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Establishing embryonic territories in the context of Wnt signaling

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ABSTRACT This review highlights the work that my research group has been developing, together with international collaborators, during the last decade. Since we were able to establish the *Xenopus laevis* experimental model in Brazil, we have been focused on understanding early embryonic patterns regarding neural induction and axes establishment. In this context, the Wnt pathway appears as a major player and has been much explored by us and other research groups. Here, we chose to review three published works which we consider to be landmarks within the course of our research and also within the history of modern findings regarding neural induction and patterning. We intend to show how our series of discoveries, when painted together, tells a story that covers crucial developmental windows of early differentiation paths of anterior neural tissue: 1. establishing the head organizer in contrast to the trunk organizer in the early gastrula; 2. deciding between neural ectoderm and epidermis ectoderm at the blastula/gastrula stages, and 3. the gathering of prechordal unique properties in the late gastrula/early neurula.

KEY WORDS: *Xenopus laevis, β-catenin, neural induction, prechordal plate, Tiki, Notum*

Introduction

It is now well documented that a handful of signaling pathways have been co-opted, throughout evolution, to regulate a huge diversity of biological phenomena. This is very clear when studying the embryogenesis of complex animals; if you study closely the development of the limbs, for example, you will learn it holds many molecular similarities to the development of the anterior-posterior body axis (Church and Francis-West, 2002; Endo *et al.*, 1997; Gilbert and Barresi, 2016; Soshnikova *et al.*, 2003). Remarkably, these molecular similarities can be spotted during not only embryogenesis, but also regulating multiple cellular functions during homeostasis on adult life. Which also means that the dysregulation of different signaling pathways is implicated in human disease (Dodge and Lum, 2011; Noah and Shroyer, 2013; Sancho *et al.*, 2004; Veland *et al.*, 2009). Within this context, the Wnt pathway might be the signaling pathway most explored throughout the evolution of metazoans for morphogenesis and cell differentiation during embryogenesis as well as in many diseases and birth defects (Amado *et al.*, 2014; Lee *et al.*, 2006; Petersen and Reddien, 2009).

Our investigation has been concentrated in the canonical Wnt signaling pathway also known as β-catenin-dependent pathway. Briefly, in the absence of Wnt ligands, a family of secreted glycoproteins, cytoplasmic β-catenin is phosphorylated and triggered for proteasomal degradation by the so-called destruction complex. This complex is formed by axin, glycogen synthase kinase 3 (GSK3β), casein kinase 1α (CK1 α) and protein that when mutated causes adenomatous polyposis of the colon (APC), which phosphorylates serine residues in β-catenin and lead to its ubiquitination by β-Trcp followed by proteasomal degradation (Fig. 1). When Wnt ligand is present in the extracellular space, it binds to the Frizzled receptor and its coreceptor low-density 5/6 receptor-related protein lipoprotein (LRP5/6), recruiting the disheveled adapter protein (Dvl) and the axin to the receptor complex (Kim *et al.*, 2013). This signal promotes the disassembly of the cytoplasmic destruction complex, leading to the accumulation and stabilization of β-catenin in the cytosol. Then, β-catenin is translocated to the nucleus, where it is

Abbreviations used in this paper: GSK3b, glycogen synthase kinase 3.

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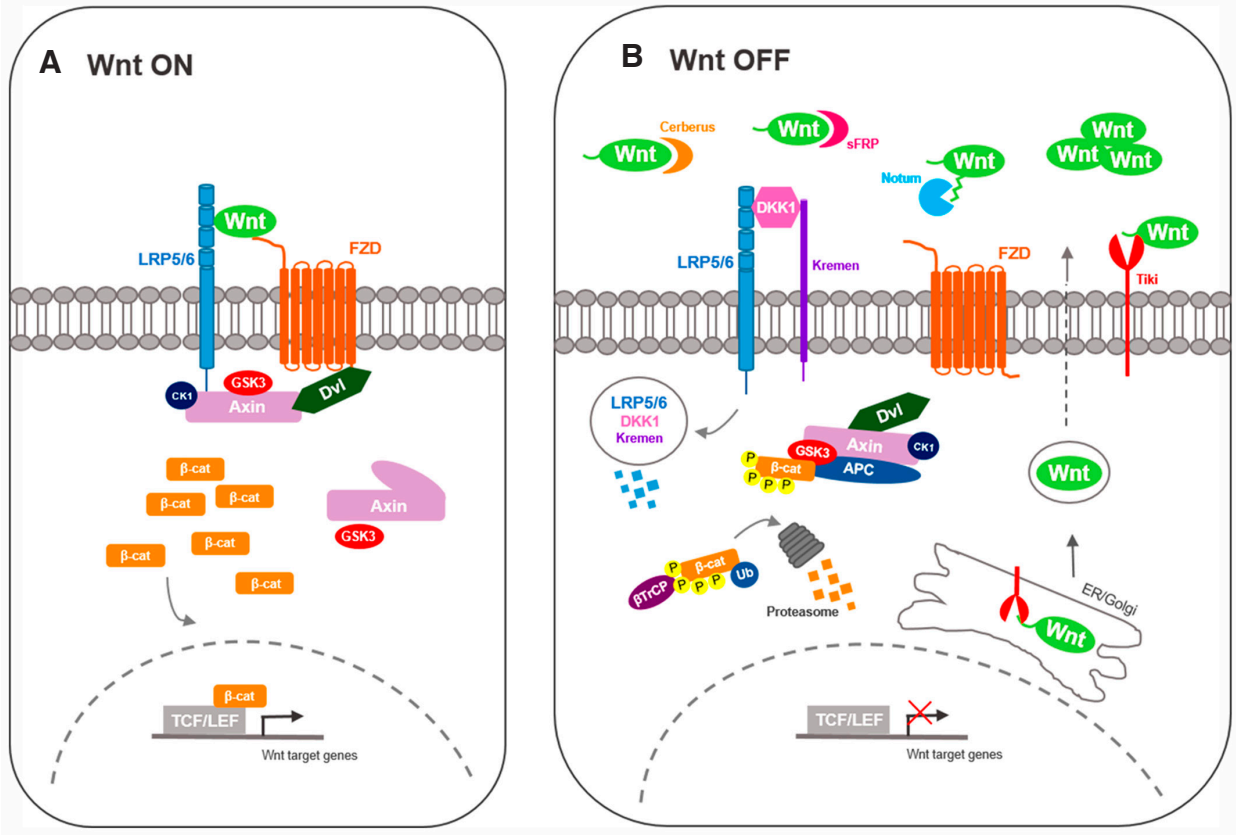

Fig. 1. Schematic representation of the Wnt/β-catenin signaling pathway and different ways it can be inhibited. (A) *Wnt ON, See text for details* **(B)** *Wnt OFF: Extracellular Wnt antagonists such as secreted frizzled-related proteins (sFRP1-5) and Cerberus sequester Wnt ligands in extracellular spaces. Secreted protein Dickkopf1 (DKK1) competitively binds to Wnt ligand-receptor LRP5/6, which leads to a ternary complex formation with the Kremen receptor (Kre), a transmembrane protein, followed by endocytosis and degradation of the LRP5/6 receptor. Notum, a Wnt deacylase, hydrolyzes the Wnt palmitoleoylate adduct extracellularly, resulting in inactivated Wnt proteins that form oxidized oligomers incapable of receptor binding. Tiki-1, a transmembrane protein with proteolytic activity, cleaves a peptide from the N-terminal part of Wnt proteins. This causes the formation of oligomers of Wnt proteins that are inactive in signal transduction. Tiki-1 can also act in the endoplasmic reticulum (ER)/Golgi, wherein it inactivates Wnt before it is secreted into the extracellular space through secretory vesicles.*

associated with T-cell factor/lymphoid enhancer factor transcription factors (TCF/LEF) to activate the target genes of the Wnt/β-catenin pathway (Fig. 1) (for a detailed review of the Wnt/β-catenin signaling pathway see Nusse and Clevers, 2017; Tortelote *et al.*, 2017). Considering the pivotal role of the Wnt/β-catenin pathway in embryonic development and adult life, its fine regulation in different contexts is a matter of great investigation. In this regard the *Xenopus* embryo has been instrumental.

In vertebrates, the Wnt pathway regulates multiple embryonic events including body axes formation and neural induction, which have been much studied under the umbrella of early embryonic inductions and the Spemann organizer concepts (Cho *et al.*, 1991; Gilbert and Saxén, 1993; Glinka *et al.*, 1997; Hemmati-Brivanlou *et al.*, 1994; Kiecker and Niehrs, 2001; Sasai *et al.*, 1994; Sasai *et al.*, 1995; Smith and Harland, 1992; Spemann and Mangold, 1924; Zimmerman *et al.*, 1996). Nowadays, many kinds of organizers have been discovered (Anderson *et al.*, 2016; Martinez Arias and Steventon, 2018), but the first neural inductive and axial inductive organizer being described was the one of Amphibians. In 1924 Spemann and Mangold claimed that the dorsal marginal zone of the pre-gastrula embryo of a Salamander holds organizer properties, once it could organize an ectopic primary embryonic induction when transplanted to the ventral marginal zone of another embryo (Spemann and Mangold, 1924). Importantly, for primary embryonic induction to be achieved (endogenous or ectopic), a dramatic morphological transformation must happen and it is precisely within this chain of transformations that our niche of research stands. Our work has been thriving on the fundamental role of molecular fine-tune regulation behind gastrulation/neurulation morphological changes in *Xenopus laevis.*

Xenopus laevis is an amphibian that lays visible eggs (1 mm of diameter), which are externally fertilized and develop into embryos that can be easily staged by naked eye or with the help of simple magnifying glass. Furthermore, and most importantly, the early embryos reveal us perceptive mesolecithal cleavage and amazingly didactic morphogenetic movements during gastrulation and neurulation (Fig. 2) (Sive, HL; Grainger, RM; Harland, 2000). One can, for instance, explant the dorsal marginal zone out of the embryo and record its morphogenesis during gastrulation; from a limited horizontal rectangle to a marvelous elongated axial tissue. Depending on how it is prepared, this explant might contemplate the prechordal plate, archenteron roof, notochord, somites, and neural plate. Importantly, many of the cells (or, in some cases, their daughter cells) that end up forming these axial structures

were originally part of the famous Spemann organizer (Sive, HL; Grainger, RM; Harland, 2000; Wilson and Keller, 1991).

Our laboratory has been able to contribute to a great extent on understanding the differentiation path of anterior neural tissue, from blastula stage, passing through gastrulation and until neurulation. Here, we will review three fundamental landmarks on this subject that we, in collaboration with Professor Xi He´s group, have published in the last few years: The role of Tiki on head organizer activity; the fundamental role of Notum on distinguishing neural plate from epidermis; and the link between Wnt/β-catenin pathway and the cholesterol-rich membrane microdomains (CRMMs) in the prechordal plate for the correct head formation. For didactic reasons we will write this review in line with the steps of embryonic development in which our research fits, but the articles published may not reflect a chronological sequence.

Tiki: a proteolytic enzyme that inhibits Wnt signaling and is essential in the Spemann organizer

A crucial dichotomy to be established during amphibian gastrulation, just as in any other vertebrate's gastrulation, is the one between the head organizer and the trunk organizer (Inui *et al.*, 2012; Onai *et al.*, 2017; Stern, 2001). The head organizer comprises the cells that involute first during gastrulation and migrates actively against the blastocoel wall to form the prechordal plate and the trunk organizer comprises more superficial cells that involute later and reveal a convergent extension behavior to form the notochord (Keller, 1984; Mangold, 1933; Martinez Arias and Steventon, 2018). Nevertheless, what is the canonical Wnt pathway take on this?

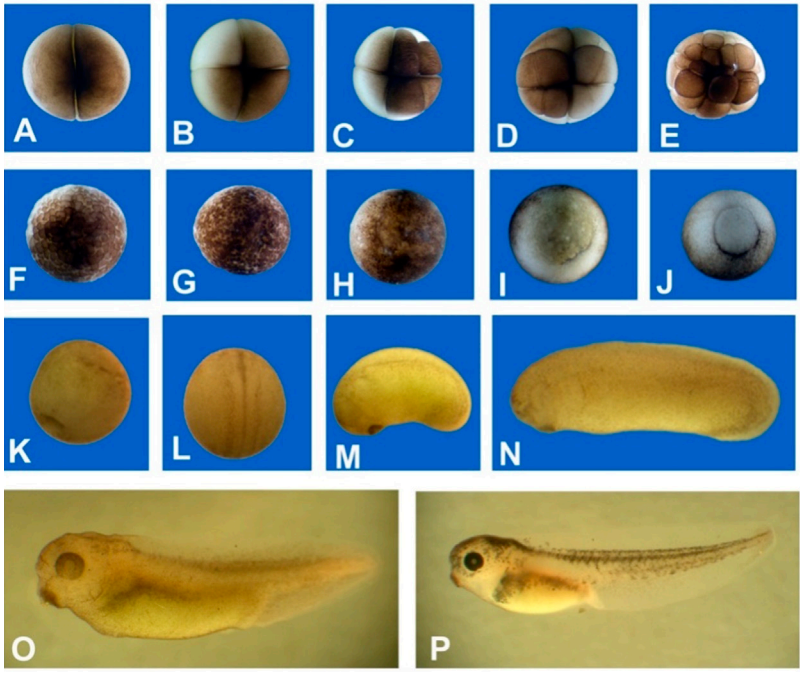

Fig. 2. *Xenopus laevis* stages. (A) *2-cell,* **(B)** *4-cell,* **(C)** *8-cell,* **(D)** *16-cell,* **(E)** *32-cell,* **(F-H)** *stage 8-9 blastula stages,* **(I)** *Stage 10,5 gastrula,* **(J)** *Stage 11,5 early neurula stage,* **(K)** *Stage 12,* **(L)** *stage 13,* **(M)** *stage 22,* **(N)** *stage 28 tailbud,* **(O)** *stage 35,* **(P)** *stage 42 tadpole. (A-H) animal pole view, (I-J) vegetal pole view, (K-L) dorsal view, (M-P), lateral view. Courtesy by Karla Almeida Coburn from her master dissertation, 2005.*

First of all, Wnt maternal wave, initiated when β-catenin is stabilized at the dorsal side of the embryo, is the trigger for Spemann organizer assembly (Larabell *et al.*, 1997; Moon and Kimelman, 1998; Yost *et al.*, 1996). Then, it is imperative that Wnt pathway becomes turned off precisely at the head organizer for it to achieve its fate during gastrulation and neurulation (Bouldin and Kimelman, 2012; Glinka *et al.*, 1997; Glinka *et al.*, 1998; Niehrs, 1999). It is exactly on this particular process that most inhibitory activity of the Wnt pathway can be fitted. Proteins encoded by genes like Cerberus, Dkk1, Sost, Notum, Tiki1 and Tiki2, among others, have been demonstrated to inhibit canonical Wnt pathway at different experimental models and different stages during development (see fig. 1 for a visual description of some Wnt inhibitors activity) (Bouwmeester *et al.*, 1996; Giráldez *et al.*, 2002; Grotewold *et al.*, 1999; Niehrs *et al.*, 2001; Reis *et al.*, 2014; Semënov *et al.*, 2001; Zhang *et al.*, 2012; Zhang *et al.*, 2015).

Professor Xi He's lab at the Boston Children Hospital, Harvard Medical School in collaboration with our group, described an Organizer-specific transmembrane protein, Tiki1 and Tiki2. These genes were discovered out of a functional cDNA expression screening assay in which the aim was to identify new players involved in *Xenopus* AP patterning/Wnt signaling phenotypes. Tiki proteins resemble a metalloprotease activity that cleaves the amino terminus of Wnt ligands either in the endoplasmic reticulum/Golgi trafficking or at the plasma membrane (Fig. 1). Tiki-cleavage of Wnts results in oxidation and oligomerization through inter-Wnt disulfide bonding without harming secretion, but inactivating signaling. The name 'Tiki' was a reference to a large-headed humanoid in Polynesian mythology. To understand the role played by Tik1 and Tiki2, we investigated the expression pattern of both of them during frog, chick and rabbit embryogenesis. In frog, Tiki1 is zygotically expressed in the Organizer, in particular in the head organizer region responsible for anterior patterning. In chicken, Tiki2 is expressed at Hensen's node and at the prechordal plate, which is the head organizer itself (or forebrain organizer). In rabbits, both Tiki1 and Tiki2 are expressed at the prechordal plate, suggesting that both are important for head patterning. Consistently, in mouse, Tiki2 is expressed in the anterior neural fold and prechordal plate (Reis *et al.*, 2014).

Functional experiments performed during *Xenopus* development shows that Tiki1 depletion entails severe anterior defects exhibiting loss of forebrain structures, including diminished or loss of the cement gland and eyes or exhibiting cyclopic eyes fused at the midline, which could be rescued by Human Tiki1. Moreover, Tiki1 depletion drastically suppressed the expression of head organizer genes: Goosecoid (Gsc), Lim1, Otx2 and Dkk1 (Fig 3A). Importantly, however, the *goosecoid* expression before gastrulation onset, which is an outcome of the maternal Wnt pathway, cannot be suppressed by the depletion of Tiki1 (Zhang *et al.*, 2012). Suggesting that Tiki1 appears to be required for Spemann organizer maintenance, but not for triggering its formation, which requires maternal Wnt signaling. In sum, Tiki1 as a founding "Wnt inactivator" thus joins known Organizer-specific Wnt antagonists such as Dkk1 and Frzb/sFRP3, and acts to ensure a "Wnt-free" zone for anteriorization.

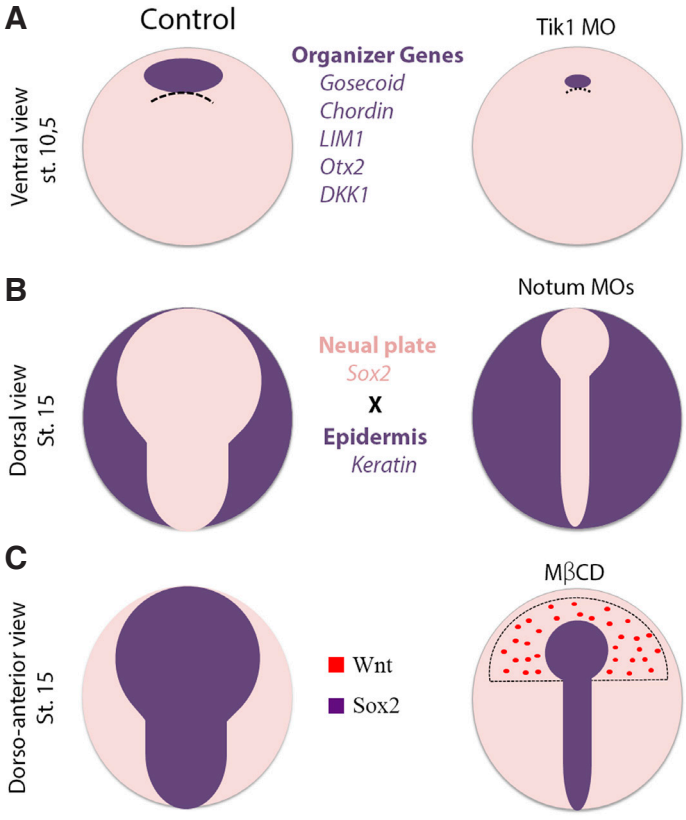

Fig. 3. Wnt inhibitors and early brain formation. (A) *Short scheme of Tiki1 loss-of-function experiments on Spemann Organizer and Head Organizer markers,* **(B)** *Short scheme of Notum loss-of-function experiments on neural plate formation,* **(C)** *Short scheme on the Methyl-β-cyclodextrin (MβCD) treatment and its phenotype. See text for details.*

Notum: to be or not to be? How a Wnt inhibitor acts regulating the decision between becoming neural or epidermal

In the late '90s and early 2000s, much has been discussed on the 'default model' of neural induction, which states that all cells that don't involute (the ectoderm) during gastrulation will become neural ectoderm if not instructed otherwise (Muñoz-Sanjuán and Brivanlou, 2002; Pera and Kessel, 1997; Stern, 2005; Stern, 2006; Wilson and Hemmati-brivanlou, 1997). The default model has been extensively explored in *Xenopus laevis* embryogenesis and it became widely known that the BMP family of secreted growth factor plays a major role in preventing ectodermal cells to fulfill its natural fate of becoming neural cells. On the other hand, numerous BMP antagonists, such as noggin, *follistatin* and *chordin* were discovered to be fundamental on allowing the ectoderm cells to reach their neural fate (Knecht *et al.*, 1995; Lamb *et al.*, 1993; Reversade *et al.*, 2005; De Robertis and Kuroda, 2004). Evidences have suggested that inhibition of Wnt signaling is also required for neural induction in *Xenopus* and chick embryos (Fuentealba *et al.*, 2007; Wilson *et al.*, 2001), but how regulation of Wnt signaling is achieved and contributes to neural induction by the Organizer was still unknown.

A breakthrough discovery of Xi He´s lab in collaboration with our group is that Notum, a canonical Wnt inhibitor, is essential for neural induction in *Xenopus laevis*. Notum is a widely conserved extracellular Wnt antagonist that was discovered in *Drosophila melanogaster* embryogenesis, in which it regulates the thoracic patterning, and wing disc development by suppressing the signaling activity, through deacetylation of Wnt proteins (Wg, *Drosophila* Wnt1) (Gerlitz and Basler, 2002; Giráldez *et al.*, 2002; Kakugawa *et al.*, 2015; Mata *et al.*, 2000). Notum belongs to the α/β hydrolase superfamily and is a carboxylesterase capable of removing an essential palmitoleoylate portion from Wnt ligand and, therefore, inhibiting its activity from the outside of the cell (Kakugawa *et al.*, 2015). During *Xenopus* embryogenesis, *notum* mRNA exhibits a dynamic expression, which is particularly important for neural induction and AP patterning. Notum is maternally expressed and, through cleavage to gastrulation stages, it is enriched in the animal (prospective ectoderm) and dorsal regions in early gastrula. At late gastrula and early neurula stages Notum mRNA was found in the forming neural plate in a noticeable A-P (high to low) gradient, with additional weaker expression in the head mesoderm (Zhang *et al.*, 2015).

We have shown that downregulation of Notum results in embryos with severe head defects (lacking forebrain, eyes and cement gland). However, head organizer markers such as *chordin, goosecoid, Xnr3* and *xNot* were not affected by Notum downregulation. This led us to believe that Notum activity regulates head patterning by acting within the prospective ectoderm, rather than prospective dorsal mesoderm. In order to validate this hypothesis, we performed a specific MO blastomere injection at 32-cell stage embryos; in the first condition Notum MO was injected into the A1-blastomere, which gives rise mostly to neural-ectoderm cells, and in the second condition Notum MO was injected into B1-blastomere, which, together with C1-blastomere, gives rise to the Spemann Organizer, following the highly detailed fate map developed by Sally Moody and collaborators (Bauer *et al.*, 1994; Moody, 1987). Notum depletion in those two conditions showed drastically different outcomes; the second condition did not entailed any abnormal phenotypes, while the first one end up revealing a significantly large number of embryos having severe head defect phenotypes, supporting our hypothesis that Notum is required primarily in the prospective ectoderm for anterior neural development. In agreement with those results, it has been shown that embryos injected with Notum MO had lost neural tissue (illustrated by Sox 2 expression) and expanded epidermis domain (illustrated by Keratin expression) (Fig. 3B).

In sum, our work with Notum showed that ectoderm cells have an 'on' state regarding Wnt/β-catenin signaling pathway that induces the differentiation on the epidermis, whereas Notum expression is fundamental to maintain the neural default on those cells that will form the neural plate (Fig. 3B) (Zhang *et al.*, 2015). Also, our results demonstrate that distinct Wnt inactivation mechanisms by Notum in naïve ectoderm and Tiki in Organizer coordinate early brain formation and these two Wnt-inactivating enzymes are each required for anterior development and not redundant.

Prechordal plate and anterior neural plate, let's form the head

Prechordal plate and notochord are essential for the neural plate to close as a neural tube and for this neural tube to be A-P patterned into forebrain, midbrain, hindbrain and spinal cord (Al-

taba, 1993; Pera and Kessel, 1997; Shinya *et al*., 2000). During development, prechordal plate induces anterior neural plate to fold into forebrain at its most anterior region and into midbrain just posterior to the forebrain (Hemmati-Brivanlou *et al*., 1994; Pera and Kessel, 1997; Rubenstein *et al*., 1998). Importantly, for any kind of intercellular communication, molecular signals must go from cell to cell, and in this particular scenario resides the importance of cholesterol-rich membrane microdomains (CRMMs). Also known as, lipid rafts, CRMMs are regions within the plasma membrane, which are highly enriched with cholesterol, sphingolipids, GM1 ganglioside and transmembrane proteins. It is also common to find GPI proteins anchored to membrane proteins and associated lectins, which have an important structural role (Brown and London, 1998). The existence of CRMMs was, for a long time, a theme of intense debate, once its idealization challenged, to some extent, the current mosaic-fluid model (Munro, 2003). That is, instead of conceiving the plasmatic membrane as a uniform ocean of lipids in which proteins would flow randomly, there would be islands of cholesterol, sphingolipids and transmembrane proteins standing still now and then (Lingwood and Simons, 2010). Studying CRMMs is also challenging because these structures are highly dynamic in the lateral plane of the plasma membrane. The properties of the lipid rafts make it arduous to visualize them in living cells. Hence, indirect methods have been carried out as the main way to prove their functions.

Later on, it became clear that those islands have a fundamental role in compartmentalizing signaling pathways receptors (Simons and Toomre, 2000). Meaning that, instead of hanging around randomly throughout the membrane, the receptors were often concentrated precisely at the CRMMs, revealing important physiological roles (Levental *et al*., 2020). Our group addressed the role of CRMMs and its regulation of signaling pathways. Two articles published by us showed CRMMs importance for gastrulation and neurulation of vertebrates.

Our first article, published in 2012, was a pioneer in showing the existence and importance of CRMMs during early embryonic development. First, we were able to isolate, through a sucrose gradient centrifugation, CRMMs from *Xenopus laevis* embryos at the 4-cell stage, at the blastula stage and the gastrula stage. The biochemical characterization proved that these microdomains are present in early frog embryos and have similar properties to those from mammals. Then, we took advantage of a known cholesterol depletion compound called methyl-beta-cyclodextrin (MβCD) to challenge the organization of CRMMs in *Xenopus laevis* embryos. We have shown that MβCD treatment through blastocoel injection was capable of disrupting CRMMs organization at the embryo's cells without affecting their architecture as a whole. Next, we showed that this specific target treatment resulted in severe head defects in frog embryos and also in chick embryos. i.e., lack of forebrain, diminished cement gland, absence of the oral cavity, and missing optic vesicles. Importantly this phenotype was not achieved when the MβCD was saturated with exogenous cholesterol, indicating high phenotype specificity (Reis *et al*., 2012).

Additionally, we could explain the molecular nature of this phenotype by showing that certain embryological territories were compromised after cholesterol depletion, whereas others remained unaffected. Molecular markers, such as *Sox2, Otx2, Six3, Krox20, Hox-B9* and Engrailed-2 showed us, through *in situ* hybridization analysis, that neither spinal cord, isthmus nor the rhombomeres 3 and 5 areas were affected by our treatment, leaving us with a very specific most-anterior problem of territory loss. Which was illustrated by Sox2 diminished expression only at the anterior neural plate region (Fig. 3C), as well as the mispatterned expression of *Otx2* and Six3 (Reis *et al*., 2012). Then, we went further on and specified, through transplantation experiments, the developmental window in which the head mesoderm/prechordal plate gets compromised. First, we have learned that the Spemann organizer explant from an MβCD treated embryo, when grafted to the ventral marginal zone of a non-treated embryo could organize a secondary axis containing head structures. This indicates that, by the beginning of gastrulation, head mesoderm behavior and its properties had not been compromised by CRMM disruption.

On the other hand, when we have grafted the whole anterior portion (anatomically know as head anlage) of an MβCD treated embryo at the very end of gastrulation and cultured it together with the animal cap of the untreated embryo, the outcome was a posterized head. This result was in contrast with the normal head that developed from the anterior graft of an untreated embryo cultured together with a control animal cap. Moreover, when the anterior graft was originated from an MβCD treated embryo and cultured with a Dkk1 secreting animal cap, head features were rescued. These findings lead to the following conclusions: 1. The head mesoderm/prechordal plate does not get compromised to its fate until some point during gastrulation and 2. Wnt pathway might be involved in the phenotype obtained after cholesterol depletion. Given these two points we could not help ourselves from digging further into this matter.

We further addressed the molecular signaling underlying de CRMM on the prechordal plate organization. Taking advantage of the same anterior neural plate (or head anlage) explant procedure, we showed that this specific region of the embryo presented very little Wnt3a expression in control conditions, but considerably high expression when the embryo had been treated with MβCD. We also showed that β-catenin loss-of-function in the head inhibits the headless phenotype caused by MβCD. Briefly, we took advantage of the morpholino oligonucleotide (MO), which is an antisense approach that specifically prevented the translation of β-catenin (Heasman *et al*., 2000). To target the anterior neural plate, we injected β-catenin MO into the dorsal animal cells (A-tier) of 32-cell stage embryos. When co-injected with MβCD, the head defects resulting from cholesterol depletion were no longer seen. This result suggests that reduced levels of cholesterol from the plasma membrane relies on overactive Wnt/β-catenin signaling in the head. Thus, β-catenin knockdown inhibited the phenotype caused by cholesterol depletion. (Reis *et al*., 2016). In order to test this we performed the following elegant experiment: we created an ectopic Wnt morphogen field by injecting Wnt8 together with β-galactosidase so we could trace the evolution of the field. Next we recognized that this ectopic field was able to displace the Otx2 expression domain a little bit away from its normal site. Remarkably, when MβCD was injected in the blastocoel of embryos that had been previously injected with Wnt8 and β-galactosidase, the Otx2 expression domain was displaced much further from Wnt source or, in some cases, completely abolished (Reis *et al*., 2016).

Taking all our results together, we felt comfortable to propose a novel model on the endogenous role of CRMMs during *Xenopus laevis* gastrulation/neurulation. In this scenario, the CRMMs are placed as an additional mechanism of anterior protection against

Wnt signaling. That is, the presence of CRMMs at Prechordal plate cells would prevent the Wnt signal secreted by posterolateral cells from spreading towards the anterior end of the embryo. We can affirm that, together with important Wnt antagonists, such as DKK and Tiki1, CRMMs play a fundamental role in controlling the Wnt morphogen field during the dramatic morphological changes happening in gastrulation. Importantly, each mechanism described here, and also those that were not described, are specific to different space-time windows during early embryogenesis and that is what makes our work so relevant. We were able to publish solid results covering a series of crucial steps within neural induction matter. Making it clearer for the developmental biology community how the molecular and cellular behavior aspects combine to entail amazing and dramatic morphological changes during gastrulation and neurulation.

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