure 23C-F). Next, we assessed if there were alterations in some populations of dorsal interneurons (dINs) in addition to those previously stained with the MN marker Islet1 (dI3), which did not show any change. For that, we used a

marker shared among dI2, 4 and 6 populations (Lhx1/5), again showing no significant changes (Figure 23G) (Bonanomi and Pfaff, 2010; Le Dreau and Marti, 2012).

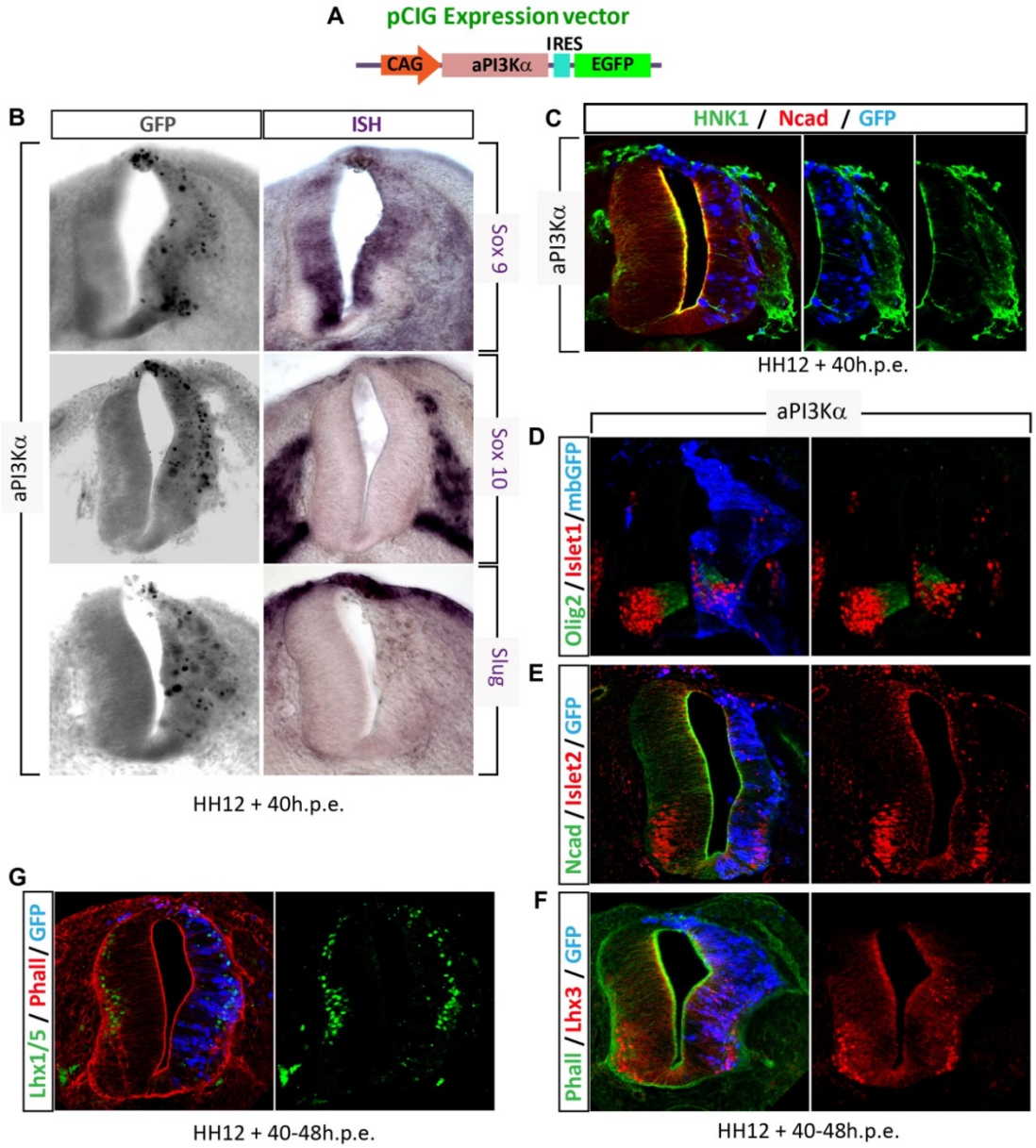


Figure 23. High PI3Kα activity does not change the cell fate of neuroepithelial cells in the neural tube. **(A)** Scheme of the pCIG vector where the p110α catalytic and mutant regulatory isoforms forming the active PI3Kα were cloned (see Materials and Methods). **(B-F)** Representative images of chicken neural tubes electroporated at HH12 with aPI3Kα and analyzed 40-48hrs later. **(B)** These images show staining against GFP (black, transfection) on the left part of the panel and in situ hybridization staining against the indicated NCC genes on the right part**. (C)** Representative image stained against GFP (blue, transfection), Ncadherin (red) and HNK-1 (green, NCCs). **(D)** Representative image stained against membrane GFP (blue, transfection), Olig2 (red, MN precursors) and Islet1 (green, all MNs except for one subtype of the Lateral Motor Column positioned laterally). **(E)** Representative image stained against GFP (blue, transfection), Islet2 (red, all MNs but preganglionic motor column) and Ncadherin (green). **(F)** Representative image stained against GFP (blue, transfection), Lhx3 (red, medial motor column projecting to the dermomyotome) and phalloidin (green). **(G)** Representative image stained against GFP (blue, transfection), phalloidin (red) and Lhx1/5 (green, dorsal interneurons 2-4-6).

6. PI3Kα does not induce neural differentiation, but it enhances beta-III tubulin expression and stabilization

Neurogenesis is initiated by the onset of asymmetric divisions that generate a stem cell daughter remaining as progenitor within the ventricular zone and another daughter cell committed to differentiation that migrates laterally towards the mantle zone (MZ). During this apico-to-basal movement, post-mitotic neurons go through an extensive and dynamic remodeling of their cytoskeleton (Singh and Solecki, 2015). In previous sections, we have demonstrated that PI3Kα activity is regulating AB polarity of NEP cells and controlling AB positioning of cells from the NT without direct changes in cell proliferation, differentiation or fate. In this section, we will study more in depth the role of PI3Kα in AB positioning and the cellular events occurring in the basal side of the tube.

6.1 PIP3 accumulates basally and colocalizes with actin-based protrusions

We have hypothesized that PI3Kα could be a basal signal needed for the neurons to correctly locate in their final position in the MZ. This is based on the fact that, in theory, uncontrolled overexpression of active PI3Kα in NEP cells should generate products (refer as PIP3) equally distributed along the whole membrane assuming that the substrate (PIP2) is homogenously disposed, which is an inaccurate simplification. In that “simple” case, absence of polarity caused by unrestricted PI3Kα product might explain NEP aberrations. However, we have also observed frequent basal migrations beyond the basement membrane after active PI3Kα overexpression in cells still maintaining their AB polarity. In order to get further insight into the cellular and molecular mechanisms behind these events, we performed gain-of-function experiments with the aPI3Kα and analyzed them short time after electroporation. On the first place, we used cytoskeletal markers such as F-actin (phalloidin) and beta-III tubulin (Tuj1) to visualized the morphology of cells slipping out after 16/24hpe (Figure 24A, B). Beta-III tubulin is an early neuronal marker, but it is also a structural protein that contributes to microtubule stability in neuronal cell bodies and axons and plays a role in axonal transport (Katsetos et al., 2003a). Interestingly, we detected actin filament-based protrusions emerging from the basal domain of transfected cells (Figure 24A), but these membrane extensions were not always positive for beta-III tubulin (Figure 24B).

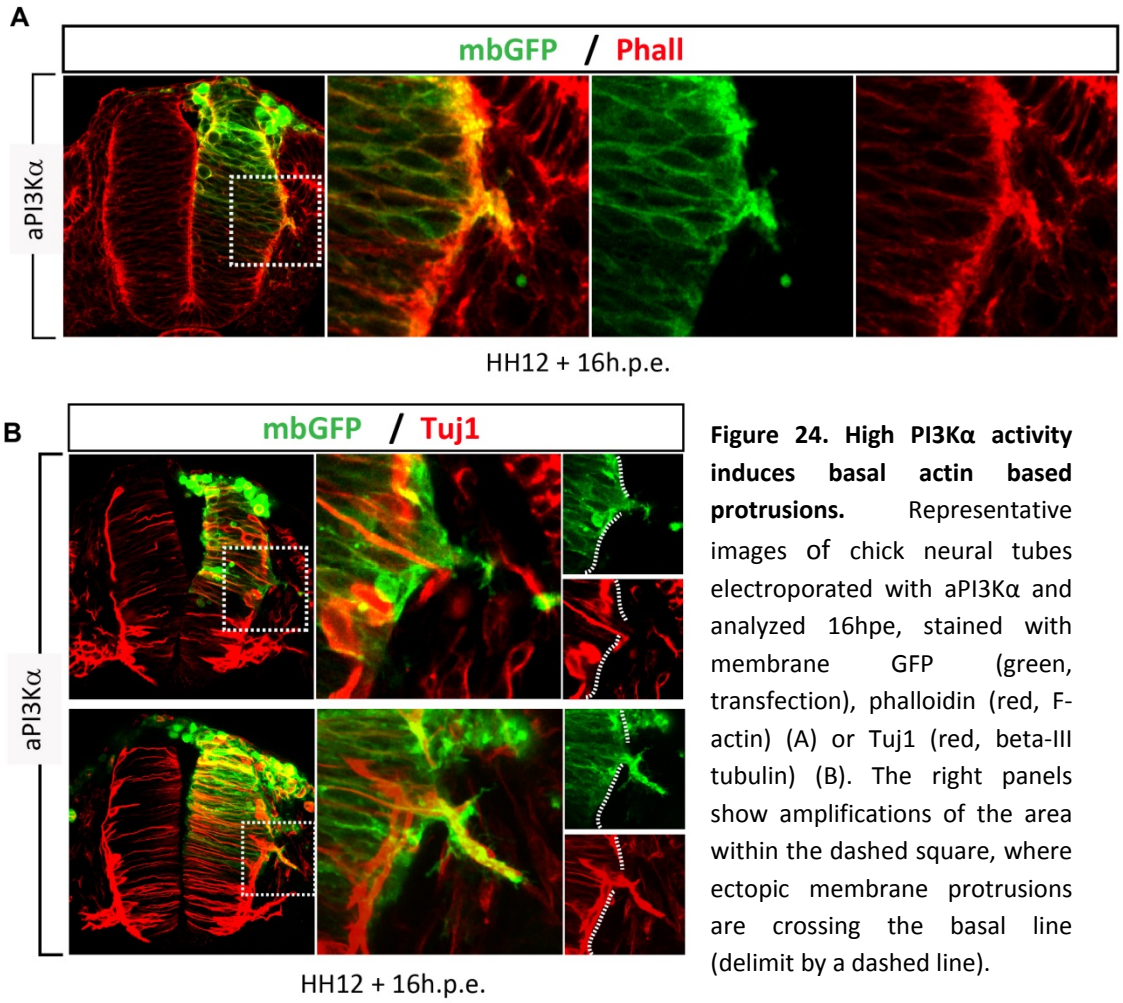


Figure 24. High PI3Kα activity induces basal actin based protrusions. Representative images of chick neural tubes electroporated with aPI3Kα and analyzed 16hpe, stained with membrane GFP (green, transfection), phalloidin (red, F-actin) (A) or Tuj1 (red, beta-III tubulin) (B). The right panels show amplifications of the area within the dashed square, where ectopic membrane protrusions are crossing the basal line (delimit by a dashed line).

Afterwards, we took advantage of a probe for PI3K products that can be transfected, formed by the pleckstrin homology (PH) domain of an important downstream protein of this pathway, Akt, coupled to a green fluorescent protein (GFP). This "PIP3 sensor" has been used in previous works (Franke et al., 1997; Martin-Belmonte et al., 2007) demonstrating high affinity of this PH domain for PIP3 and providing a useful tool for visualizing the cellular distribution of certain phosphoinositides through confocal microscopy.

First of all, we optimized the DNA concentrations (10-50ng/ul) and tested the functionality of the sensor in our system by comparing its cellular distribution with a cytoplasmic red fluorescent protein (tdTomato, Figure 25A). To contrast the distribution and intensity of both fluorescent proteins, we displayed each channel as a pseudo-color image using a "Look Up Table" (LUT) which assigns a color to each pixel based on its intensity (from 0 to 256 for 8-bit images). As it was previously published in other systems, distribution of the PIP3 sensor in the chick NT was specific, since it had more affinity for some locations than others, and was mainly concentrated in the membrane.

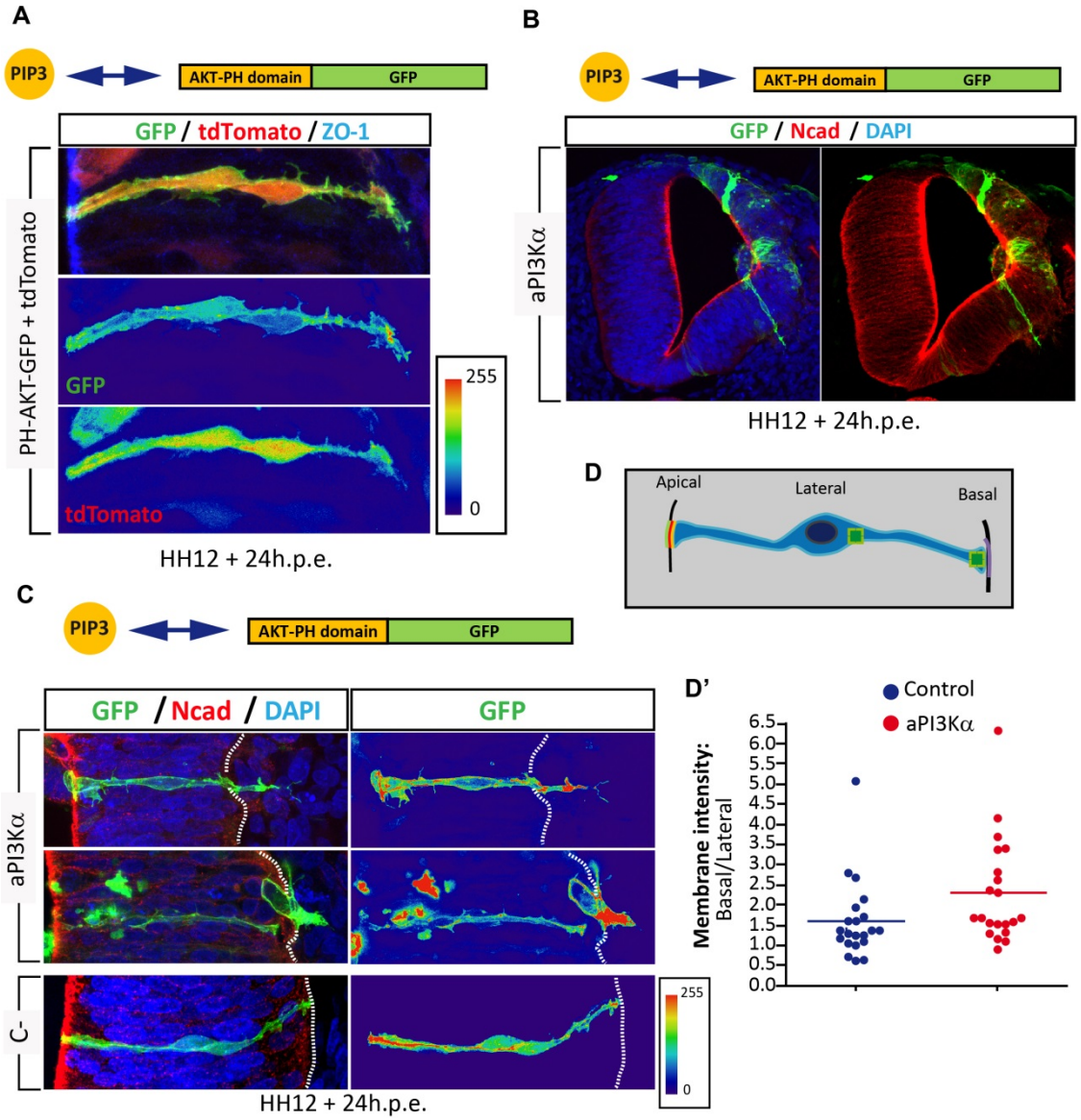


Figure 25. PIP3 is enriched in the basal side of NEP cells and in basal membrane protrusions.
(A) Representative image of a cell from chick neural tubes electroporated with PH-Akt-GFP (green) and tdTomato (red) stained against ZO-1 (blue) to visualize the apical membrane on the top of the panel. Lower images are displayed in pseudo-color using the physics LUT (scale on the right square) for the green and red channels. **(B)** Representative image of chicken neural tubes electroporated with aPI3Kα (without GFP) and PH-Akt-GFP showing a cell extending a protrusion through the basement membrane. **(C)** Representative images of cells from the experiment (B) on the top panel and a control cell transfected with empty vector and PH-Akt-GFP on the bottom panel. The dashed line delimits the basement membrane. **(D)** Drawing of a NEP cell showing the orientation of the cell-images and indicating with green squares the areas quantified in (D'). **(D')** Dispersion graph showing the relative quantification obtained from dividing pixel intensity of basal and lateral membranes from electroporated cells. The number of cells analyzed was n=21 for aPI3K and n=21 for empty vector.

We next assessed PIP3 distribution after electroporating aPI3Kα (without GFP) to test its localization in relation to these early detected actin based protrusions compared to wild-type conditions (Figure 25B-D'). Since the GFP distribution was not exclusive of

one specific membrane domain in contrast to other polarity models such as the MDCK (Madin-Darby canine kidney) cells (Martin-Belmonte et al., 2007), we decided to quantify this experiment comparing the GFP intensity among similar areas of basal and lateral transfected NEP cells (Figure 25D). Two striking results were obtained from this quantification (Figure 25D'): firstly, PIP3 tended to concentrate more in the basal than in the lateral domain in wild-type conditions; and secondly, the overproduced PIP3 generated after aPI3Kα transfection also concentrated basally and co-localized with the basal membrane protrusions, suggesting that high accumulation of basal PIP3 could be the cause of the membrane protrusions and posterior "invasive" basal migrations.

6.2 PI3Kα activity promotes beta-III tubulin expression and stabilization

In order to gain some knowledge about the basal function of PI3Kα avoiding the apical depolarizations, we designed an experiment based on our findings about PI3Kα expression pattern at later stages. We had previously shown that around HH stage 20-22, p110α expression got restricted to the intermediate and marginal layers, where neural differentiation is taking place. Accordingly, overexpressed wild-type p110α was specifically stabilized in these areas due to the presence of regulatory subunits with similar expression pattern. Therefore, to assess in particular the effect of high PI3Kα activity in differentiating neurons, we repeated the same experiment, but using the oncogenic form of p110α (H1047R). We performed electroporation of HH stage 12 chicken embryos with p110αH1047R alone and analyzed after 48hrs finding an important increase in the F-actin and beta-III tubulin staining in the mantle zone and also ectopic beta-III tubulin expression in sections of electroporated Roof Plate (RP) (Figure 26). The RP is a dorsal midline organizing center involved in specification of dorsal interneurons during spinal cord development (Chizhikov and Millen, 2004), however RP cells do not have neural identity and, therefore, they should not express beta-III tubulin.

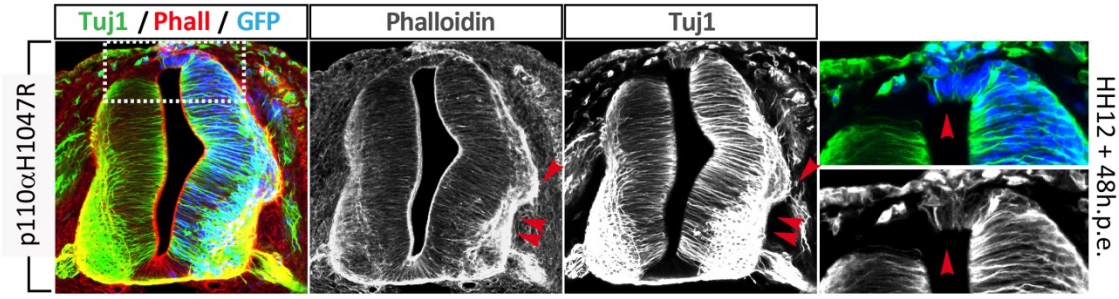
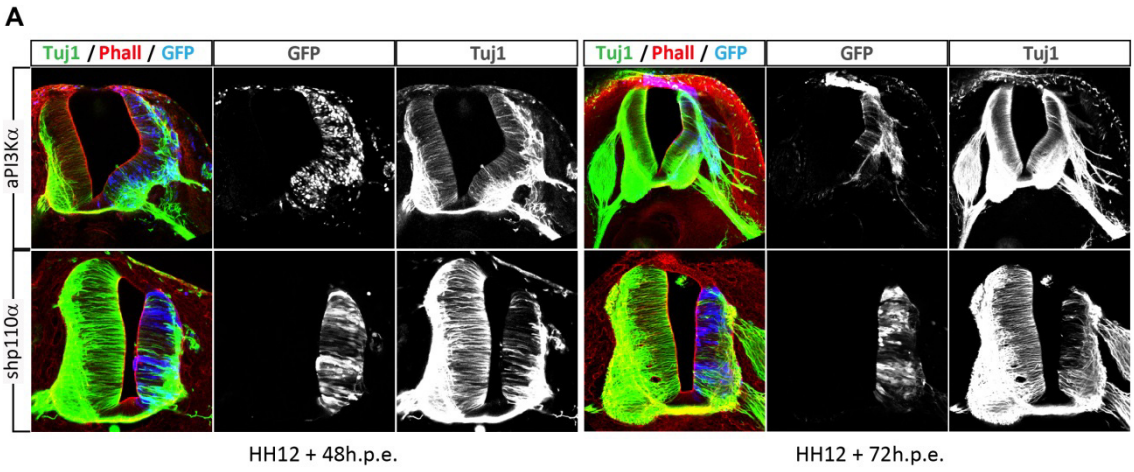


Figure 26. Oncogenic p110α induces F-actin and beta-III tubulin accumulation in differentiating neurons and ectopic beta-III tubulin expression in the Roof Plate. Representative image of chick neural tubes electroporated with oncogenic p110αH1047R stained against GFP (blue, transfection), phalloidin (red, F-actin) and Tuj1 (green, beta-III tubulin). Red arrowheads point to F-actin and beta-III tubulin accumulations on the electroporated side. The two right panels show amplifications of the dorsal area within the dashed line, showing ectopic expression of beta-III tubulin in transfected cells from the RP indicated by the red arrowhead.

Our next step was investigating if PI3Kα activation could be promoting beta-III tubulin expression and/or stabilization. NEP cells have to switch from an AB polarity to a transitory migratory cell polarity from which they have to grow an axon and develop a mature morphology. Local activation of PI3K has been linked to induction of axon specification and growth in several neuronal types through local cytoskeletal dynamics (Menager et al., 2004; Polleux and Snider, 2010), so the fact that PI3Kα can have a role in neural differentiation is not totally new, but it has not been related to beta-III tubulin expression. Thereby, we performed gain and loss of function experiments by transfecting the aPI3Kα used in previous sections and the shp110α in parallel, and we analyzed beta-III tubulin protein expression by immunohistochemistry after 48hpe and 72hpe (Figure 27A, B). Increased PI3Kα activity resulted in the appearance of axons emerging through the basal breaches and growing inside the mesoderm already at 40-48hpe and, notably, these axons remained stable along time despite their wrong location since they were visualized after 72hrs (Figure 27A, top panel). Coherently, p110α knock down caused a reduction of beta-III tubulin staining in the remaining transfected cells, apart from the reported loss of neurons (Figure 27A, low panel). Picture quantification of Tuj1 pixel intensity pondered by the phalloidin staining allowed us to measure the amount of beta-III tubulin protein in transfected cells by comparing the electroporated side with the control one, reaching up to 50% of beta-III tubulin reduction at 72hpe (Figure 27B). In order to confirm this result, we performed a similar experiment by implementing the recent targeted gene editing technology CRISPR/Cas9, which has been demonstrated to be an efficient tool for knocking out genes also in the chick embryo (Veron et al., 2015). For this experiment, it was used a vector containing a GFP as reporter, the Cas9 nuclease under control of a constitutively active promoter and the cloning site for the guide RNA (gRNA) all in the same backbone (Figure 27D). We obtained similar phenotype by using this technique (Figure 27E), but since in theory the edition occurring in each cell varies a lot among cells and embryos, we continued to use the shRNA technology.



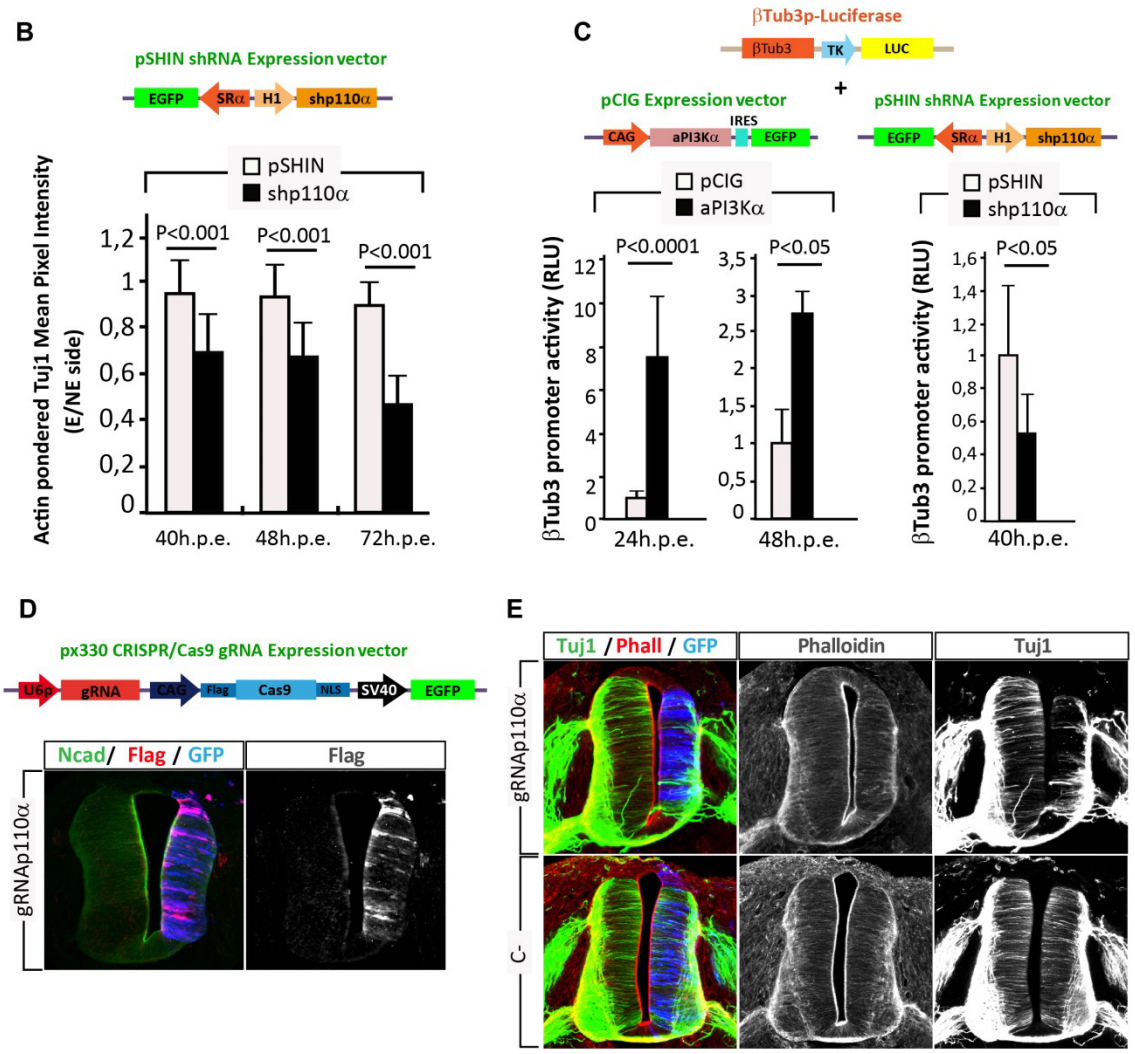


Figure 27. βtubulin III expression partially depends on PI3Kα. (A) Representative images of chick neural tubes electroporated with aPI3Kα (top panel) or shp110α (lower panel) and stained against GFP (blue, transfection), phalloidin (red, F-actin) and Tuj1 (green, beta-III tubulin) at 48hpe and 72hpe. **(B)** Pixel intensity quantification of beta-III tubulin staining pondered by F-actin staining comparing electroporated and control sides from experiment (A), but only the shp110α condition. The number of sections studied was pSHIN, 40hpe=9, shp110α, 40hpe=13; pSHIN, 48hpe =9, shp110α, 48hpe =24; pSHIN, 72hpe =12, shp110α, 72hpe=25. Significant differences were tested by one-way ANOVA followed by the Tukey's test. **(C)** Luciferase expression driven by the beta-III tubulin promoter was assayed at 24, 40 or 48hpe after aPI3Kα or shp110α transfection. Significant differences were tested by the Student's t-Test. **(D-E)** The px330 CRISPR/Cas9 expression vector containing a guide sequence against the chick p110α was electroporated at HH12 and analyzed at 48hpe to check: (D) Cas9 expression, with staining against GFP (transfection, blue), Ncadherin (green) and Flag (red, tagged Cas9); and Tuj1 levels after eliminating the *Pik3ca* gene, with staining against GFP (blue, transfection), Tuj1 (green) and phalloidin (red).

Since microtubule regulation depends on both tubulin expression and microtubule stabilization, we studied whether those PI3Kα dependent changes in beta-III tubulin levels were partially originated by gene expression modulation. For that, we electroporated a luciferase reporter under the control of beta-III tubulin promoter at

HH12 and measured luciferase levels at 24 and 48hpe (Figure 27C). Interestingly, aPI3Kα overexpression resulted in significant increase of beta-III tubulin promoter driven luciferase expression at both time points while suppression of p110α showed a weaker but consistent tendency to reduced expression.

We confirmed the luciferase assay result by using the same promoter, but this time controlling the expression of fluorescent proteins. Analyzing the number of GFP+ (Figure 28A, A') or RFP+ (Figure 28B, B') cells under the control of beta-III tubulin promoter for gain and loss of function experiments respectively, we found significantly similar tendencies to the previous experiments. An additional read-out from this result is that increased PI3Kα activity induces partial ectopic beta-III tubulin expression, yet it mainly seems to be boosting beta-III tubulin expression in differentiating neurons.

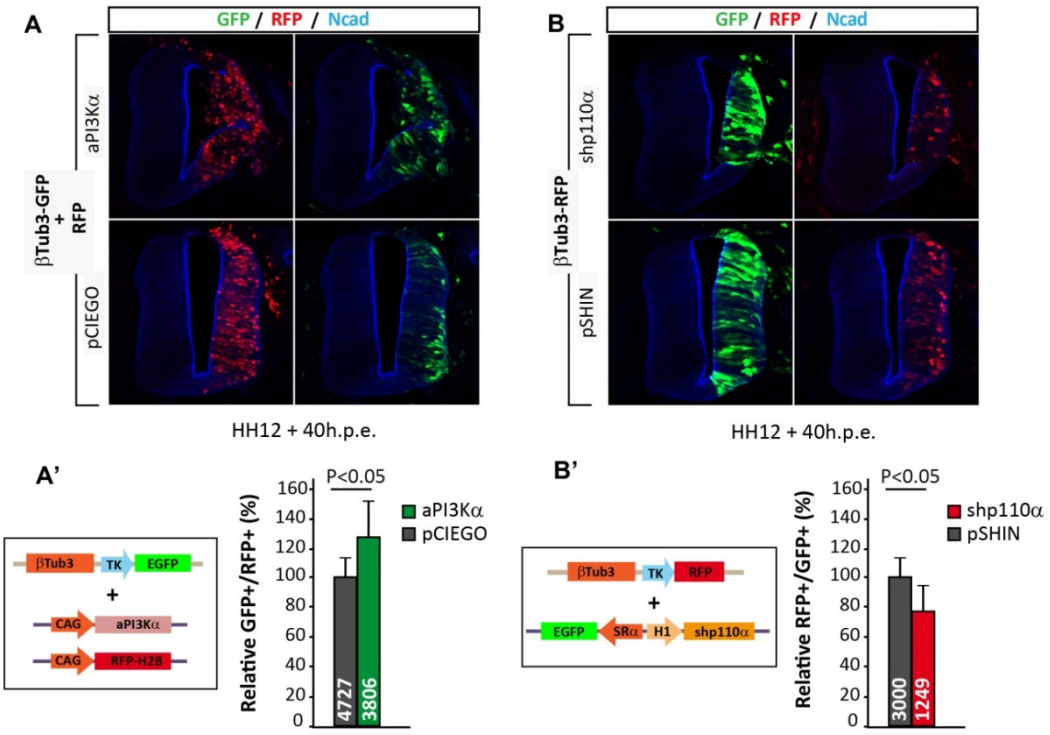


Figure 28. PI3Kα promotes beta-III tubulin expression. (A-B) Representative images of chick neural tubes electroporated with a fluorescent reporter controlled by the beta-III tubulin promoter and aPI3Kα (A) or shp110α (B) that were stained against GFP (green), RFP (red) and Ncadherin (blue) at 40hpe. **(A'-B')** Bar graphs show the relative mean ±s.d. of the ratio obtained from dividing the number of EGFP+ (A') or RFP+ (B') cells by the total number of transfected cells (RFP+ in A' and GFP+ in B') and compare it to the control. The number of transfected cells counted (n) is displayed within each bar. Differences were tested by the Student's t-Test.

Together, these results propose PIP3 as a basal signal and suggest that PI3Kα activity might be somehow promoting beta-III tubulin expression.

7. PI3Kα controls apico-basal polarity and basal migration through control of Rho GTPases

In previous sections, we have seen that PI3Kα activity is required for AB polarity of the NEP, modulates AB positioning of progenitors and neurons and participates in neural cytoskeleton maturation through promotion of actin membrane protrusions and beta-III tubulin expression/stabilization. We have also demonstrated that high PI3Kα activity produces an excess of PIP3 which concentrates basally promoting invasive actin-based membrane protrusions at early stages, basement lamina breaches, enhanced basal migration and ectopic axonal growth. Our next step is finding out the molecular mechanisms behind these events.

7.1 Akt activation is not sufficient for explaining the PI3Kα dependent phenotype

The protein serine/threonine kinase AKT is a principal target of PIP3. Binding of Akt to PIP3 through its PH-domain leads to the membrane recruitment of AKT and subsequent phosphorylation by PDK1 (3-phosphoinositide-dependent kinase-1) at the catalytic phosphorylation site T308. Later on, AKT is also phosphorylated at the hydrophobic motif, S473, by TORC2 (target of rapamycin complex 2) and possibly by other proteins, further increasing its enzymatic activity and broadening substrate scope (Fayard et al., 2010; Vasudevan and Garraway, 2010). Akt is critical for neuronal polarity as reported, for example, when a myristoylation site is added to AKT (myr-AKT) generating a form constitutively targeted to the membrane and active that is sufficient to induce multiple axon formation (Yoshimura et al., 2006). It is also upstream of several substrates that are important for neurite outgrowth, such as GSK3β, peripherin, mTOR or δ-catenin (Read and Gorman, 2009). Furthermore, it appears to undergo selective degradation when non-phosphorylated by the ubiquitin (Ub)–proteasome system, which selectively targets the inactive pool of AKT in neurites resulting in a net enrichment of activated AKT in a single process becoming the axon (Yan et al., 2006). Finally, active Akt has been related to actin-rich membrane structures and concentration at the leading edge in motile cells suggesting a role for Akt in cell polarity still non-well defined (Higuchi et al., 2008). Thereupon, we decided to investigate whether Akt might be responsible for the apical depolarizations or the enhanced basal migrations induced by excess of PI3Kα activity. For that, we designed two approaches: firstly, we assessed whether an active form of Akt, N-terminally myristoylation signal-attached Akt (myr-Akt) (Grider et al., 2009) alone was sufficient to mimic the effect caused by excess of PI3K activity in the neural tube; and secondly, if we could neutralize the aPI3Kα phenotype with a catalytically inactive Akt (kinase-dead K179M) (Aoki et al., 1998).

Regarding the first approach, we transfected the myr-Akt form in HH stage 12 embryos and analyzed them 40hpe. As controls, we used two myristoylated mutant forms affecting the main phosphorylation sites (S473F and T308I) and wild-type Akt (Figure 29A). Interestingly, overexpressed myr-Akt generated severe apical depolarizations as seen by the disturbed apical aPKC and F-actin distribution and the presence of ectopic mitosis (Figure 29C), however it did not alter the basal side of the tube. These depolarization events required full Akt activation, since none of the myristoylated mutants or the wild-type Akt showed similar effects. Notably, the myr-AktS473F mutant, but not the myr-AktT308I, was able to mimic the ectopic beta-III tubulin expression in the Roof Plate previously seen after oncogenic p110α transfection in Figure 26 (Figure 29D). An additional observation we made was that overexpressed wild-type Akt showed very low expression levels (detected with an anti-HA antibody) compared to the membrane targeted forms, denoting a specific degradation process in agreement with Yan et al. 2006 (Figure 29E). HA staining in the myristoylated mutants such as S473F (the active Akt had too much effect, Figure 29E) also suggested a higher affinity of membrane targeted Akt for the most apical and basal domains of electroporated cells, but we did not deepened into Akt subcellular localization or stability mechanisms. Additionally, when we co-electroporated aPI3Kα and wild-type Akt resulted in increased Akt levels/stability and more aggressive depolarizations (Figure 29B), which are consistent with the previous observations. These results suggest that the effects caused by PI3Kα misexpression might partially go through Akt activation regarding AB polarity, but they do not explain the “basal” events.

The second approach consisted of neutralizing aPI3Kα-induced aberrations with a kinase-dead Akt (K179M) that has been reported to act as dominant negative (Guo et al., 2011; Zhou et al., 2000) by co-electroporating both together in HH stage 12 embryos. Misexpression of AktK179M alone seemed to partially reduce beta-III tubulin expression in some embryos (Figure 29F) and it softly diminished the apical depolarization events when combined with aPI3Kα, but it did not significantly impede either of the described structural effects (Figure 29B). We obtained similar results using a myristoylated form of AktK179M (data not shown).

The conclusion obtained from these results is that Akt might be involved in AB polarity of the NEP and it is probably needed for the correct development of the neural tube, but its activation does not seem responsible for the PI3Kα-dependent phenotype.

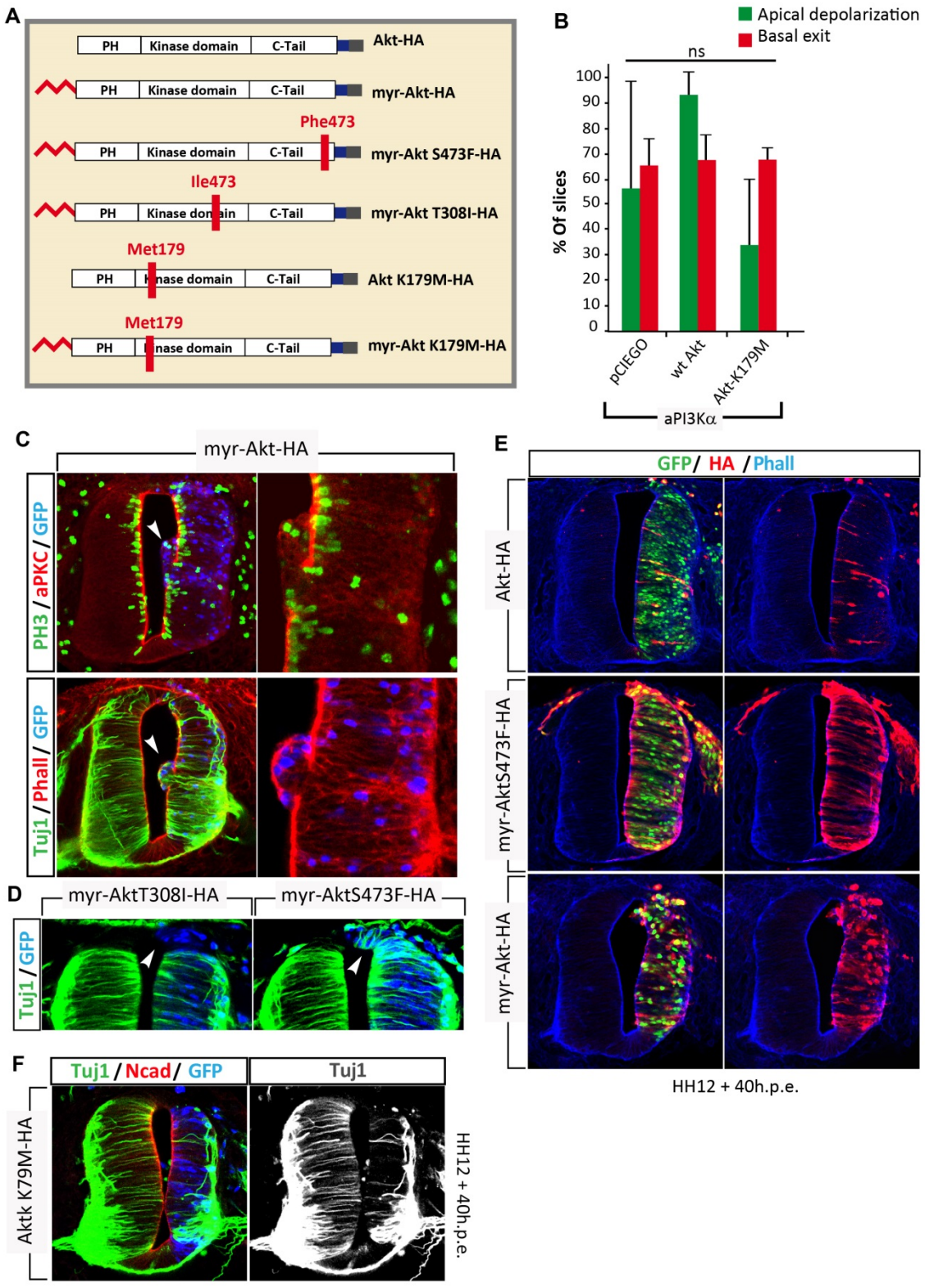


Figure 29. Akt does not reproduces the PI3Kα dependent phenotype. (A) Representations of the different mutants used in figures (B-F) cloned in pCMV6 vector. **(B)** The bar graph shows the percentage of sections from chicken embryos electroporated with the indicated DNAs that presented loss of apical polarity (green) and/or basal exits (red) for each treatment. The number of sections studies was aPI3Kα/pCIEGO=28, aPI3Kα/wt Akt=52, aPI3Kα/AktK179M=52. Significant differences were tested by one-way ANOVA followed by the Tukey's test. **(C-F)** The different constructs were analysed 40h after electroporation into HH-12 chicken neural tubes. Transverse sections were stained with antibodies

against: (C) GFP (blue, transfection), aPKC or phalloidin (red, F-actin) and Phis3 or Tuj1 (green, beta-III tubulin) to study the effects of overexpressed active Akt (arrows indicate apical depolarizations); (D) GFP (blue, transfection), and Tuj1 (green) to see induction of Tuj1 in the RP (arrows point to transfected RP); (E) GFP (green, transfection), HA (red, tagged Akt) and phalloidin (blue) to analyze expression levels of transfected Akts; (F) GFP (blue, transfection), Ncadherin (red) and Tuj1 (green) to see effects from the kinase-dead Akt.

7.2 Rho GTPases are regulated by PI3Kα activity in the neural tube and they are necessary for the PI3Kα induced epithelial aberrations

Afterwards, we focused our attention in another downstream protein family, the Rho GTPases, involved in cell polarity and actin cytoskeleton organization and whose activation can be also mediated by PIP3 through interaction with PH-containing GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins) (Gassama-Diagne and Payrastre, 2009). The main members of the Rho family are Cdc42, Rac1 and RhoA and, in a highly summarized way, they regulate filopodial extensions, lamellipodia protrusions and stress fibers respectively. In migrating cells, Cdc42 and Rac1 are localized at the leading edge (front) mediating cellular protrusions and RhoA localizes mainly at the trailing edge (rear) driving retraction of the membrane through acto-myosin contraction. In the CNS, Rho GTPases have been reported to participate in neuronal development and differentiation by controlling migration and neurite outgrowth. An example of their function in neurons is that Cdc42 and Rac1 promote the formation of lamellipodia and filopodia at the axon growth cone and interference with both can block axon formation. Instead, RhoA mediates growth-cone collapse and neurite retraction. Likewise, Cdc42 and RhoA coordinate adherent junction assembly and maintenance respectively in the luminal surface of the neuroepithelium (Azzarelli et al., 2014; Iden and Collard, 2008). Therefore, they were good candidates to explain the structural aberrations caused by excess of PI3Kα activity and to further understand the endogenous processes behind the NT development. We studied them following similar approaches to the previous Akt section.

In the first place, we examined whether Cdc42, Rac1 or RhoA were required for generating the described phenotype or, in other words, we tried to hamper the aPI3Kα induced malformations in the chick NT by co-electroporating with myc-tagged dominant-negative (DN) forms of each Rho GTPase: Cdc42T17N, Rac1T17N and RhoAT19N (Etienne-Manneville and Hall, 2001) (Figure 30C). It is worth noting that individual transfection of these mutants in the chick NT barely caused structural defects: mild and rare depolarizations when using DN Cdc42 or Rac1, and no phenotype for RhoA DN. Nevertheless, when combined they did induce apical alterations revealing their dominant-negative activity (data not shown).

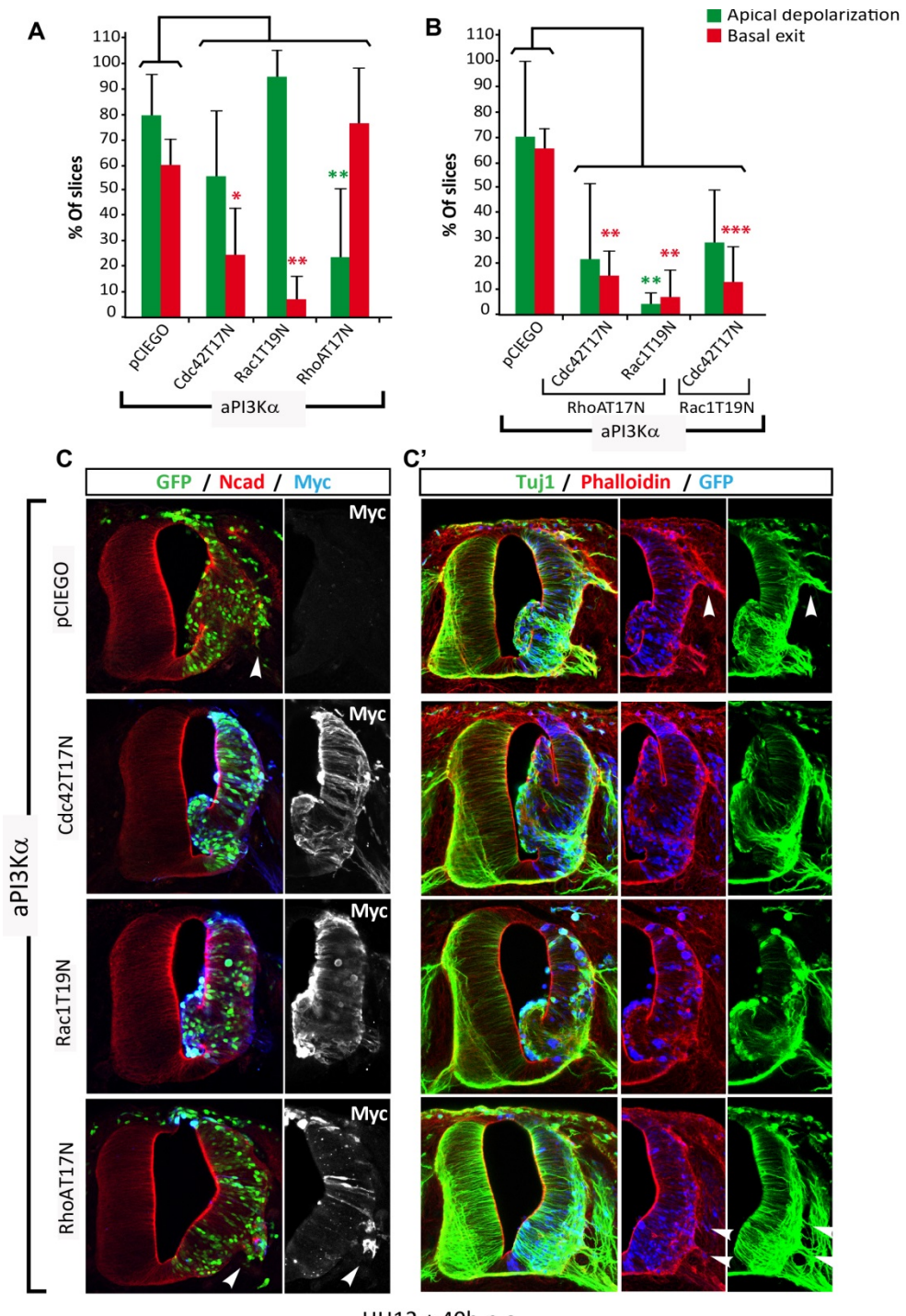


Figure 30. PI3Kα promotes βtubulin III expression. (A-B) The bar graph shows the percentage of sections from chicken embryos electroporated at HH12 with the indicated DNAs that presented loss of apical polarity (green) and/or basal exits (red) for each treatment after 40hrs. The number of sections studied was: (A) aPI3Kα/pCIEGO=59, aPI3Kα/Cdc42T17N=56, aPI3Kα/Rac1T19N=33, aPI3Kα/RhoAT17N=56; (B): aPI3Kα/pCIEGO=36, aPI3Kα/RhoAT17N/Cdc42T17N=52, aPI3Kα/RhoAT17N/ Rac1T19N=25, aPI3Kα/ Cdc42T17N/Rac1T19N=69. Significant differences were tested by one-way ANOVA followed by the Tukey's test (*=P<0.05; **=P<0.01; ***=P<0.001). **(C-C')** Representative images from experiment (A) stained with antibodies against: (C) GFP (green, transfection), Ncadherin (red) and Myc (blue, tag) to see Rho GTPase expression levels; (C') GFP (blue, transfection), phalloidin (red) and Tuj1 (green) to study the NT structure (arrows indicate basal breaches).

When we performed the aforementioned experiment to neutralize the aPI3Kα induced phenotype, we got two interesting results: on the one hand, inhibition of RhoA activity almost neutralized all apical depolarizations, defined as disruptions of the apical line; and on the other hand, inhibition of Cdc42 and Rac1 significantly decreased the number of basal breaches made by axons and/or cell bodies (Figure 30A, C').

Afterwards, we attempted to completely neutralize the PI3Kα induced aberrations by combining RhoAT19N, which avoids apical depolarizations, with either Cdc42T17N or Rac1T17N whose inhibition seemed to stop basal exits. As an extra experimental condition, we put Cdc42T17N and Rac1T17N together (Figure 30B). As expected, both apical and basal events were significantly erased, although further conclusions are hard to obtain from this experiment since Rho GTPases can regulate each other's activity through crosstalk: Cdc42 can activate Rac1 and Rac1 can downregulate RhoA activity (Iden and Collard, 2008).

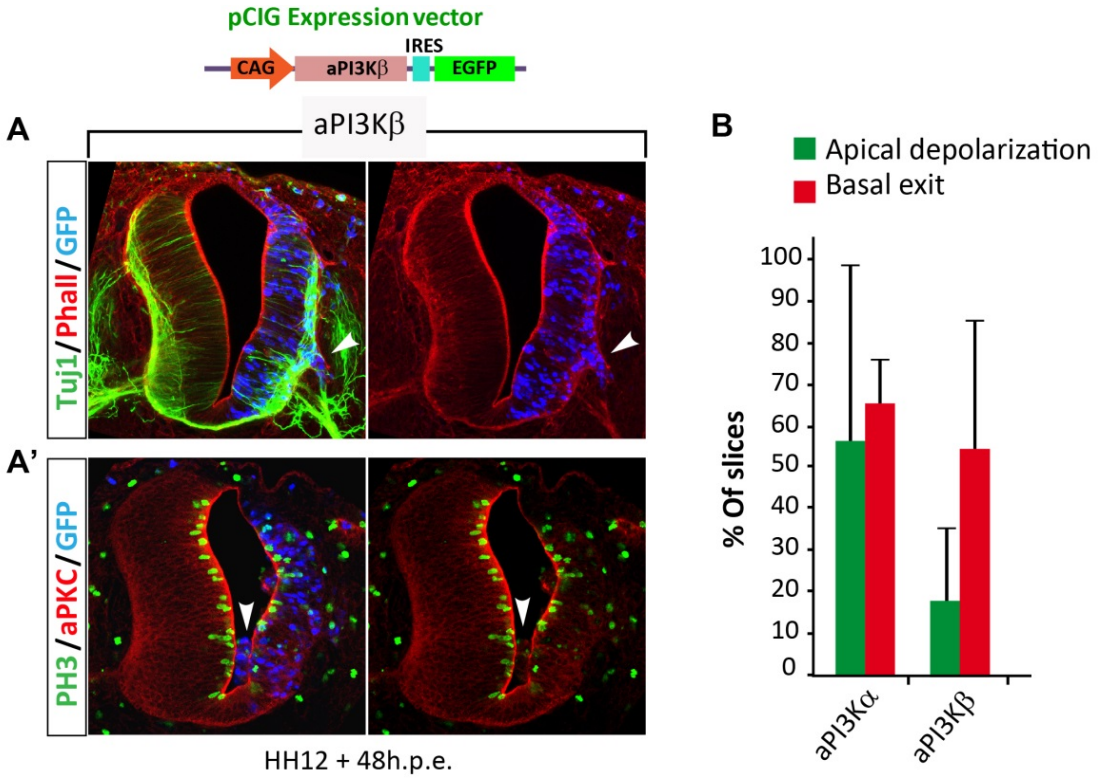
Afterwards, we tried to mimic PI3Kα dependent phenotype by transfecting GTPase-defective constitutively active forms of Cdc42 (G12V), Rac1 (Q61L) or RhoA (Q63L) (Vanni et al., 2005) in HH12 embryos and analyzing them 48hrs later. Results from this experiment are not shown since the effects on the NT were too severe and even lethal in the case of RhoAQ63L. This is not surprising due to the fine regulation in space and time they normally have and their role as molecular switches in diverse signaling pathways, which complicate the interpretation of the effects. Consequently, a finer experiment should be designed in this case.

In any manner, these results support the role of Rho GTPases as the main downstream factors regulated by PI3Kα regarding AB polarity, positioning and cytoskeletal changes during neuronal differentiation in wild-type conditions.

8. p110β might have specific functions in the neural tube compared to p110α (preliminary)

Despite their high homology, most reports agree on non-overlapping functions for p110α and β linked to differential preferences for membrane receptors, different levels of specific activity and different ages at which homozygous mouse embryos with deletions for either PIK3CA or PIK3CB genes die (Bi et al., 2002; Burke and Williams, 2015). In order to study the role of p110β in the developing spinal cord after defining functions concerning the p110α subunit, we performed gain and loss of function experiments as previously done for the alpha subunit in HH stage 12 chick embryos and analyzed the phenotype 48hrs later (Figure 31). Regarding the gain of function, we overexpressed a wild-type form of p110β with the same non-inhibitory regulatory subunit (p85αW333R) used to perform most of the previous experiments. This active form will be named as aPI3Kβ. Preliminary results obtained from this experiment revealed a phenotype apparently similar to the one caused by the active alpha subunit consisting of apical depolarization events with ectopic cell masses in the ventricle and mislocalized mitosis (Figure 31A') as well as disruption of the basement membrane followed by the exit of axons and cell bodies (Figure 31A). However, we also observed that these malformations were significantly less aggressive, as observed by the reduced magnitude and number of depolarization events (Figure 31A', B). Concerning the p110β knock down using specific shRNAs, preliminary results showed a differential outcome characterized by the appearance of rosettes in the most dorsal region of the NT formed by groups of cells organized concentrically around a new lumen. Additionally, the Roof Plate appeared often thickened. None of these phenotypes was ever observed after knocking down p110α (Figure 31C).

These results suggests that both isoforms could have overlapping functions, but they also support that they are not functionally identical and imply possible exclusive functions for the p110β isoform in the dorsal NT that might shed some light upon the unresolved issues concerning p110α/β specific roles.



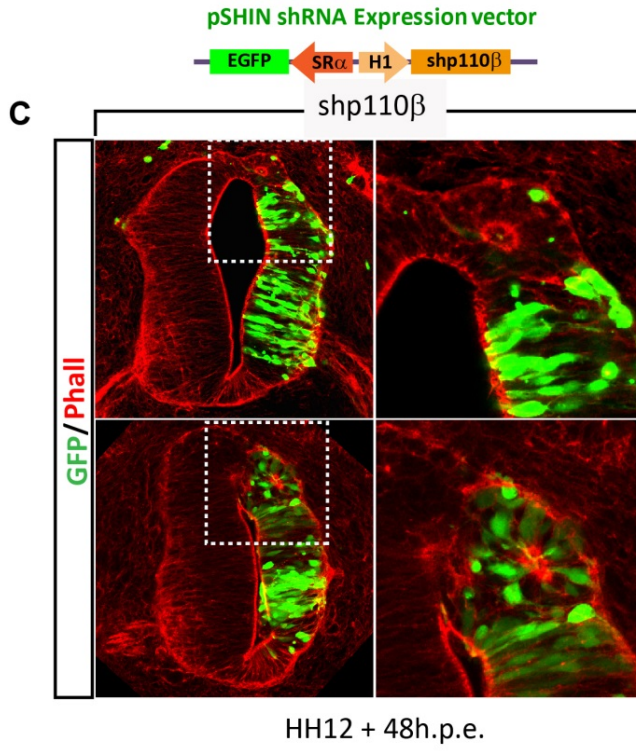


Figure 31. p110β gain and loss of function phenotypes suggest differential functions in the neural tube. (A,A') Representative images of chick neural tubes electroporated with aPI3Kβ and analyzed 48hpe, stained against: (A) GFP (blue, transfection), phalloidin (red, F-actin) and Tuj1 (green); (B) GFP (blue, transfection), aPKC (red) and PH3 (green). Arrows indicate a basal breach (A) and apical depolarizations (A'). **(B)** The bar graph shows the percentage of sections from experiment (A,A') that presented loss of apical polarity (green) and/or basal exits (red). The number of sections studied was: aPI3Kα=83, aPI3Kβ=36. **(C)** Representative images of chick neural tubes electroporated with shp110β and analyzed 48hpe, stained against: (A) GFP (blue, transfection), phalloidin (red, F-actin).

9. PI3Kα affects cell size of neuroepithelial cells during M phase (preliminary)

In the section 1.2 of this thesis, we notified the accumulation of overexpressed p110α in absence of exogenous regulatory isoform in some mitotic cells (Figure 32A). Hence, we hypothesized that p110α might have a specific role during M phase in neural progenitors of spinal cord and we carried out some experiments to study its role. Phosphatidylinositol lipid members are essential for defining membrane domain identity, which is ultimately controlling cytoskeleton and membrane dynamics during different stages of mitosis in certain cell types (Campa et al., 2015), but there are no studies addressing the role of class IA PI3K in mitotic NEP cells. Consequently, we performed electroporation at HH10 overexpressing wild-type PI3Kα to force stable expression of p110α, resulting in the presence of significantly oversized spherical mitotic NEP cells at 24hpe (Figure 32B). The size of these cells was quantified by measuring the area of p110α overexpressing ventricular cells compared to non-electroporated ventricular cells. Absolute numbers are displayed in Figure 32B' confirming the significant increase in cell size when overexpressing wtPI3Kα. It is worth noting two observations from experiments carried out at later post-electroporation time points (40hpe, data not-shown): firstly, transfected p110α alone only stabilized in mitotic cells very occasionally at later times; and secondly, mitotic cells overexpressing wtPI3Kα were less oversized, almost similar to non-electroporated cells.

Since cell shape and size are very important for both spindle positioning and stability (Cadart et al., 2014), and it has been reported that PIP3 concentrates at the cell midcortex to regulate dynein/dynactin recruitment and spindle orientation (Silio et al., 2012), our next step was analyzing the mitotic spindle of wtPI3Kα overexpressing cells using a co-transfected α-tubulin fused to GFP to visualize the mitotic spindle (Figure 32C). Preliminary results showed apparently normal spindle formation in most of the cells suggesting that PI3Kα overexpression is altering cell shape and/or size and, consequently, that sometimes leads to aberrant mitotic progression, but it is not directly disturbing mitotic spindle formation.

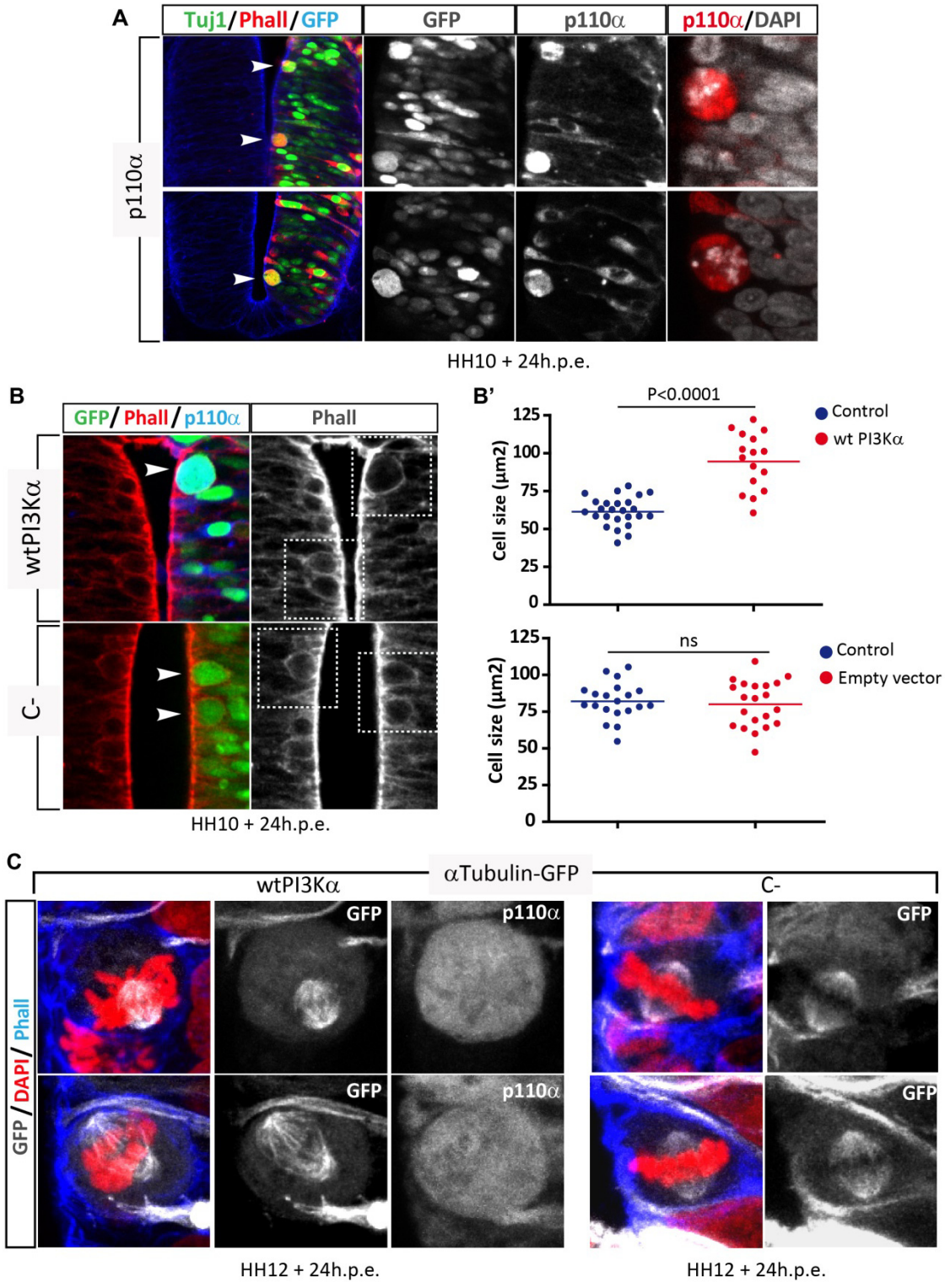


Figure 32. Overexpressed p110α stabilizes in cells undergoing mitosis. (A) Neural tubes electroporated at HH12-14 and analyzed 24h later were stained with antibodies against GFP (green transfection), p110α (red) and phalloidin (blue). Arrowheads point to electroporated cells in the VZ stabilizing high levels of p110α. The middle panels show amplifications of the electroporated area. Enlarged images at the right of the panel show staining with DAPI (grey) and p110α (red) of celsl undergoing mitosis. **(B,B')** Representative images of chick neural tubes electroporated with wtPI3K or empty vector (C-) and stained against GFP (green transfection), p110α (blue) and phalloidin (red). Cells within the squares

were measured (area in μm^2) to compare electroporated vs non-electroporated sides (B'). The number of cells counted (n) was: wtPI3Kα=16 & C-=25; empty vector=21 & C-=20. Differences were tested by the Student's t-Test. **(C)** Representative images of chick neural tubes electroporated with wtPI3K or empty vector (C-) and α-tubulin-GFP to study the mitotic spindle and stained against GFP (gray, transfection), p110α (gray separately), DAPI (red) and phalloidin (blue).

Later on, we wondered about the subcellular distribution of PI3Kα substrate (PIP2) and product (PIP3) in mitotic cells, so we took advantage of the "PIP3 sensor" previously used in the 6.1 section (PH-Akt) and a "PIP2 sensor" formed by the the pleckstrin homology domain of phospholipase Cδ1 (PHD), a high-affinity marker for PtdIns(4,5)P2, fused to GFP (Janetopoulos et al., 2005; Martin-Belmonte et al., 2007). These two sensors were transfected at HH stage 12 at very low concentration (50ng/ul) and analyzed 24hrs later (Figure 33) revealing striking differences in the distribution of both sensors during mitosis. For PIP3 subcellular localization, around 50% of cells in prophase and/or metaphase were accumulating PIP3 exclusively on the most basal part of the cell body, just on the base of the basal process (Figure 33A-B) whereas this basal concentration was not so clear on cells undergoing ana/telophase or cytokinesis (Figure 33C). Relative to the "PIP2 sensor" distribution, a general feature detected was its almost exclusive membrane location compared to the other sensor. Moreover, PIP2 was also basally enriched in prophase/metaphase, but in this case simultaneous apical and lateral accumulations were additionally detected (Figure 33D, E). Interestingly, during ana/telophase/cytokinesis, PIP2 concentrated on the cleavage furrow (Figure 33F) coinciding with the model reported by Janetopoulos et al., 2005 in *D. discoideum* where PTEN moves to the furrow to promote acto-myosin-based contractions.

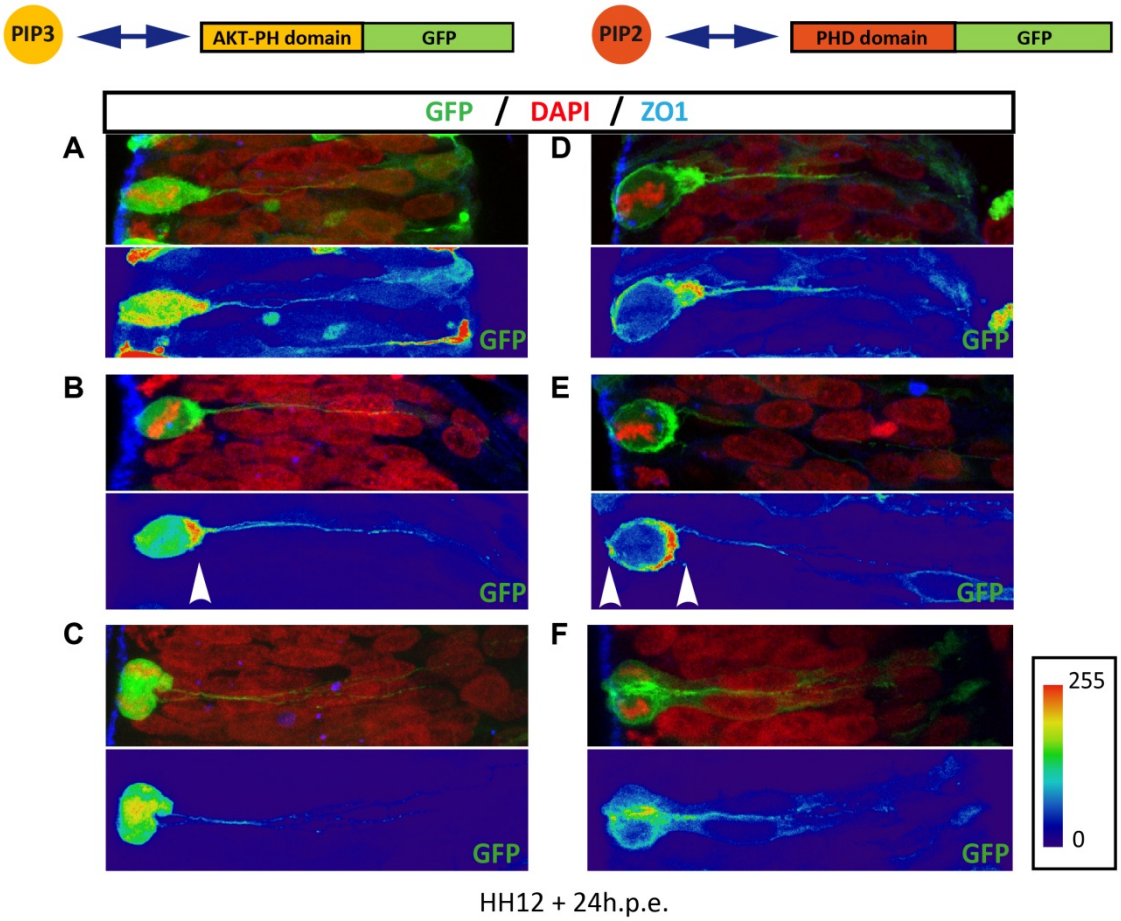
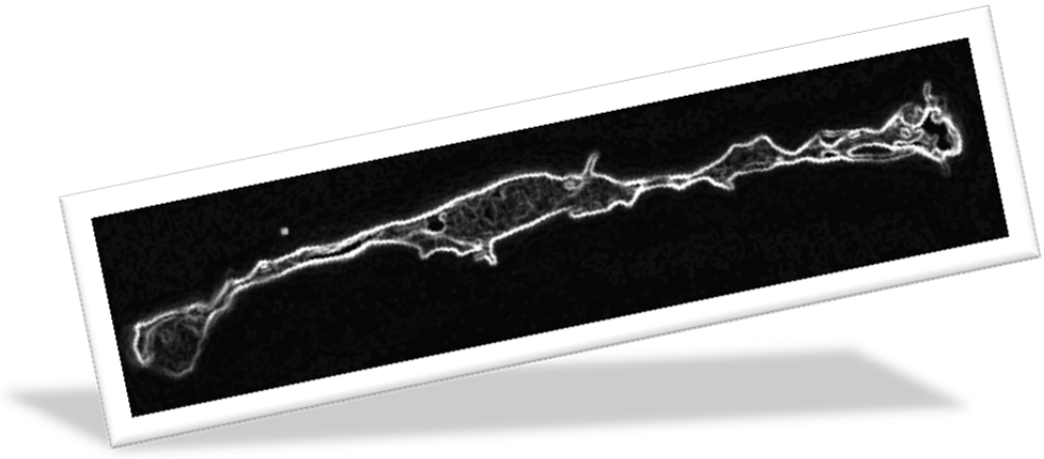


Figure 33. PIP2 and PIP3 have specific localizations during mitosis in NEP cells. (A-F) Neural tubes electroporated with PH-Akt-GFP (A-C) or PHD-GFP (D-F) at HH12 and analyzed 24h later were stained with antibodies against GFP (green transfection), DAPI (red) and ZO1 (blue, apical side). **(A,B,D,E)** Mitotic cells in metaphase show spread (A) or basal accumulation (B, arrowhead) of PIP3, and basal and apical enrichment of PIP2 (D-E, arrowheads). **(C,F)** Mitotic cells in ana/telophase/cytokinesis showing intracellular distribution of PIP3 (C) and PIP2 concentration at the cleavage furrow (F).

From these results, we concluded that PIP2 and PIP3 have a spatial and temporal regulation along the M phase and misregulation of PI3Kα seems to have important effects over the size and shape, but there are still many open questions to address.

DISCUSSION



Discussion

The development of the neural tube is a complex process that requires coordinated morphogenetic forces together with localized extracellular signaling and controlled activation of genes involved in neural induction. In this work, we intend to decipher the role of Class IA PI3K proteins as signal transducers during early developmental stages of the spinal cord, which is anatomically the simplest and most conserved region of the vertebrate CNS and, therefore, it is a good model for studying neural polarity, cell division, neurogenesis or patterning among other processes.

Although Class IA PI3Ks are known to be important for the development of the central nervous system, it has not been described in detail their expression pattern and molecular functions in the embryonic spinal cord *in vivo.* In this work, we provide evidences of the participation of PI3Kα in (1) survival and in (2) apico-basal polarity of the neuroepithelium during progenitor expansion stages, the latter through modulation of RhoA activity. Additionally, upon the onset of neurogenesis, we propose it as a basal signal involved in (3) neural migration and (4) differentiation, based on its effects on neural lamination and axonal growth, via activation of Cdc42 and/or Rac1. These functions seem to be mainly mediated by regulated PIP3 production, and not protein-protein interaction, since PTEN overexpression was able to efficiently rescue the gain of function phenotypes.

Since PI3Ks are a numerous family of proteins with overlapping expression patterns, interactors and functions, it is important to gain further knowledge about the tissue-specific role of each isoform in order to understand their pathogenic effects in disease or their potentiality as therapeutic targets. Regarding the CNS, the high dynamism of PIP3 production and degradation combined with organization of the neural membrane into functionally distinct and non-static microdomains during development complicate the task of unraveling PIP3 cellular distribution as well as PIP3-dependent mechanisms involved in neural survival or maturation.

1. Class IA PI3Ks and the neural tube

Due to the high homology among Class IA catalytic or regulatory subunits, it has been difficult to decode the patterns of expression of specific isoforms at the protein level because of the lack of antibodies. That is why the previous studies and the present thesis have mostly performed analysis of mRNA expression to obtain that information. It was already known that Class IA PI3Ks were expressed in the central nervous system (CNS) and it had been also reported that this expression was not fixed during time. For example, p110α decreased in expression during postnatal development and in adult

tissue in the mouse nervous system (Waite and Eickholt, 2010). Interestingly, our results also confirmed Class IA PI3K expression in the chick neural tube with changes in expression pattern at different developmental stages. More specifically, we show differential expression of catalytic and regulatory subunits before and after the onset of neurogenesis in the neural tube. Notably, at early stages their expression is abundant in the neuroepithelium (NEP) while, at later stages, Class IA PI3K expression gets enriched in the basal neural population while disappears from the germinal layer. We propose that this different expression is associated to different functions along time, such as survival, mitosis and polarity at early stages and roles in migration and neural morphogenesis at later stages as we will discuss in the next sections.

In addition, we confirm results from previous studies reporting that conformational stabilization of p110α catalytic subunit requires the presence of regulatory subunits since NEP cells of the spinal cord were able to efficiently degrade p110α in absence of regulatory subunit when overexpressed. Notably, the stabilization of overexpressed p110α by the chick endogenous regulatory subunits revealed the same pattern than the mRNA expression (Geering et al., 2007; Yu et al., 1998). The coincidence between the mRNA and protein distribution in the neural tube is also in agreement with the conclusion from Geering et al., 2007 pointing to transcriptional regulation as an important parameter in the regulation of the relative levels of p85 and p110.

2. Neuroprotective role of PI3Kα in the neural tube

We provide evidence that the p110α is needed for survival of progenitors and neurons of the developing spinal cord. Most cells in an organism are fated to die unless their survival is maintained by trophic factors produced from neighbouring cells (Raff, 1992; Suzanne and Steller, 2013). PI3K has been reported to be one of the dominant survival mechanisms in many different neuronal types precisely through signaling transduction of different growth factors stimulating RTKs, such as neurotrophic factors, insulin, FGF or IGF-1, also present in the neural tube (Bartlett et al., 1991; Jungbluth et al., 1997). However, most of the studies have been performed in *in vitro* cultures because homozygous PIK3CA or PIK3CB mouse knockouts were not viable (Brunet et al., 2001; Gross and Bassell, 2014; Segal, 2003). Here, we demonstrated that *in vivo* transient knockdown of p110α in the chick embryonic spinal cord induces massive apoptosis at different stages supporting an isoform-specific survival role for PI3Kα in spinal cord. Results from analyzing PTEN overexpression (data not shown) showed similar outcomes confirming PI3K signaling as a necessary signal in the embryonic spinal cord.

Additionally, we observed that progenitors and neurons showed different sensitivity to the lack of p110α at different developmental times, with higher neural apoptosis at

later stages compared to progenitors, concomitant with the PI3K restricted expression in neurons. That could be explained by the fact that the behavior and the mechanisms for apoptosis differ among dividing cells and neurons, so alteration of the same molecule could have different impacts in both populations. However, the different expression patterns suggest that, after the onset of neurogenesis, Class IA PI3K stops being such an important signal for NEP cells. Moreover, we propose that p110α is needed for survival of differentiated neurons and for cytoskeletal changes during neural migration-differentiation. Consequently, reduction of PI3K signaling in neurons would result in induced apoptosis due to attenuation of the neurotrophic stimuli and/or alteration of the final position or the neural morphology, eventually leading to cell death of misrouted neurons, as it occurs naturally for adjusting cell populations (Hidalgo and ffrench-Constant, 2003).

Consistently, an excess of PI3K signaling has been reported to result in an excess of progenitors and neurons that have severe consequences in the neural tissue architecture. For instance, in a mouse model presenting high PI3K signaling caused by PTEN loss, it was described the formation of enlarged, histoarchitecturally abnormal brains generated by (1) enhanced proliferation and self-renewal in progenitors and (2) augmented soma size leading to brain hypertrophy following PTEN loss only in post-mitotic neurons (Chalhoub et al., 2009; Kwon et al., 2001; Li et al., 2002). In another neural tissue, the retina, it was similarly reported an aberrant development after overexpression of an active form of PI3K, a phenotype partially explained in this case by increased neural survival (Pimentel et al., 2002). In the chick neural tube, high PI3K signaling also generated neuroepithelial aberrations, but we never quantified the final cell numbers after increasing PI3Kα activity or whether we were altering the developmental cell death. Interestingly, although we did not measure the neuronal soma size either, we detected an increased size in PI3Kα overexpressing cells undergoing mitosis. This is in agreement with previous studies supporting a role of PTEN in the regulation of cell size, but in this case it might be also part of the mitotic machinery (Cadart et al., 2014; Campa et al., 2015; Kozma and Thomas, 2002). In any manner, evidences point to p110α as an important survival signal in neurons.

3. PI3Kα is a molecular regulator of apico-basal cell polarity in the neural tube

3.1 PI3Kα and apico-basal polarity

In order to study Class IA PI3K functions in the neural tube, we performed loss and gain of function studies, the latter to avoid apoptosis and mimic what normally occurs in

pathological conditions, which is an increase in Class IA PI3K signaling (Wymann and Marone, 2005). For that, we took advantage of PI3Kα active forms based on oncogenic mutations that alter the inhibition exerted by the regulatory subunit on the catalytic one, but preserving the interaction to avoid degradation of the catalytic subunit (Berenjeno and Vanhaesebroeck, 2009; Gabelli et al., 2010).

This way, we have described how alterations in the amount, location and activity of PI3Kα and, for extension, of PIP3 generate severe abnormalities in the spinal cord architecture, which is a NEP tissue characterized by AB polarity. Previous articles have linked PIP3 production with establishment of AB polarity in epithelial cells and linked PIP3 concentration to basolateral membrane determination. Segregation of PIP2 and PIP3 in the plasma membrane seems to be crucial to define apical and basolateral membrane identity in some epithelial cells. For instance, in MDCK (Madin-Darby canine kidney) cysts PIP2 becomes enriched at the apical membrane domain due to strong PTEN localization, delimiting the lumen during cyst formation by recruitment of apical proteins such as annexin 2 or Cdc42, which activate the Par complex. In contrast, PIP3 is restricted to and specifies the basolateral surfaces of these cells through localization/interaction of PI3K with basal protein complexes like Dlg or after contact with laminin surfaces (Gassama-Diagne et al., 2006; Martin-Belmonte and Mostov, 2007; Roignot et al., 2013). Concerning neuroepithelial tissues, there is very little work done addressing how the NEP establishes and maintains AB polarity through phospholipid asymmetry, but some evidences support similar mechanisms of membrane segregation and protein recruitment in establishing neuroepithelial AB polarity (Ghosh et al., 2008; Grego-Bessa et al., 2016; Roy et al., 2015). For example, Ghosh et al., 2007 propose that simultaneous activation of Rac/Cdc42 and PI3K/PDK1 regulates aPKCζ-dependent neural progenitor cell adhesion in the embryonic spinal cord. And Grego-Bessa et al., 2016 demonstrate a link between PI3Kα activity and PDK1 activation in neural epithelium.

We add more evidences in this line, since we showed that alterations in PI3Kα activity severely disrupted neural tube architecture. Active PI3Kα overexpression at HH12 disrupted the AB polarity of the NEP as well as the distribution of the accumulated apical complex proteins (Ncadherin, aPKC, ZO1) already after 17hpe. At later post-electroporation time points, maintenance of high and mislocalised PI3Kα activity induced cell-autonomous neuroepithelial malformations characterized by the presence of cell masses with disrupted polarity inside the ventricle. Disorganization of apical markers, normally concentrated or even restricted to the most apical membrane, seemed to cause also mislocalization of mitotic cells at any position in the NEP, as previously reported after ectopic accumulation of apical markers as Par3 and aPKC (Afonso and Henrique, 2006; Herrera et al., 2014). This likely occurs because the INM is a delicate process and it is affected by changes in polarity, so the nucleus cannot move properly and the cell enters mitosis at any place. More severe depolarization events

even showed generation of new ventricles inside the neural tissue originated from the original one by bending of the most apical side of the tube. Importantly, these lumens were not newly generated like the rosettes created by Par3 overexpression inducing new apical junctional complexes (Afonso and Henrique, 2006), but apparently by reorganization of the existing polarity complexes.

Because of these structural aberrations, the laminar organization characteristic of the neuroepithelial tissue, with an apical germinal layer (ventricular zone) and basal layer of neural differentiation (mantle zone), was no longer preserved. Notably, we noticed that the general structure of the neural tube was somehow maintained because most of mitotic cells were still found around the ventricle and differentiated neurons migrated away of the new ventricle without significant changes in the total number of mitotic cells. We discarded alterations in the cell cycle because the total number of cells undergoing M phase was unaltered. Nevertheless, we observed accumulation of p110α in mitotic cells as well as increased size of those cells overexpressing PI3Kα during mitosis, so we have reasons to think of a role for PI3Kα during mitosis, already reported for other cell types (Campa et al., 2015; Kumar and Carrera, 2007). For that, it could be possible that the number of cells in M phase does not increase because they finally die. Additionally, we ruled out direct changes in proliferation, differentiation or cell fate as initial promoters of the gain of function phenotype in agreement with previous studies in other neural tissues (Marino et al., 2002; Roy et al., 2015). And we also observed similar loss of polarity when downregulating p110α expression, concluding that PI3K is mainly a regulator of AB polarity in the neuroepithelium.

3.2 RhoA as an effector of PI3Kα in apico-basal polarity

The ability of PI3Kα to modulate AB polarity at the membrane of NEP cells might involve various mechanisms. Segregation of PIP2 and PIP3 might contribute to define membrane identity by inducing the relocalization of polarity components, such as the apical Par complex (Par3/Par6/aPKC), in which aPKC needs to be phosphorylated by the PIP3 recruited PDK1 to be fully activated (Ghosh et al., 2008). In addition, phospholipids regulate both endocytic and exocytic processes, such as PIP2 which controls targeting of the exocyst to the plasma membrane (Roignot et al., 2013; Thapa and Anderson, 2012). An additional mechanism might involve the regulation of Rho GTPases, crucial in cell polarization through regulation of cytoskeleton remodeling (Iden and Collard, 2008). The main family members of Rho GTPases are RhoA, Cdc42 and Rac1. In the developing spinal cord, RhoA is essential for neurulation (Kinoshita et al., 2008) and, afterwards, to maintain AB polarity by maintaining Ncadherin-based cell-cell adhesions and the apical actin belt through localization of mDia1 (an actin nucleator) to the AJCs (Herzog et al., 2011; Katayama et al., 2011). Cdc42 has been also linked to the apical localization of AJC proteins such as Par6, aPKC, E-cadherin or beta-catenin in more cranial areas of the neural tube, but its role seems more important in

the initial formation of AJC than in the maintenance (Cappello et al., 2006; Chen et al., 2006). Although Rac1 and Cdc42 share many effectors, Rac1 does not seem specifically required for AB polarity in the embryonic NEP (Leone et al., 2010). Interestingly, our results support previous observations concerning the role of Rho GTPases in NEP polarity and propounding PI3Kα activity as an upstream regulator, while discarding Akt activation as the main downstream event. Our data in the chick neuroepithelium showed that inhibition of RhoA activity was sufficient to rescue the apical depolarization phenotype promoted by an excess of PI3Kα activity suggesting a requirement and tight modulation of PI3Kα/PTEN activity in the maintenance of AJCs through control of RhoA and, probably, the modulation of the AJ-associated actomyosin ring (Takeichi, 2014).

Concerning the role of Akt in this complex phenotype, it was surprising to find out that Akt might be participating, but it was not the main PI3Kα downstream effector in this system. Transfection of targeted membrane Akt in the neural tube unveiled an interesting capability of active Akt to induce loss of polarity in neuroepithelial cells, but it was not able to reproduce the active PI3Kα dependent basal phenotype. Likewise, a dominant negative form of Akt was not able to rescue either apical or basal neuroepithelial malformations. It is worth noting that the Akt isoform used for these experiments was Akt1, which has ubiquitious expression and crucial roles in cell survival (Gonzalez and McGraw, 2009). Instead, it could be that the same experiments perfomed with Akt3, the main isoform expressed in brain (Easton et al., 2005; Gonzalez and McGraw, 2009), might result in a different outcome. But independently of a potential participation of Akt3 in the described roles of PI3Kα, we have demonstrated that PI3Kα take part in the development of the neural tube through localized regulation of Rho GTPases.

4. PI3Kα and neural lamination

4.1 PI3Kα activity might promote basal migration during neural maturation

Another cellular phenotype due to active PI3Kα overexpression in chick neuroepithelium was the alteration of basal markers such as laminin (staining the basement membrane). This is not unexpected since disruption of AB polarity would likely lead to alterations in the basal side, although it does not happen in all phenotypes involving neuroepithelial depolarization events. For example, after active aPKC misexpression, an instructive signal of apical compartimentalization of adherens junctions, the chick neural tubes showed disruptions in the continuity of the basal

lamina and abnormal delamination of progenitors and neurons streaming out of the neural tube (Ghosh et al., 2008). However, disruption of RhoA in the neuroepithelium led to loss of VZ integrity without alteration of the basal morphology (Herzog et al., 2011). After transfection with active PI3Kα, we also observed abnormal basal events, like in the active aPKC mutant, but slightly different. It seems that in the aPKC phenotype, alteration of progenitor AB polarity might result in basal lamina alterations and cells could stream out of the neural tube at sites where the basal lamina was affected simply because they no longer had a physical barrier. This ability of neural cells to "run away" from the neural tube has been also reported in other cases where that basal lamina is affected supporting an intrinsic capacity of these cells to migrate in absence of barriers. For example, overmigration is the mechanism of cerebral cortical malformations in syndromes of congenital muscular dystrophies caused by defects in O-mannosyl glycosylation, important for basement membrane integrity (Hu et al., 2007). Likewise, elimination of the BC (Boundary Cap) cells at the Motor Exit Points results in motor neuron cell bodies migrating out of the spinal cord (Chauvet and Rougon, 2008). Alternatively, we propose another explanation for the basal PI3Kα dependent phenotype not based directly on basement membrane disruptions or absence of barriers, but in an enhanced migratory capacity. We support this hypothesis in three observations: (1) shortly after PI3Kα hyperactivation, we found NEP cells still polarized, without apical disruptions, but extending F-actin based membrane protrusions throughout the basal membrane, (2) subsequent disruption of basal lamina and cell body migration did not required apical depolarization and (3) migratory events were often concomitant with bending of the unaffected apical side of the tube, probably because cells streaming out express Ncadherin, so they were attached to the wild-type progenitors and pulled the neural tissue basally. We did not check for protease expression or performed in vitro cell invasion assays to discard protease activity as the reason for the lamina breaches, but the shapes of aPI3K overexpressing neural tubes suggest that those membrane breaches could be originated also from mechanical forces, as shown by O'Toole et al., 2015 in the growth cones. Notably, the mouse model conditionally expressing an oncogenic form of p110α in radial glial progenitors (hGFAP-cre;H1047R) from Roy et al., 2015 also showed disruptions in the pial surface (equivalent to the basement membrane, but in the brain cortex) associated with irregular clusters of enlarged radial glial end-feet as well as NeuN-positive mature cortical neurons within the normally cell-sparse marginal zone. These findings support a role of PI3Kα in neural lamination in the embryonic CNS.

We confirmed this "basal role" of PI3Kα through detection of a significant and consistent basal accumulation of PIP3 in PI3Kα overexpressing cells using the PH domain of Akt fused to a GFP. Basal actin membrane protrusions traversing the basal membrane were coincident with the basal PIP3 enrichment, supporting PIP3 as the promoter. We did not analyzed PIP3 levels in the apical membrane, so we cannot propose a model for PIP2/PIP3 distribution in the membrane as in other epithelial

models (Gassama-Diagne et al., 2006; Martin-Belmonte and Mostov, 2007), but our observations would suggest a more dynamic role for PIP3 in the neuroepithelium, dependent on the cell identity (progenitor vs neuron) and the maturation stage of the post-mitotic neuron. It would be really interesting to analyze the PIP3 membrane distribution in wild-type migrating post-mitotic neurons through *in vivo* imaging in the neural tube and deciphering the molecular mechanisms modulating local PIP3 production. For the second question there are already two proposed mechanisms, briefly mentioned in the introduction referring to the local production of PIP3 and the directional transport of PIP3-containing vesicles, but these have been observed specifically in axonal specification in hippocampal neurons (Horiguchi et al., 2006; Toriyama et al., 2006).

Notably, our results suggest that these basal effects caused by PI3Kα hyperactivation are mainly generated by progenitors and immature neurons, possibly because they are receiving this "basal signal" to migrate before time (progenitors) or in high dose (immature neurons). And these effects seem to have a critical embryonic period coincident with the beginning of the neurogenesis, which sets off in the motoneuron domain (Saade et al., 2013). Electroporation at HH stage 18 (24hrs later than HH12, when most of the experiments were performed) caused cell depolarizations, but significantly less ectopic cell migration events and only dorsally. These could be explained by the different plasticity of the tissue, which become more rigid at later developmental stages due to the growth of the mantle zone and marginal layer as neurogenesis proceeds and the progressive thickening of the basement membrane which strengthen the neural tube borders. Moreover, it is consistent with the explanation that the basal membrane disruptions are generated by progenitors and immature neurons. When the transfection is performed at HH stage 12, all the cells are progenitors, but at HH stage 18 there are already lots of neurons differentiating and localizing at their final positions, such as the ventral motoneurons. If all cells had the same potential ability to migrate and make basal breaches, the phenotype in both stages would be more similar. This data is relevant for the severity of PI3Kα induced malformations, since earlier alterations in PI3K signaling would have a higher impact in the neural tube development.

Analysis of PI3Kα function as modulator of apico-basal positioning in the neural tube was confirmed by analysis of the neural tube at later developmental stages, when neurogenesis is more advanced. Interestingly, slight increases in PI3Kα expression resulted in cell bodies embedded in the marginal zone in the motoneuron domain, normally occupied by axonal fibers. Higher induction of PI3Kα signaling brought about the presence of neurons embedded in the somite at any point along the whole dorso-ventral axis as well as the presence of long axons growing throughout ectopic places in the mesoderm never occupied by efferent axons (Bonanomi and Pfaff, 2010). On the contrary, when p110α expression was reduced, the space between the motoneurons

and the edge of the tube was wider than in the control side, explained by a reduction in the apico-basal migration and/or higher neural apoptosis. And additionally, decrease in p110α levels often resulted in the presence of surviving differentiated neurons misplaced apically, in the germinal zone.

4.2 PI3Kα regulates apico-basal positioning and axonal growth through activation of Cdc42 and Rac1

The idea of PI3K as a mediator of cell migratory/invasive events has already been reported in several cell types, as explained in the introduction and reviewed in several articles (Cain and Ridley, 2009; Rorth, 2011). In neurons it is less studied, but there are also evidences supporting a role for PI3K in neural migration, for example in Reelin-dependent migration, although its role in this pathway has not been fully clarified yet (Arcangelo, 2014; Kubasak et al., 2004; Valiente and Marin, 2010). In this line, our results support a role of PI3Kα as a basal signal during neurogenesis for proper lamination in the embryonic spinal cord, although the external stimulus activating this signal basally in the proper moment remains unknown. Moreover, our data show a link between high PIP3 and cytoskeletal modifications involving F-actin and the neuronal beta-III tubulin. It is tempting to think of this mechanism as a possible explanation for the axonal outgrow of motor axons when they exit the spinal cord, a question not elucidated yet, since the gain-of-function phenotype reminds of this neural population.

The competence of PI3Kα/PIP3 to promote enhanced migration and/or actin based protrusions might comprise several molecular mechanisms. Our data in the chick embryonic spinal cord, however, attribute these basal functions, at least partially, to basal activation of Cdc42 and Rac1. These two molecules of the small family of the Rho GTPases have been classically associated to extension of membrane protrusions through actin polymerization and capture of microtubules, processes involved in cell polarization and migration in several cell types and also in neurons (Azzarelli et al., 2014; Iden and Collard, 2008). Interestingly, co-expression of dominant-negative forms of Cdc42 and Rac1 impeded membrane protrusions, lamina breaches and ectopic basal migration induced by magnified basal PIP3 accumulation without neutralizing apical loss of polarity.

It is highly curious the spatial distribution of RhoA on the apical side, and Cdc42/Rac1 in the basal side regarding the downstream pathways modulated by PI3Kα in the neural tube, because it resembles their disposition during cell motility in a very simplified way. Generally, the generation of front-rear polarity is mediated by the localized activation of Cdc42 and Rac1 at the front of the cell, resulting in rapid and dynamic assembly of actin filaments, and RhoA activation at the back of the cell,

promoting the assembly and activation of contractile actomyosin networks (Nelson, 2009). Nevertheless, more studies are needed to understand exactly how these cytoskeletal regulators are adapted to jump from AB polarity regulation to front-rear polarity during migration since, for instance, Cdc42 is needed apically for AJC formation (Cappello et al., 2006; Chen et al., 2006) and, subsequently, in basal axonal growth cones (Grunwald and Klein, 2002; Meyer and Feldman, 2002). In this work, we offer evidences that link basal PIP3 accumulation and Cdc42/Rac1 activation to basal neural migration during differentiation and axonal growth in the embryonic spinal cord.

The next question would be how this PIP3 dependent basal migration and axonal growth events are endogenously regulated to avoid overmigration respecting the CNS boundaries. A good candidate to counteract this signal and delimit the CNS border would be the transmembrane guidance cue Semaphorin6A. Previous work has related Semaphorin expression with control of axonal growth in DRG neurons and spinal MNs (Mauti et al., 2007). Semaphorins are a large family of secreted or membrane-bound proteins shown to regulate axonal pathfinding during development of the nervous system through growth cone collapse, axon repulsion, or growth cone turning by regulating Rho GTPases and suppressing PI3K signaling pathway (Menager et al., 2004; Nakamura et al., 2000). During chick spinal cord development, Sema6A has been found at the transition zone between the peripheral and the central nervous system avoiding emigration of motoneurons (Mauti et al., 2007). Therefore, we hypothesize that in normal conditions, PI3Kα basal signal is controlled by the Semaphorin expression outside the neural tube, avoiding neural somas to stream out throughout the DREZs (Dorsal Root Entry Zone) or MEPs (Motoneuron Exit Poins). However, PI3Kα hyperactivation might overcome the physical (basement membrane) and signaling based (Sema6A) barriers allowing cells to move farther.

5. PI3Kα and neural differentiation

Our results concerning the influence of PI3Kα in the development of the neural tube revealed that uncontrolled PI3Kα activation mainly resulted in polarity alterations, enhanced migration and ectopic axonal growth, without modifying cell fate or neural differentiation. Interestingly, we obtained apparently opposite results when studying neural differentiation using different neuronal markers (beta-III tubulin vs NeuroD or HuC/D). It is important to take into account what type of protein is being used as a marker for the correct interpretation of the results. In our case, the basic helix-loop-helix transcription factor NeuroD and the RNA-binding protein HuC/D did not show alterations when increasing PI3Kα signaling. Surprisingly, we detected a significant rise when using the neuron specific beta-III tubulin, which is a cytoskeletal component

already present in neurogenic divisions and immature neurons (Katsetos et al., 2003a; Memberg and Hall, 1995). Coherently, we observed a significant reduction of beta-III tubulin when knocking down p110α.

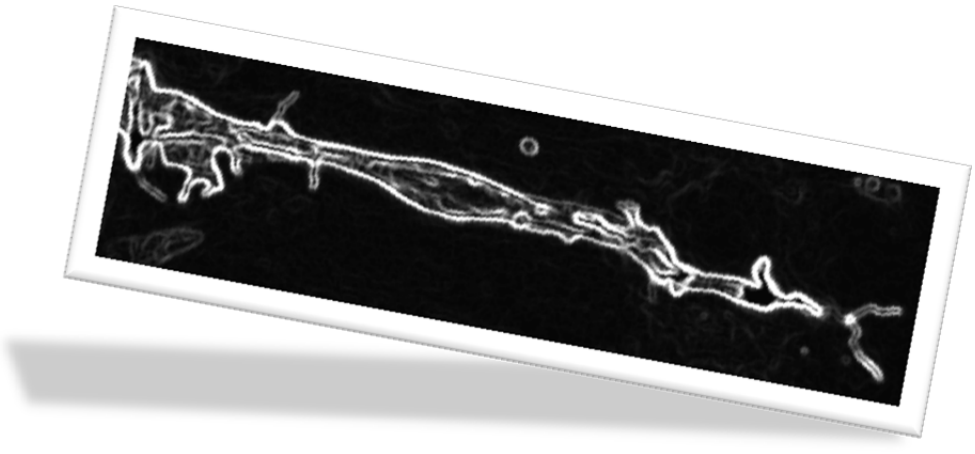
There is little information about the regulation of the beta-III tubulin gene or protein. For instance, the transcription factors AP-2 or Scratch (Scrt) have been linked to promotion of beta-III tubulin expression (Dennis et al., 2002; Nakakura et al., 2001). Interestingly, at the protein level, the regulatory subunits p85α, p55α and p55ɣ (and so p110) have been reported to interact with beta tubulin in brain samples (Inukai et al., 2000; Itoh et al., 1996). More recently, recruitment of active PI3K in the growth cone associated with microtubules after IGF-1 stimulation was demonstrated in hippocampal neurons, in agreement with our observations (Laurino et al., 2005). Nevertheless, more studies should be needed to understand the genetic and proteomic codes that orchestrate the acquisition of neural cell fate and the concomitant morphological changes. Notably, our results demonstrated modulation of beta-III tubulin by PI3Kα activity at the transcriptional and the protein level, unveiling an additional function for PI3K signaling during neural differentiation. This is also consistent with the expression pattern that we described after the onset of neurogenesis, restricted to the mantle zone, and supported by the ectopic beta-III tubulin induction in the non-neural roof plate.

It would be interesting to deepen into the molecular link between PI3K and beta-III tubulin in normal tissues and tumor samples to understand how they influence each other. We have some controversial results with a conditional luciferase reporter (not shown in this report) revealing a significant decrease of the neuroepithelial Sox2 promoter activity and, in parallel, increase of the neurogenic Tis21 promoter activity when increasing PI3Kα activity, which is also confronted with the results presenting unaffected neurogenesis, but in agreement with the increased beta-III tubulin. The ability of PI3Kα to promote beta-III tubulin without inducing neural differentiation (assessed with other neuronal markers such as NeuroD or HuC/D) would imply that neural differentiation involves various mechanisms affecting cytoskeletal and cell identity elements whose intermingled regulation is still not well understood. More neuronal markers should be used to test the effects of PI3Kα activity on neural differentiation. For instance, it could be interesting to analyze whether the transfected roof plate expressing beta-III tubulin is also expressing another neural marker.

A further controversial result was that reduction of p110α did not avoid motor axon exit and growth. This could be expected since the gain of function phenotype showed ectopic emergence of axons crossing the basal lamina border at any point along the dorso-ventral axis and subsequent axonal growth through the mesoderm. Therefore, we had hypothesized that maybe the PI3Kα activity could be involved in the cytoskeletal changes needed for initial motor axon exit and/or in the posterior axonal

growth (Bravo-Ambrosio and Kaprielian, 2011). Nevertheless, inhibition of p110α did not impede motor axon projection. This suggests that (a) PI3K activity might promote axonal growth, but it is not an obligatory signal. Another explanation is that (b) inhibition by shp110α transfection was not strong enough or (c) that the lack of p110α isoform could be compensated by other Class IA catalytic subunit (p110β or δ). A fourth option is that (d) p110α is not the main isoform responsible for axonal growth, but p110δ as proposed by Eickholt et al., 2007. Therefore, increase of p110α activity could promote axonal elongation by production of PIP3, but its absence would not have such a big impact. Finally, high beta-III tubulin expression has been observed in a wide variety of tumors with different implications (Katsetos et al., 2003b). It may be intriguing to see if there is any link between disruptions in the PI3K/Akt pathway and the enhanced expression of beta-III tubulin in regards to therapeutic targets.

CONCLUSIONS



Conclusions

- The catalytic subunit p110α and the regulatory subunits p85α, p55α, p50α and p55ɣ of the Class IA PI3Ks are expressed in the chick embryonic neural tube. They are highly abundant in cycling progenitors before neurogenesis while they get restricted to neurons at later developmental stages.

- The p110α catalytic subunit requires the presence of a regulatory subunit to be stabilized in the neural tube.

- The first aminoacid of the nSH2 domain of the Class IA regulatory subunits is highly important for inhibition of p110 activity.

- PI3Kα is necessary for survival of neuroepithelial cells and neurons of the embryonic spinal cord.

- High PI3K activity generates structural malformations of the neural tube, characterized by apical depolarizations, ectopic mitosis and basal overmigration of progenitors and neurons thoughout the basement membrane.

- Active forms of PI3K induce accumulation of PIP3 at the basal tip of the neuroblasts most likely causing the observed membrane protrusions and enhanced migration.

- PI3Kα regulates apico-basal polarity of neuroepithelial cells partially through regulation of RhoA activity. PI3Kα enhances apico-to-basal cell migration in the neural tube through regulation of Cdc42 and Rac1 activity.

- PI3Kα promotes beta-III tubulin expression and/or stabilization.

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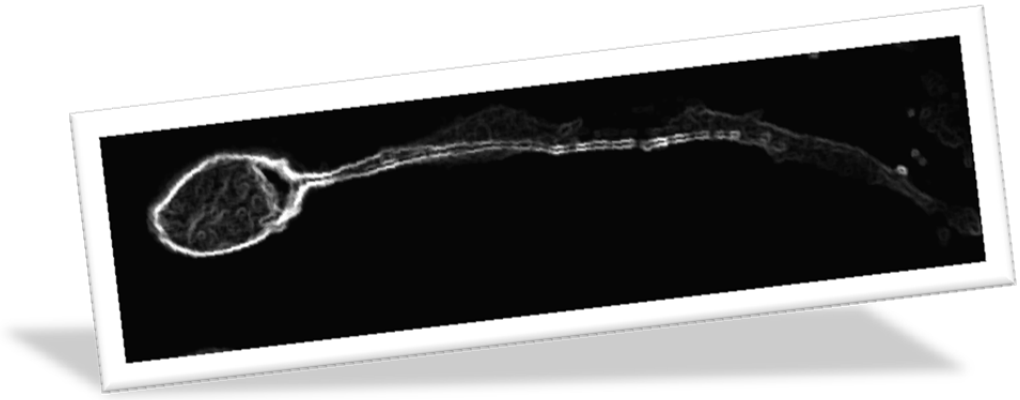

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