Int. J. Dev. Biol. 61: 697-722 (2017)
doi: 10.1387/ijdb.170202sb

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Developmental cell-cell communication pathways in the cephalochordate amphioxus: actors and functions

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ABSTRACT During embryonic development, cells of metazoan embryos need to communicate in order to construct the correct bodyplan. To do so, they use several signals that usually act through interactions between ligands and receptors. Interestingly, only a few pathways are known to be fundamental during animal development, and they are usually found in all the major metazoan clades, raising the following question: how have evolution of the actors and of the functions of these pathways participated in the appearance of the current diversity of animal morphologies? The chordate lineage comprises vertebrates, their sister group the urochordates, and the cephalochordates (i.e. amphioxus). Urochordates are quite derived relative to the chordate ancestor, whereas cephalochordates and vertebrates share many morphological traits. Thus, comparing embryonic development between vertebrates and cephalochordates should give us some insight into the ancestral characters present in chordates and into the morphological evolution in this clade. However, while much is known about the function of different signalling pathways in vertebrates, data are still scarce in the literature for cephalochordates. In this review, we summarize the current state of the field concerning the expression of actors and the function of the major cell-cell communication pathways, including Hedgehog (Hh), Notch, Nuclear Receptor (NR), Receptor Tyrosine Kinase (RTK), Transforming Growth Factor-β (TGF-β) and Wingless/Int (Wnt), in amphioxus.

KEY WORDS: *chordates, evolution, development, signalling pathways*

Introduction

Vertebrates belong to the chordate group together with the urochordates, their sister group, and the cephalochordates (i.e. amphioxus). Although urochordates are more closely related to vertebrates, they show derived features making difficult the inference of ancestral characters by comparing their traits with those of the other clades (Delsuc *et al.*, 2006). On the other hand, amphioxus seem to have retained many ancestral chordate characters at both developmental and genomic levels, making these small marine animals a good model for chordate evo-devo studies (Bertrand and Escriva, 2011). Most cephalochordates live in the sand in shallow waters and are filter feeders. They are gonochoric, and reproduction occurs through external fertilization in the sea water column after males and females have released their gametes at sunset (Bertrand and Escriva, 2011). The transparent zygote divides to form a morula, and then a blastula consisting of a single cell layer surrounding a cavity, the blastocoel (Conklin, 1932, Hatschek, 1893, Holland and Holland, 2007, Kowalevsky, 1867). The vegetal region flattens and forms the vegetal plate, which will invaginate to form a gastrula with two germ layers. The external layer, the ectoderm, will develop to form both the epidermis on the ventral side, and the neural plate in the dorsal region (Fig. 1). The mesendoderm is the internal layer. Its axial dorsal region is fated to become the

Abbreviations used in this paper: EPH, erythropoietin-producing human hepatocellular receptor; FGFR, fibroblast growth factor receptor; Hh, hedgehog; INSR, insulin receptor; NR, nuclear receptor; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; TGF, transforming growth factor; TRK, tropomyosin receptor kinase; VEGFR, vascular endothelial growth factor receptor.

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Submitted: 13 August, 2017; *Accepted:* 22 September, 2017.

ISSN: Online 1696-3547, Print 0214-6282

notochord, whereas the dorsal paraxial mesendoderm gives rise to the somites. Finally, the ventral mesendoderm will form the embryonic gut. At the end of gastrulation, the epidermis that has detached from the neural plate covers the whole embryo. During neurulation, the neural plate curves to form the neural tube, which is enlarged in the most anterior region, forming the so-called cerebral vesicle (Fig. 2). The somites start to form by enterocoely from the dorsal paraxial mesendoderm while the embryo elongates. In the late neurula stage embryo, several regions of the anterior endoderm begin to form different structures. Two diverticula are established in the most anterior region, the Hatschek's left and right diverticulum, which will later develop into the pre-oral pit and the rostral coelom, respectively. On the right side, the future club-shaped gland and the region where the first gill slit will open can be detected, whereas the endostyle starts to develop from the ventral anterior endoderm. Finally, at the larval stage, the formation of these structures is achieved and the mouth opens on the left side (Fig. 2).

Many questions remain as to how cell-cell communication mediates the processes taking place in these different developmental stages. In metazoans, few intercellular signals are known, and deciphering their specific functions in each lineage is crucial for our understanding of morphological evolution in animals. In this review, we will therefore briefly present the mode of action for the main signalling pathways (Hedgehog (Hh), Notch, Nuclear Receptor (NR), Receptor Tyrosine Kinase (RTK), Transforming Growth Factor-β (TGF-β), Wingless/Int (Wnt) pathways), as well as describe the embryonic expression of known actors in amphioxus. We will then detail available information on the embryonic function of each signal. Table 1 summarizes the bibliography concerning gene expression patterns, whereas Table 2 presents the main results obtained using pharmacological treatments to modulate these different pathways.

Hedgehog pathway

How does it work?

The role of the Hedgehog (Hh) signal during embryogenesis was first discovered in *Drosophila*. The name was derived from the phenotype of null mutants for *Hh*, which presented hair-bristles resembling the spines of the hedgehog (Nusslein-Volhard and Wieschaus, 1980). This signalling pathway is very complex and requires the participation of many actors in addition to the ligands and receptors. The Hedgehog ligand proteins contain two regions. The N-terminal region is the active peptide whereas the C-terminal region, which shows some similarity with inteins, possesses autocatalytic activity. After autocleavage of Hh, the N-terminal peptide is linked to a cholesterol molecule and to a palmitic acid and is recruited at the plasma membrane. From there it can be released in different ways into the extracellular space in order to spread through tissues (Briscoe and Therond, 2013, Lee *et al.*, 2016) (Fig. 3). The first identified receptor of Hh is a 12-pass transmembrane protein called Patched (Ptc), which acts as a constitutive repressor of the pathway (Hooper and Scott, 1989, Nakano *et al.*, 1989). In the absence of Hh, Ptc represses Smoothened (Smo), a GPCR family protein, resulting in the proteolysis of the zinc-finger domain transcription factor Gli (or Cubitus interruptus (Ci) in *Drosophila*), which is processed to its repressor form (Fig. 3). Following Hh binding to Ptc, Smo is activated and Gli translocates into the nucleus in its active form, leading to the transcription of target genes (Briscoe and Therond, 2013, Lee *et al.*, 2016) (Fig. 3).

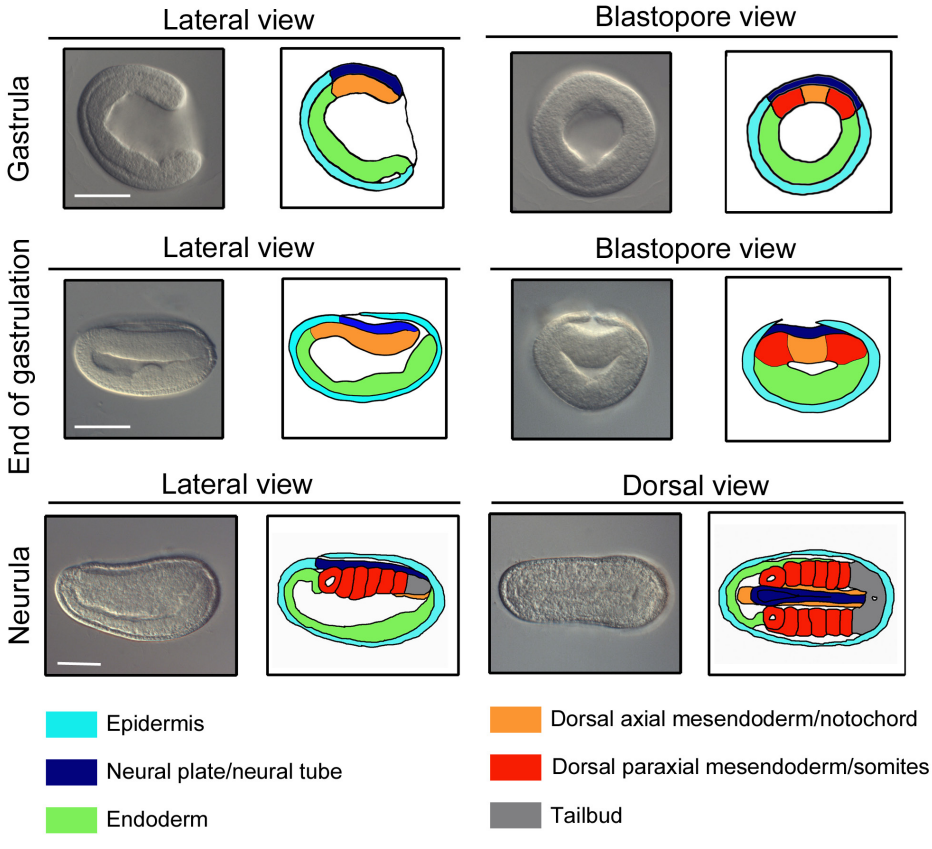

Fig. 1. Early embryonic development in amphioxus. *Three early developmental stages are presented, from gastrula to neurula. For each, two pictures of the same fixed embryo are shown together with small schemes indicating the different presumptive, or already formed, territories/structures. Scale bar, 50 μm.*

Receptors and ligands in amphioxus

Amphioxus possesses a unique *Hh* gene, orthologous to vertebrate *Sonic*, *Indian* and *Desert Hedgehog* (Shimeld, 1999). Its expression pattern has been described in three amphioxus species, *Branchiostoma floridae*, *B. belcheri* and *B. lanceolatum* (Shimeld, 1999, Shimeld *et al.*, 2007, Somorjai *et al.*, 2008, Zhang *et al.*, 2002). *Hh* transcripts are first detected in the dorsal mesendoderm at the early gastrula stage (Somorjai *et al.*, 2008, Zhang *et al.*, 2002). As gastrulation proceeds, expression is observed in the presumptive notochord and floor plate

TABLE 1

AMPHIOXUS ACTORS OF THE DIFFERENT SIGNALLING PATHWAYS CITED IN THIS REVIEW AND REFERENCES OF ARTICLES IN WHICH THEIR EXPRESSION PATTERN HAS BEEN DESCRIBED

Pathways	Actors	Genes in amphioxus	Orthologs in vertebrates (mammal/teleost common ancestor)	Amphioxus developmental expression pattern
Hedgehog				
	Receptors	Patched (Ptc)	Ptc1, Ptc2	(Lin *et al*., 2009)
		Smoothened (Smo)	Smo	(Lin *et al*., 2009)
	Ligand	Hedgehog (Hh)	Sonic Hedgehog, Indian Hedgehog, Desert Hedgehog	(Shimeld, 1999, Somorjai *et al*., 2008, Zhang *et al*., 2002)
	Others	Gli	Gli1, Gli2, Gli3	(Shimeld *et al*., 2007)
		Sufu	Sufu	(Lin *et al*., 2009)
NR				
	Receptor family NR0	NR0B	DAX1, SHP	
	Receptor family NR1	TR	TRα, TRβ	(Paris *et al*., 2008)
		RAR	RARα, RARβ, RARγ	(Escriva *et al*., 2002)
		PPAR	PPARα, PPARβ, PPARγ	
		REV-ERB	REV-ERBα, REV-ERBβ, REV-ERBγ	
		ROR	RORα, RORβ, RORγ	
		NR1H1-10	LXRα, LXRβ, FXRα, FXRβ	
		NR1I/J/K	VDR, PXR, CAR	
	Receptor family NR2	HNF4	HNF4α, HNF4β, HNF4γ	
		RXR	RXRα, RXRβ, RXRγ	(Escriva *et al*., 2002)
		TR2/4	TR2, TR4	(Escriva *et al*., 2002)
		TLX	TLX	
		PNR	PNR	
		NR2E		
		COUP-TF	COUP-TFα, COUP-TFβ, COUP-TFγ, EAR2	(Langlois *et al*., 2000)
	Receptor family NR3	ER	ERα, ERβ	(Bridgham *et al*., 2008)
		ERR	ERRα, ERRβ, ERRδ, ERRγ	(Bardet *et al*., 2005)
		SR	AR, GR, MR, PR	(Bridgham *et al*., 2008)
	Receptor family NR4	NR4A	NGFI-B, NURR1, NOR1	(Candiani *et al*., 2009)
	Receptor family NR5A	NR5A	SF1, LRH1	
	Receptor family NR6	GCNF	GCNF	
	Others	NRa		
		NRb		
		NRc		
Notch				
	Receptor	Notch	Notch1, Notch2, Notch3, (Notch4?)	(Holland *et al*., 2001, Somorjai *et al*., 2008)
	Ligands	Delta	DLK1, DLK2, DLL3	(Rasmussen *et al*., 2007)
		JaggedA	JAG1, JAG2	
		JaggedB	JAG1, JAG2	
	Others	Fringe	Lunatic Fringe, Manic Fringe, Radical Fringe	(Mazet and Shimeld, 2003)
RTK				
EPH	Receptors	EPH1	EPHA1-10, EPHB1-6	(Bosne, 2010)
		EPH2	EPHA1-10, EPHB1-6	(Bosne, 2010)
	Ligands	Efn1	EFNA1-5, EFNB1-3	(Bosne, 2010)
		Efn2	EFNA1-5, EFNB1-3	(Bosne, 2010)
		Efn3	EFNA1-5, EFNB1-3	
FGFR	Receptor	FGFR	FGFR1, FGFR2, FGFR3, FGFR4	(Bertrand *et al*., 2011)
	Ligands	FGF1/2	FGF1, FGF2	(Bertrand *et al*., 2011)
		FGF8/17/18	FGF8, FGF17, FGF18, FGF24	(Bertrand *et al*., 2011; Meulemans and Bronner-Fraser, 2007)
		FGF9/16/20	FGF9, FGF16, FGF20	(Bertrand *et al*., 2011)
		FGFA	(FGF3, FGF7, FGF10, FGF22?)	(Bertrand *et al*., 2011)
		FGFB	(FGF4, FGF5, FGF6?)	(Bertrand *et al*., 2011)
		FGFC	(FGF19, FGF21, FGF23?)	(Bertrand *et al*., 2011)
		FGFD		(Bertrand *et al*., 2011)
		FGFE		(Bertrand *et al*., 2011)
INSR	Receptor	INSR	INSR, INSRR, IGF1R	(Pashmforoush *et al*., 1996)
	Ligands	Ilp	Insulin, IGF1, IGF2	(Guo *et al*., 2009, Holland *et al*., 1997, Lecroisey *et al*., 2015)
		Ilp2	Insulin, IGF1, IGF2	
PD/VEGFR	Receptor	PD/VEGFR	PDGFRA, PDGFRB, FLT3, CSF1R, KIT, VEGFR1, VEGFR2, VEGFR3	(Pascual-Anaya *et al*., 2013)
TRK	Receptor	Trk	NTRK1, NTRK2, NTRK3	(Benito-Gutierrez *et al*., 2005)
	Ligand	NT	NGF, BDNF, NT-3, NT-4	
all RTKs	Others	AKT	AKT1, AKT2, AKT3	(Bertrand *et al*., 2009)
		PDK	PDK	(Bertrand *et al*., 2009)
		PLCγ	PLCγ1, PLCγ2	(Bertrand *et al*., 2009)
		PKCα/β/γ	PKCα, PKCβ, PKCδ	(Bertrand *et al*., 2009)
		RAF	RAF1, ARAF, BRAF	(Bertrand *et al*., 2009)
		H/K/NRASa	HRAS, NRAS, KRAS	(Bertrand *et al*., 2009)
TGF-β				
	Receptors type I	Alk1/2	Alk1, Alk2	
		Alk3/6	Alk3, Alk6	
		Alk4/5/7	Alk4, Alk5, Alk7	
	Receptors type II	TGF-βRII	TGF-βRII	
		ActRII	ActRII, ActRIIB	
		BMPRII	BMPRII, AMHR2	
	Ligands BMP family	ADMP	ADMP	(Kozmikova *et al*., 2013, Yu *et al*., 2007)
		BMP2/4	BMP2, BMP4	(Kozmikova *et al*., 2013, Panopoulou *et al*., 1998, Yu *et al*., 2007)
		BMP3/3b	BMP3, GDF10	(Sun *et al*., 2010)
		BMP5/8	BMP5, BMP6, BMP7, BMP8	(Kozmikova *et al*., 2013, Yu *et al*., 2007)
		BMP9/10	BMP9, BMP10	
		GDF5/6/7	GDF5, GDF6, GDF7	

and in two endodermal regions adjacent to the paraxial mesoderm (Shimeld, 1999, Somorjai *et al.*, 2008). In neurula stage embryos and in larvae, *Hh* is expressed in the notochord, the tailbud, the endostyle and the pre-oral pit (Shimeld, 1999, Shimeld *et al.*, 2007, Somorjai *et al.*, 2008, Zhang *et al.*, 2002), as well as transiently in some ventral neural tube cells (Shimeld, 1999). The expression of both *Ptc*, orthologous to *Ptc1* and *Ptc2* in vertebrates, and *Smo* was analyzed in *B. belcheri* (Lin *et al.*, 2009). The *Ptc* transcripts are first detected in the dorsal region of gastrula stage embryos. In neurulae, *Ptc* is expressed in newly formed somites and in the anterior endoderm (Lin *et al.*, 2009). As the embryo elongates, expression is also detected in the notochord, the presumptive gill slit and the pre-oral pit. Finally, at the larval stage, *Ptc* is expressed in the cerebral vesicle, the somites, the gill slits, the pre-oral pit, the club-shaped gland and in the gut and pharynx epithelium (Lin *et al.*, 2009). *Smo* has a similar expression pattern. It is first expressed later than *Ptc* in the newly formed somites and anterior endoderm in early neurula stage embryos (Lin *et al.*, 2009). Expression is observed at later stages in the somites, the gut and the anterior notochord (Lin *et al.*, 2009).

Other actors

The expression of two other major players of the Hh pathway has been described in amphioxus. A single *Gli* gene, orthologous to *Gli1*, *2* and *3* in vertebrates, was found in the amphioxus genome (Shimeld *et al.*, 2007). This gene produces at least two splice variants and the presence of transcripts was first analyzed by using a probe able to recognize both (Shimeld *et al.*, 2007). *Gli* expression is first detected in the dorsal ectoderm and mesendoderm of late gastrula embryos. In neurulae, *Gli* is expressed in the lateral neural plate and in the paraxial mesoderm, in cells alongside the *Hh* expressing regions. Expression then fades in these territories and is observed in the cerebral vesicle, in the Hatschek's left diverticulum and in the forming gill slits. In the larva, labelling is observed in the cerebral vesicle, the pre-oral pit, the club-shaped gland as well as in the gill slits. Using a probe only recognizing the

TABLE 1 (CONTINUED)

AMPHIOXUS ACTORS OF THE DIFFERENT SIGNALLING PATHWAYS CITED IN THIS REVIEW AND REFERENCES OF ARTICLES IN WHICH THEIR EXPRESSION PATTERN HAS BEEN DESCRIBED

Pathways	Actors	Genes in amphioxus	Orthologs in vertebrates (mammal/teleost common ancestor)	Amphioxus developmental expression pattern
	Ligands	Activin/Inhibin 1	(Inhibinβ-A, Inhibinβ-B, Inhibinβ-C, Inhibinβ-E ?)	
	TGFβ/Nodal/Activin family	Activin/Inhibin 2	(Inhibinβ-A, Inhibinβ-B, Inhibinβ-C, Inhibinβ-E?)	
		Lefty	Lefty1, Lefty2	(Morov *et al.*, 2016, Onai *et al.*, 2010, Yu *et al.*, 2007)
		Myostatin	Myostatin, GDF11	
		Nodal	Nodal	(Morov *et al.*, 2016, Onai *et al.*, 2010, Yu *et al.*, 2002, Yu *et al.*, 2007)
		TGF-β	TGF-β1, TGF-β2, TGF-β3	
		Vg1	GDF1	(Onai *et al.*, 2010)
	Others	BAMBI	BAMBI	(Yu *et al.*, 2007)
		Chordin	Chordin	(Somorjai *et al.*, 2008, Yu *et al.*, 2007)
		Cerberus	Cerberus, DAND5	(Le Petillon *et al.*, 2013, Onai *et al.*, 2010)
		Gremlin	Gremlin1, Gremlin2	(Le Petillon *et al.*, 2013)
		NBL1	NBL1	(Le Petillon *et al.*, 2013)
		Tolloid	BMP1, Tolloid like 1, Tolloid like 2	(Yu *et al.*, 2007)
		Tsg	Tsg	(Yu *et al.*, 2007)
Wnt				
	Receptors	Frizzled 1/2/7	Fzd1, Fzd2	(Qian *et al.*, 2013)
		Frizzled 4	Fzd4	(Qian *et al.*, 2013)
		Frizzled 5/8	Fzd5, Fzd8	(Qian *et al.*, 2013)
		Frizzled 9/10	Fzd9, Fzd10	(Qian *et al.*, 2013)
	Antagonists	sFRP1/2/5	SFRP1, SFRP2/sizzled, SFRP3/FrzB, sFRP5	(Kong *et al.*, 2012, Yu *et al.*, 2007)*
		sFRP3/4	sFRP4	(Yu *et al.*, 2007)
		Dkk1/2/4	Dkk1 Dkk2, Dkk4	(Yu *et al.*, 2007, Zhang and Mao, 2010)
		Dkk3	Dkk3	(Yu *et al.*, 2007, Zhang and Mao, 2010)
	Co-Receptors	LRP5/6	LRP5, LRP6	(Wang *et al.*)
		Kremen1, Kremen2, Kremen3, Kremen4	Kremen1, Kremen2	(Zhang and Mao, 2010)
	Ligands	Wnt1	Wnt1	(Holland *et al.*, 2000)
		Wnt2	Wnt2, Wnt2b/Wnt13	(Somorjai *et al.*, in prep)
		Wnt3	Wnt3, Wnt3a	(Albuixech-Crespo *et al.*, 2017, Schubert *et al.*, 2001, Somorjai *et al.*, 2008, Yu *et al.*, 2007)
		Wnt4	Wnt4	(Schubert *et al.*, 2000b)
		Wnt5	Wnt5a, Wnt5b	(Albuixech-Crespo *et al.*, 2017, Schubert *et al.*, 2001, Somorjai *et al.*, 2008, Somorjai *et al.*, 2012)
		Wnt6	Wnt6	(Schubert *et al.*, 2001, Somorjai *et al.*, 2008)
		Wnt7	Wnt7a, Wnt7b	(Albuixech-Crespo *et al.*, 2017, Schubert *et al.*, 2000b, Somorjai *et al.*, 2008)
		Wnt8	Wnt8a, Wnt8b	(Albuixech-Crespo *et al.*, 2017, Morov *et al.*, 2016, Schubert *et al.*, 2000c, Yu *et al.*, 2007)
		Wnt9	Wnt9a, Wnt9b	(Somorjai *et al.*, in prep)
		Wnt10	Wnt10a, Wnt10b	(Somorjai *et al.*, in prep)
		Wnt11	Wnt11	(Schubert *et al.*, 2000a, Yu *et al.*, 2007)
		Wnt16	Wnt16	(Somorjai *et al.*, in prep)
	Others	Dsh	Dvl1, Dvl2, Dvl3	(Wang *et al.*, 2016)
		Axin	Axin1, Axin2	(Beaster-Jones *et al.*, 2008, Wang *et al.*, 2016)
		GSK3β	GSK3β	(Wang *et al.*, 2016)
		β-catenin	β-catenin, Plakoglobin	
		TCF	TCF1, TCF3, TCF4, LEF1	(Lin *et al.*, 2006)
		CK1α	CK1α	(Wang *et al.*, 2016)
		CK1δ	CK1δ	(Wang *et al.*, 2016)
		Groucho	Groucho	(Wang *et al.*, 2016)
		APC	APC	(Wang *et al.*, 2016)

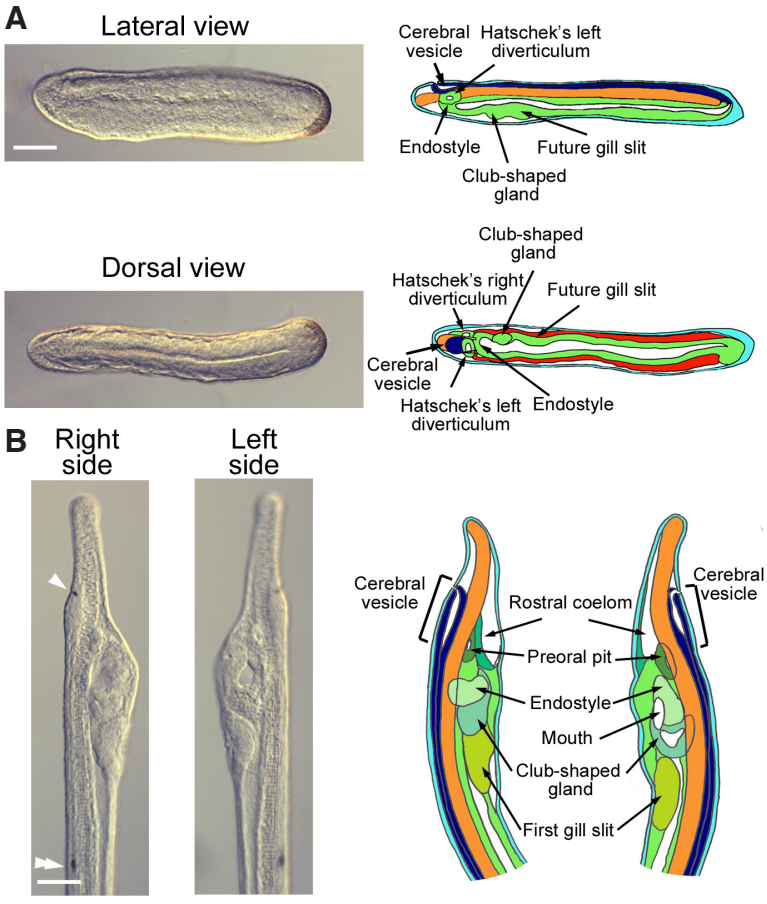

Fig. 2. Late embryonic development in amphioxus. (A) *Pictures of lateral and dorsal view of the same late neurula stage embryo are shown together with small schemes presenting the different presumptive structures that are found in the anterior region. Scale bar, 50μm.* **(B)** *Pictures of the anterior region of a larva on its left and right side are shown together with schemes highlighting the different pharyngeal structures that are formed at this stage. The frontal eye is indicated by a white arrowhead and the first pigment spot by a double arrowhead. Scale bar, 50μm. Colour code is according to Fig. 1.*

large splicing isoform, the authors showed that it is present only in neurulating embryos in the somitic mesoderm and in the neural plate (Shimeld *et al.*, 2007).

Another actor for which embryonic expression has been described is Sufu (Suppressor of Fused homolog). Sufu is a negative regulator of Hh signalling (Fig. 1). It binds to Gli or Ci and impedes its translocation into the nucleus (Briscoe and Therond, 2013, Lee *et al.*, 2016). In *B. belcheri*, *Sufu* is expressed in the same regions as *Smo* (Lin *et al.*, 2009).

Functional studies

Amphioxus mutants for the *Hh* gene were generated using TALEN (Wang *et al.*, 2015). 25% of the F1 embryos showed deformities with a curled tail, no mouth and a ventral endostyle and gill slits, suggesting a role of Hh pathway in the control of L/R asymmetry in amphioxus (Wang *et al.*, 2015). Purmorphamine (Sinha and Chen, 2006) and Cyclopamine (Chen *et al.*, 2002) are molecules that are known to activate and inhibit the Hh pathway, respectively, by interacting with Smo. However, up to now there is no study reporting the effects of such molecules in amphioxus.

Functional studies have been undertaken in heterologous assays to test for the activity of the two Gli isoforms. Using different approaches such as mammalian cell transfection, chick neural tube electroporation and expression of both isoforms in *Drosophila* imaginal discs, the authors showed that the long isoform is an activator of Hh signalling, whereas the small isoform acts as a repressor (Shimeld *et al.*, 2007).

Notch pathway

How does it work?

The first *Notch* gene sequence was described in the 80's in *Drosophila* (Kidd *et al.*, 1986, Wharton *et al.*, 1985). The Notch receptor is a one-pass transmembrane protein with extracellular EGF repeats, LIN-12-Notch repeats (LNR) and an intracellular specific domain called NICD (Notch IntraCellular Domain) containing a RBP-jk association module (RAM), nuclear localization signals, ankyrin repeats and PEST motifs (proline, glutamic acid, serine, threonine) (Wang, 2011). Canonical ligands of Notch are likewise transmembrane proteins forming the Delta/Serrate/lag-2 (DSL) family, which also present EGF repeats in their extracellular region as well as a conserved so-called DSL domain (Wang, 2011). The fact that both receptors and ligands are transmembrane proteins implies that the Notch signal can only be achieved between contacting cells. Following ligand binding, the Notch receptor is first cleaved by metalloproteases releasing the ectodomain, and then by a γ-secretase complex that frees the NICD, which can translocate into the cell nucleus and interact with the DNA-binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1) (Kopan and Ilagan, 2009) (Fig. 4). Together, NICD and CSL recruit the coactivator Mastermind/Lag-3, leading to transcriptional activation of target genes (Kopan and Ilagan, 2009) (Fig. 4). Although a panel of genes coding for regulators of the Notch pathway exists in the amphioxus genome (Gazave *et al.*, 2009), we will mainly focus our attention on genes for which expression patterns have been described.

Receptors and ligands in amphioxus

In vertebrates, four receptors are known, at least in mammals. In amphioxus, one *Notch* receptor gene has been described in *B. floridae*, which is orthologous to vertebrate *Notch1/2/3/4* (Holland *et al.*, 2001). The Notch protein contains 36 EGF repeats, 3 LNR, a transmembrane region, a RAM domain, 6 ankyrin repeats and several sites for proteolytic cleavage, which are required for Notch signalling pathway activation. Transcripts are first detected by *in situ* hybridization at the gastrula stage, in a ring of presumptive mesendoderm (Holland *et al.*, 2001). Subsequently, a gradient in the dorsal mesendoderm is observed with higher expression in the posterior region (Holland *et al.*, 2001). In the neurulating embryo, strong *Notch* expression is detected in the posterior mesoderm and in the dorsal part of the three most anterior somites (Holland *et al.*, 2001). While the embryo is elongating, *Notch* transcripts are expressed in the second-youngest somites and in the neural plate and forming notochord (Holland *et al.*, 2001). Thereafter, expression in the somites gets more intense in their posterior part, and becomes stronger in the forming neural tube (Holland *et al.*, 2001).

TABLE 2

SUMMARY OF PHENOTYPES OBTAINED AFTER PHARMACOLOGICAL TREATMENTS INTERFERING WITH DIFFERENT SIGNALLING PATHWAYS

Pathways	Molecules	Targets	Concentrations, stages	Effects	References
Notch					
	DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester)	γ-secretase inhibitor	50μM (continuous from late gastrula)	Ectopic epidermal sensosry cells	(Lu *et al.*, 2012)
			50–100μM (continuous from late blastula)	Incomplete formation of the somites segmental boundary Incorrect dorso-ventral segregation of somites	(Onai *et al.*, 2015)
	Compound E	γ-secretase inhibitor	not specified	Ectopic epidermal sensosry cells	(Lu *et al.*, 2012)
	L-685458	γ-secretase inhibitor	not specified	Ectopic epidermal sensosry cells	(Lu *et al.*, 2012)
RAR					
	all-trans Retinoic Acid (ATRA)	Agonist of RAR	1nM to 1μM (continuous from blastula stage)	Posteriorization of the embryo	(Holland and Holland, 1996)
			1μM (continuous from blastula stage)	Posteriorization of the embryo	(Escriva *et al.*, 2002)
			1μM (continuous from blastula stage)	Posteriorization of epidermal sensory neurons	(Schubert *et al.*, 2004)
			1μM (continuous from blastula stage)	Posteriorization of the pharynx	(Schubert *et al.*, 2005)
			1μM (continuous from early gastrula stage)	Anteroposterior patterning defects in gastrula	(Koop *et al.*, 2010)
			1μM (continuous from blastula stage)	Hox genes expression expansion anteriorly, less motor neurons.	(Schubert *et al.*, 2006)
			1μM (continuous from 6-8 gill slit larvae to 8-10 gill slit larvae)	Tail regression	(Koop *et al.*, 2011)
			1μM (continuous from early gastrula stage)	Inhibition of hematopoiesis	(Pascual-Anaya *et al.*, 2013)
			1μM (continuous from early-mid neurula, mid-neurula and late neurula to larval stage)	Shortening of the pharynx and loss of pharyngeal structures	(Koop *et al.*, 2014)
	BMS009	Antagonist of RAR	1.5μM (continuous from blastula stage)	Anteriorization of the embryo	(Escriva *et al.*, 2002)
			1μM (continuous from blastula stage)	Anteriorization of epidermal sensory neurons	(Schubert *et al.*, 2004)
			1μM (continuous from blastula stage)	Anteriorization of the pharynx	(Schubert *et al.*, 2005)
			1μM (continuous from early gastrula stage)	Anteroposterior patterning defects in gastrula	(Koop *et al.*, 2010)
			1μM (continuous from blastula stage)	Hox genes expression anterior limit shifted posteriorly, more motor neurons	(Schubert *et al.*, 2006)
			2μM (continuous from early-mid neurula, mid-neurula and late neurula to larval stage)	Expansion of the pharynx	(Koop *et al.*, 2014)
			1μM (continuous from late blastula stage)	Enlarged pharynx and expansion of pharyngeal structures	(Carvalho *et al.*, 2017)
	BMS493	Antagonist of RAR	1μM (continuous from late blastula stage)	Enlarged pharynx and expansion of pharyngeal structures, tail fin elongation	(Carvalho *et al.*, 2017)
	R115866	Inhibitor of Cyp26	500nM (continuous frome eraly gastrula stage)	Shortening of the pharynx and loss of pharyngeal structures	(Koop *et al.*, 2014)
			0,1 to 0,5μM (continuous from late blastula stage)	Shortening of the pharynx and malformation of pharyngeal structures, tail fin reduction	(Carvalho *et al.*, 2017)
TR					
	Triiodothyronine (T3)	Agonist of TR	10-100nM (continuous treatment from premetamorphic larval stage)	Induction of metamorphosis	(Paris *et al.*, 2008)
	Thyroxine (T4)	Agonist of TR	10-100nM (continuous treatment from premetamorphic larval stage)	Induction of metamorphosis	(Paris *et al.*, 2008)
	Triiodothyroacetic acid (TRIAC)	Agonist of TR	10nM (continuous treatment from premetamorphic larvae)	Induction of metamorphosis	(Paris *et al.*, 2008)
TGF-β					
	Dorsomorphin	Inhibitor of BMP Type I receptor	8/16μM (continuous from late gastrula stage)	Reduction of the number epidermal sensosry cells	(Lu *et al.*, 2012)
			20μM (continuous from early blastula stage)	Partial dorsalization of the embryo	(Kozmikova *et al.*, 2013)
			5/10/25μM (continuous from Knife shaped larval stage)	Mouth formation/opening affected	(Kaji *et al.*, 2016)
			35μM (continuous from early blastula stage)	Dorsalization of the mesendoderm and absence of ectodermal cells specification	(Le Petillon *et al.*, 2017)
	LDN-193189	Inhibitor of BMP Type I receptor	3μM (continuous from early blastula stage)	Partial dorsalization of the embryo	(Kozmikova *et al.*, 2013)
	zBMP4		250ng/ml (continuous from early blastula stage)	Ventralization of the embryo	(Lu *et al.*, 2012, Yu *et al.*, 2008, Yu *et al.*, 2007)
				Loss of neural pate border genes expression	(Yu *et al.*, 2008)
			250ng/ml (continuous from late gastrula stage)	Ectopic epidermal sensosry cells	(Lu *et al.*, 2012)
	mBMP2		250ng/ml (continuous from early blastula stage)	Ventralization and posteriorization of the embryo	(Kozmikova *et al.*, 2013)
	hActivin		10ng/ml (continuous from early blastula stage)	Dorsalization and anteriorization of the embryo	(Onai *et al.*, 2010)
			50ng/ml (treatment before early gastrula stage on ectodermal explants)	The whole ectoderm expresses neural markers	(Le Petillon *et al.*, 2017)
	mNodal		8μg/ml (continuous from early gastrula stage)	Loss of asymmetrical expression Nodal pathway genes	(Li *et al.*, 2017)
	SB505124	Inhibitor of TGF-β/Nodal/Activin Type I receptor	5/10μM (continuous from late gastrula stage)	Loss of asymmetry	(Bertrand *et al.*, 2015, Soukup *et al.*, 2015)
			50μM (continuous from early gastrula stage)	Loss of asymmetrical expression of Nodal pathway genes	(Li *et al.*, 2017)
			5μM (from prehatching neurula to hatching neurula stage)	Loss of orobranchial structures	(Kaji *et al.*, 2016)

TABLE 2 (CONTINUED)

SUMMARY OF PHENOTYPES OBTAINED AFTER PHARMACOLOGICAL TREATMENTS INTERFERING WITH DIFFERENT SIGNALLING PATHWAYS

Pathways	Molecules	Targets	Concentrations, stages	Effects	References
			50µM (continuous from early gastrula stage)	Ventralization of the embryo	(Onai *et al.*, 2010)
			50µM (continuous from early blastula stage)	Ventralization of the embryo	(Kozmikova *et al.*, 2013)
			1µM (continuous from early gastrula stage)	Loss of dorsal structures	(Onai *et al.*, 2012)
			50µM (continuous from one cell stage)	Reduced blastopore diameter	(Morov *et al.*, 2016)
	SB431532	Inhibitor of TGF-β/Nodal/Activin Type I receptor	5/10/20µM (continuous from late gastrula stage)	Loss of asymmetry	(Bertrand *et al.*, 2015, Soukup *et al.*, 2015)
FGFR					
	SU5402	Inhibitor of FGFR	50µM (continuous from 8-cell stage)	Gastrulation defects	(Bertrand *et al.*, 2011)
			50µM (continuous from blastula stage)	Absence of anterior somites	(Bertrand *et al.*, 2011)
			50µM (continuous from late neurula stage)	Malformation of the notochord	(Bertrand *et al.*, 2011)
PD/VEGFR					
	SU5416	Inhibitor of PD/VEGFR	0,1-20µM (continuous from early gastrula stage)	Curved tail	(Pascual-Anaya *et al.*, 2013)
Wnt					
	Li^+/LiCl	Inhibitor of GSK3-β	30 min at onset of gastrulation; Na^+ substituted for Li^+ in seawater	Loss of anterior markers expression; forebrain truncation, posteriorization	(Onai *et al.*, 2009)
			400mM (30 min from early blastula stage)	Exogastrula	(Holland *et al.*, 2005)
			Various concentrations (from early blastula stage)	Posteriorization and lack of neural plate; severity is concentration dependent	(Holland *et al.*, 2005)
			50 to 100mM (1-128 cell stage)	Early effect: abnormal cell divisions and apparent loss of polarity; late effect: gastrulation and elongation defects	(Yasui *et al.*, 2002)
	BIO	Inhibitor of GSK3-β	0.5 µM for 30min at early gastrula stage	Disruption of early polarity, posteriorization and elongation defects, downregulation of anterior ectoderm and CNS markers	(Onai *et al.*, 2012)
	Alsterpaullone	Inhibitor of GSK3-β	0.75-1.5µM	Reduction of forebrain and pharynx as assessed by *Otx* expression; more severe elongation defects than with lithium	(Onai *et al.*, 2009)
	Azakenpaullone	Inhibitor of GSK3-β	10 µM (continuous from late gastrula to early neurula or larva)	Loss of *Sp5* expression and expansion of *Bra2* posterior expression; loss of anterior-most structures by larval stages	(Dailey *et al.*, 2017)
	CHIR99021	Inhibitor of GSK3-β	10 µM; cleavage to gastrula	Increase of *Sp5* expression	(Dailey *et al.*, 2017)

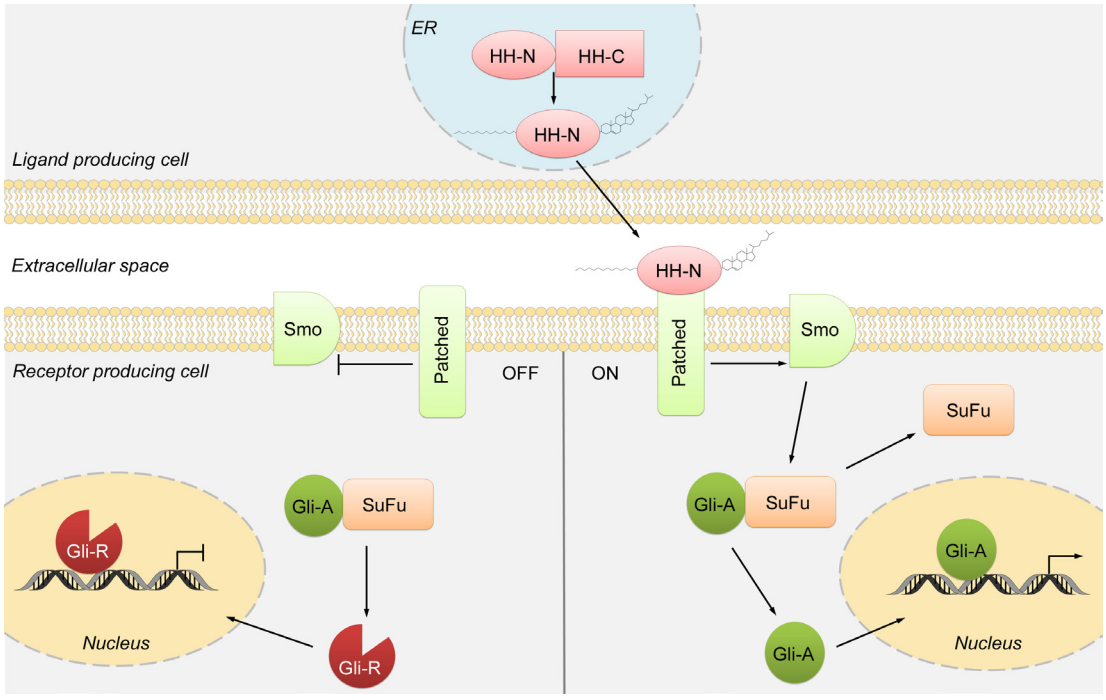

Fig. 3. Simplified schematic view of the canonical hedgehog (Hh) signalling pathway. *In the ligand producing cell, Hh undergoes autoproteolytic cleavage releasing the N-terminal domain (HH-N) from the C-terminal domain (HH-C). HH-N is subsequently linked to a palmitate in its N-terminus and to a cholesterol molecule in its C-terminus, probably in the endoplasmic reticulum (ER). In the absence of Hh (OFF), the transmembrane receptor Patched inhibits the orphan GPCR Smoothened (Smo), leading to the proteolysis of the transcription factor Gli, sequestered by Sufu (Suppressor of Fused homolog), which is converted into its repressor form (Gli-R). Gli-R acts by repressing the transcription of Hh pathway target genes in the nucleus. When Hh binds to Patched (ON), Smoothened inhibition is released leading to the dissociation of Gli from Sufu. Gli is therefore in its active form (Gli-A) and enters the nucleus to activate the transcription of Hh pathway target genes.*

At the late neurula stage, a new expression domain is observed in the anterior pharyngeal endoderm (Holland *et al.*, 2001). At later stages, Notch is expressed in the tailbud, the cerebral vesicle and Hatcheck's left diverticulum (Holland *et al.*, 2001). The expression of *Notch* has also been analyzed in *B. lanceolatum*, where it was shown to be quite similar to that in the Florida species. However, expression in the pharyngeal endoderm and cerebral vesicle can also be detected at an earlier developmental stage (mid-neurula) in the European species (Somorjai *et al.*, 2008).

In vertebrates at least 5 ligands are known: Jagged1 and 2 and Delta-like 1, 3, and 4 (Wang, 2011). Although two genes for ligands of the Jagged family have been found in the *B. floridae* genome (Gazave *et al.*, 2009), only cloning and expression of the single *Delta* gene, orthologous to vertebrate *Delta-like* genes (Gazave *et al.*, 2009), was reported (Rasmussen *et al.*, 2007). The Delta protein of amphioxus contains a signal peptide, a DSL domain and nine EGF repeats (Rasmussen *et al.*, 2007). *Delta* expression, as for *Notch*, is first observed at the gastrula stage, in the dorsal mesendoderm region (Rasmussen *et al.*, 2007). Then, expression becomes restricted to the paraxial mesoderm and is observed after hatching in the two first pairs of somites before they begin to form, and in some cells of the neural plate (Rasmussen *et al.*, 2007). As development proceeds, transcripts are also detected in some epidermal cells in the ventral region (Rasmussen *et al.*, 2007). At the late neurula stage, *Delta* is expressed in the posterior part of somites, in some neural plate cells, in ectodermal cells on both sides of the embryo, and in the pharyngeal endoderm (Rasmussen *et al.*, 2007). In the larva, expression is mainly observed in the forming somites budding from the tailbud, in some cells of the club-shaped gland and in the most anterior pharyngeal cells (Rasmussen *et al.*, 2007).

It is interesting to note that the expression patterns of *Notch* and *Delta* are not fully overlapping. Concerning the expression of *Notch* in territories in which *Delta* is not expressed, we might expect another ligand coding gene (from the Jagged family possibly) to be transcribed, although we cannot exclude Notch activation through a non-canonical pathway (Andersen *et al.*, 2012) in such regions.

Other actors

The only other actor of the Notch pathway for which expression data are available is *Fringe* (Mazet and Shimeld, 2003), the ohnolog of *lunatic fringe*, *radical fringe* and *manic fringe* in vertebrates. Fringe proteins are glucosaminyltransferases that have been shown to modulate Notch activity by initiating elongation of O-linked fucose residues attached to the EGF repeats of its ectodomain (Bruckner *et al.*, 2000, Moloney *et al.*, 2000). In a first study, amphioxus *Fringe* expression was detected at the late gastrula stage in the anterior neural plate (Mazet and Shimeld, 2003). Then, neural expression becomes segmented as somites start to form, and *Fringe* transcripts are detected in the posterior endoderm and in the endodermal cells that are fated to become the dorsal midline of the embryonic gut (Mazet and Shimeld, 2003). In late neurula stage embryos, *Fringe* is expressed in the cerebral vesicle and in the anterior and posterior endoderm (Mazet and Shimeld, 2003). In a more recent study, *Fringe* expression was detected earlier during gastrulation, in the ventral mesendoderm, with a dorsal boundary just adjacent to the *Delta* expressing paraxial mesoderm territory (Onai *et al.*, 2015).

Functional studies

Notch pathway function has mainly been studied using treatments with DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester), a γ-secretase inhibitor (Dovey *et al.*, 2001) that inhibits Notch receptor cleavage, thereby impeding Notch pathway activation by any ligand. Amphioxus possesses a dorsal central nervous system and a peripheral nervous system composed of several populations of epidermal sensory neurons (ESNs) (Wicht and Lacalli, 2005). Among these cells, the type I solitary neurons are the most abundant, and their progenitors express *Delta*, together with the neural bHLH factor *achaete-scute homologue* at the neurula stage (Lu *et al.*, 2012). Lu and colleagues showed that inhibiting the Notch pathway using DAPT, and other γ-secretase inhibitors, induces the formation of more ESN progenitors, which are then grouped in clusters containing more cells than in control embryos (Lu *et al.*, 2012). This experiment suggests that Delta/

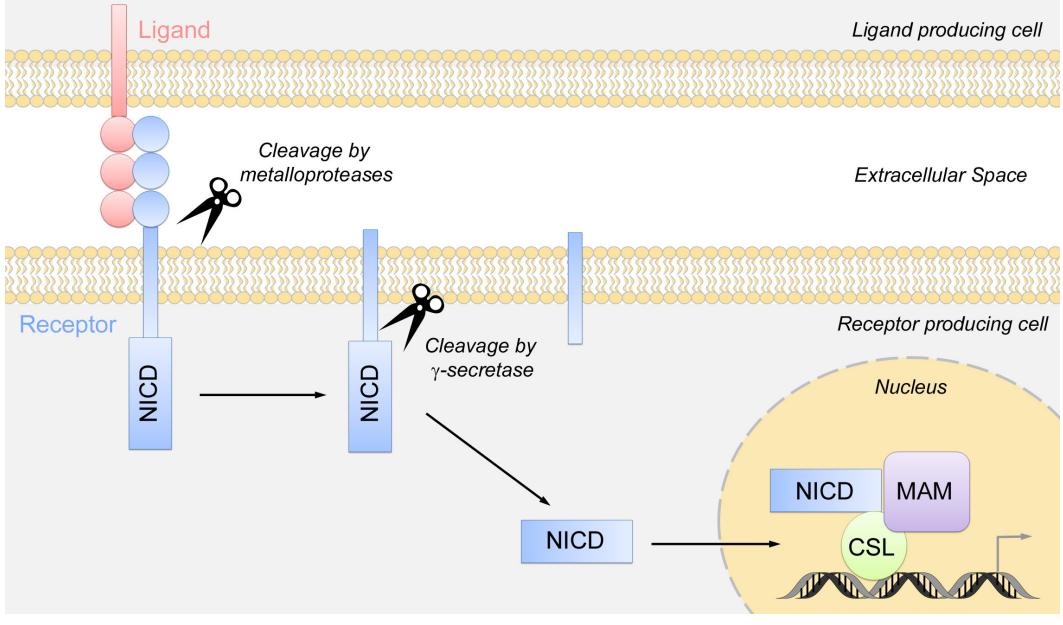

Fig. 4. Simplified schematic view of the canonical Notch signalling pathway. *When a Notch family receptor interacts with its transmembrane ligand produced by a neighbouring cell, the extracellular domain is first cleaved by extracellular metalloproteases. The Notch intracellular domain (NICD) is then released after intracellular cleavage by a γ-secretase complex. NICD then enters the nucleus where it interacts with a DNA binding protein, CSL (CBF1/RBPjκ/Su(H)/Lag-1). Together they recruit a co-activator called Mastermind (MAM) to activate the transcription of target genes.*

Notch-mediated lateral inhibition would be required in amphioxus for the specification of individual sensory neurons, as is the case in other animals like *Drosophila* (Lu *et al.*, 2012). In vertebrates, the Delta/Notch pathway is implicated in the segmentation of the paraxial mesoderm, which forms somites. Onai and colleagues studied the role of Notch signalling during somitogenesis in amphioxus (Onai *et al.*, 2015). They showed that after DAPT treatments, the enterocoelic somites, which normally form from the paraxial dorsal mesendoderm, are not completely separated from the archenteron roof, and that the boundaries between somites are not apparent (Onai *et al.*, 2015). The authors suggest that in amphioxus, the Notch signal would be important for the correct dorso-ventral segregation of somites as well as for antero-posterior boundary formation between somites (Onai *et al.*, 2015).

Nuclear receptors

How do they work?

Nuclear receptors (NRs) are ligand-activated transcription factors that consist of a modular structure comprising two major functional domains. These include the N-terminal domain (i.e. DNA binding domain, DBD), which is responsible for the interaction between the NRs and the response elements present in the promoter region of their target genes, and the C-terminal domain (i.e. ligand binding domain, LBD), which interacts with the ligands and with co-regulators to control gene expression (Gronemeyer *et al.*, 2004). Thus, NRs occupy a special position in gene regulation since they provide a direct link between the ligand, which they bind, and the target genes, whose expression they regulate. However, not all NRs have a ligand and some of them, the so-called orphan receptors, either function through the binding of an unknown ligand, or control expression of their target genes without binding any ligand (Benoit *et al.*, 2006). Known ligands in vertebrates include many different lipophilic molecules of different chemical natures, such as steroids, thyroid hormones, retinoic acid, or even fatty acids (Robinson-Rechavi *et al.*, 2003). Mammalian genomes contain 48-49 different NRs, grouped into 7 subfamilies (NR0-NR6) including 19-20 paralogy groups (Escriva Garcia *et al.*, 2003). The *B. floridae* genome contains 33 NRs, including members of all the mammalian paralogy groups except two (NR1I/J/K and NR2F) (Schubert *et al.*, 2008). However, a NR2F gene has been cloned and studied (COUP-TF), indicating that genome data are incomplete (Langlois *et al.*, 2000). Moreover, amphioxus also has a member of the NR5B subfamily, which is not present in vertebrates and was only known in ecdysozoans; and a member of a completely new subfamily, NR7, which is not present in vertebrates but that has been found in echinoderms. Furthermore, amphioxus shows some lineage specific duplications for the NR1H and the NR2E subfamilies. Analyses of gene expression and functional studies have only been undertaken for a few of the amphioxus NRs. We will detail below expression and functional data for these receptors.

Expression patterns of nuclear receptors

Gene expression has been studied for only a few NRs in amphioxus, including NR1A (Paris *et al.*, 2008a) and NR1B (Escriva *et al.*, 2002) (thyroid hormone receptor – TR, and retinoic acid receptor – RAR, respectively), NR2B (RXR) (Escriva *et al.*, 2002), NR2C (TR2/4) (Escriva *et al.*, 2002), NR2F (COUP-TF) (Langlois *et al.*, 2000), NR3A, B and C (ER, ERR and SR, respectively) (Bardet *et al.*, 2005, Bridgham *et al.*, 2008) and NR4A (Nurr1/NGFIB/NOR1) (Candiani *et al.*, 2009).

The biological functions of retinoic acid (RA) are mediated by heterodimers of two NRs: RAR and RXR. RXR also acts as an heterodimer of other nuclear receptors in accordance with its broad and weak expression throughout all developmental stages in amphioxus (Escriva *et al.*, 2002). In contrast, by the mid-gastrula stage, *RAR* is expressed throughout the mesendoderm and more weakly throughout the ectoderm. At the early neurula stage expression becomes downregulated anteriorly in the different tissues where it is expressed (i.e. the neural plate, the anterior endoderm and the non-neural ectoderm), but remains high in the posterior mesoderm, somites and in the posterior three-quarters of the neural plate and endoderm (Escriva *et al.*, 2002). Later on, at the mid-neurula stage, expression is also downregulated in the posterior third of the endoderm, and the only remaining strong expression is in the nerve cord, posterior to the cerebral vesicle, in the somites of the middle third of the embryo and in a small region of the endoderm. In late neurulae, *RAR* expression is limited to the nerve cord posterior to the cerebral vesicle and to the endoderm at low levels. Finally, in larvae, *RAR* is expressed only in the middle third of the nerve cord, somites and gut (Escriva *et al.*, 2002).

TR2/4 shows an expression pattern complementary to *RAR* (Escriva *et al.*, 2002). Thus, expression is first detected at the early neurula stage in the anterior neuroectoderm, particularly in the future cerebral vesicle, and in the dorso-anterior endoderm. Later on, at the mid-neurula stage, expression is most intense posteriorly and anteriorly, especially in the posterior mesoderm, cerebral vesicle and Hatcheck's left diverticulum, which is homologous to part of the vertebrate pituitary. In late neurulae, expression is strong in the cerebral vesicle, in Hatschek's left diverticulum and in the primordia of the mouth and first gill slit. There is also weak expression in the gut. At the late neurula stage, before the mouth opens, expression persists in the tailbud, cerebral vesicle and in pharyngeal structures (in the forming mouth, first gill slit and Hatschek's left diverticulum). This expression remains until the larval stage except in the tailbud (Escriva *et al.*, 2002).

Gene expression of the amphioxus thyroid hormone receptor (TR) has only been studied quantitatively using RT-qPCR. The authors showed that *TR* expression is low during embryonic development, increases in pre-metamorphic larvae just before metamorphosis, and then decreases in juveniles and adults (Paris *et al.*, 2008a).

The amphioxus orthologue of COUP-TF NRs is not expressed during early development and transcripts only start to be detected by *in situ* hybridization at the late larval stage (4-5 gill slit larvae) in the neural tube posterior to the cerebral vesicle, the homologue of the vertebrate hindbrain and spinal cord, and in dorsal and lateral groups of cells which form dorso-ventral stripes in the nerve cord (Langlois *et al.*, 2000).

The estrogen related receptor gene (*ERR*) shows an interesting expression pattern during amphioxus embryonic development (Bardet *et al.*, 2005). Transcripts are first detected at the neurula stage in the dorsal part of somites 2 to 6, and then in the newly formed somites when the neurula elongates. From the mid-neurula stage, *ERR* starts to be expressed in individual mesodermal and epidermal cells, but this expression disappears in later stages when *ERR* starts to be weakly expressed in the pharyngeal endoderm. Other expression sites at mid-neurula stage are the frontal eye, the presumptive photoreceptor of Hesse and other neuronal derivatives.

ERR is expressed from this developmental stage in paired cells in the neural tube, from the border between somites 1 and 2 up to somite 4, and this pattern is extended in later stages until labelling is observed in six pairs of cells. This segmented pattern remains until the larval stage when a new expression domain appears in the posterior part of the cerebral vesicle (Bardet *et al.*, 2005).

Expression studies for other NRs of subfamily 3 have also been published, but only in adults. Thus, while estrogen receptor (*ER*) and steroid receptor (*SR*) genes are co-expressed in the cytoplasm of the oocytes in the female gonads, only *ER* is expressed weakly in the gills. In males, *SR* is expressed broadly throughout the testes, at all stages of spermatogenesis, and *ER* is expressed in the germinal epithelium of the testis in a narrow band of cells that are likely to be early spermatogonia (Bridgham *et al.*, 2008).

The last NR for which gene expression has been documented is the orthologue of *Nurr1/NGFIB/NOR1*, which shows a restricted expression pattern in late neurula stage embryos in the ventromedial wall of the Hatschek's left diverticulum and in the posterior region of the pre-oral pit of larvae (Candiani *et al.*, 2009).

Other actors

Nuclear receptor-mediated transactivation of target genes implies direct contact between the receptor and co-regulators (co-activators or co-repressors). Although orthologues of vertebrate co-regulators are present in the *B. floridae* genome (Schubert *et al.*, 2008), no expression or functional data have been published. However, several studies have shown that mammalian co-regulators are able to interact with several amphioxus NRs. Thus, amphioxus TR in the presence of its ligand TRIAC (3,5,3'-triiodothyroacetic acid) interacts with the co-activator SRC-1 (Steroid Receptor Coactivator 1) (Paris *et al.*, 2008a), amphioxus RXR interacts with the co-activator hTIF2 (human Transcriptional Intermediary Factor 2) in the presence of its ligand 9-*cis* RA (Tocchini-Valentini *et al.*, 2009) and amphioxus ERRs (there are two isoforms of this receptor in amphioxus, long and short ERR), interact with the mammalian SRC-1, GRIP1 (Glutamate Receptor Interacting Protein 1), TIF1 (Transcriptional Intermediary Factor 1) and RIP140 (Receptor-Interacting Protein 140) (Horard *et al.*, 2004).

Other actors playing important roles in NR signalling pathways are enzymes implicated in ligand anabolism and catabolism. However, while the presence of many genes for these enzymes has been described in amphioxus (e.g. enzymes implicated in steroid metabolism such as cytochrome P450 (CYP11A, CYP17, and CYP19) and the 17β-hydroxysteroid dehydrogenase) (Castro *et al.*, 2005, Mizuta and Kubokawa, 2007), gene expression and/or function has only been characterized for enzymes involved in the synthesis and degradation of RA, and for CYP19 aromatase. Using semi-quantitative RT-PCR, it was shown that the *CYP19* is expressed in the middle third of amphioxus adults and preferentially in females (Callard *et al.*, 2011, Castro *et al.*, 2005). Other enzymes for which the expression pattern has been characterized include the retinoic acid degradation enzyme CYP26. Three CYP26 genes (*CYP26 1-3*), which originated by tandem duplication, are found in amphioxus (Carvalho *et al.*, 2017). The expression profiles of *CYP26-1* and *CYP26-3* are quite similar and weak, starting at the mid-gastrula stage in the lateral anterior mesoderm. Then, in mid-neurulae, expression is detected in the anterior somites and in the anterior central nervous system. A slight difference is then found in the larvae where *CYP26-1* is more strongly expressed in central and posterior regions, whereas *CYP26-3* labelling is more intense in central and anterior regions. The expression of *CYP26-2* starts at the mid-gastrula stage around the blastopore and, later, an expression domain appears in the presumptive lateral mesoderm and anterior neuroectoderm. At the mid-neurula stage, expression is detected in the anterior somites and neuroectoderm, but also in the most anterior and posterior tips of the ectoderm and endoderm and in the tailbud. In late neurulae, before the mouth opens, it is expressed in all anterior germ layers and at the posterior end in the ectoderm. Finally, in larvae, the overall domains of *CYP26-2* expression are maintained, with conspicuous labelling anteriorly and posteriorly and a weaker signal in the centre (Carvalho *et al.*, 2017). RA producing enzyme (aldehyde dehydrogenases, ALDHs) expression patterns have also been studied in amphioxus (Sobreira *et al.*, 2011), which possesses 6 ALDH1 genes and one ALDH2 (Canestro *et al.*, 2006). Amphioxus *ALDH1a* is expressed caudally close to the developing tailbud with a sharp anterior boundary at the mid-neurula stage (Sobreira *et al.*, 2011). Later, it is detectable in the posterior gut endoderm of late neurulae. The expression of *ALDH1d* overlaps with that of *ALDH1a* at the mid-neurula stage, but in late neurulae, expression is broad and slightly stronger in the posterior gut (Sobreira *et al.*, 2011). Other ALDH1 genes, such as *ALDH1b*, *ALDH1c*, *ALDH1e*, and *ALDH1f* are weakly expressed in posterior domains overlapping with that of *ALDH1a* in mid-neurula stage embryos (Sobreira *et al.*, 2011). However, by the late neurula, they are expressed diffusely and weakly throughout the amphioxus embryo with a weak to moderate concentration of the signal for *ALDH1b*, *ALDH1c*, and *ALDH1e* in the posterior gut (Sobreira *et al.*, 2011). *ALDH2* expression is restricted to posterior mesendodermal tissues at the mid-neurula stage and, subsequently, spreads throughout the embryo in late neurulae (Sobreira *et al.*, 2011).

Functional studies

Pharmacological treatments with molecules that are agonist or antagonist ligands of NRs have been used to study the function of NRs during embryonic development in amphioxus. In this context, the most studied function has been the role of RA during embryonic development using all-*trans* RA itself as well as RA antagonists (BMS009, BMS493). An excess of RA induces a posteriorization of the embryo, whereas the use of antagonists of RAR induces anteriorization (Escriva *et al.*, 2002). This role of RA in antero-posterior patterning starts at the early gastrula stage (Koop *et al.*, 2010) and affects different tissues, such as the ectoderm, through the control of antero-posterior patterning of epidermal sensory neurons (Schubert *et al.*, 2004); or the endoderm, where RA establishes the posterior limit of the pharynx through control of *Hox1* expression (Schubert *et al.*, 2005). Besides this role in patterning, RA also controls pharynx segmentation (Koop *et al.*, 2014), neuronal specification (Schubert *et al.*, 2006), or even tissue remodelling in the amphioxus tail (Koop *et al.*, 2011) and tail fin formation (Carvalho *et al.*, 2017). Finally, treatment of neurula stage embryos with RA was shown to inhibit hematopoiesis in amphioxus (Pascual-Anaya *et al.*, 2013).

Another receptor for which the developmental function has been studied is TR. It has been shown that amphioxus metamorphosis is controlled by a thyroid hormone, the 3,5,3'-triiodothyroacetic acid (TRIAC), through its binding to TR and its activation (Paris *et al.*, 2008a).

Other functional studies on amphioxus NRs concern their molecular capacities to bind DNA and to homo- or heterodimerize. Using *in vitro* EMSA (Electro Mobility Shift Assay) approaches, TR has been shown to bind both as homo- or heterodimer with RXR to DR4 (Direct repeat 4) and HREpal (Hormone Response Element palindrome) elements (Paris *et al.*, 2008a). RAR heterodimerizes with RXR and binds classical DR5 response elements, as well as an IR7 (Inverted Repeat 7) element found in the 5' non coding region of *TR2/4*. This receptor, TR2/4, also binds to this IR7 and to DR5 elements (Escriva *et al.*, 2002). In addition, RXR binds as a heterodimer with TR and RAR to DR4 and DR5 elements, respectively, and as a homodimer to DR1 elements (Escriva *et al.*, 2002). COUP-TF can bind elements ranging from DR0 to DR5 with different affinities (Langlois *et al.*, 2000). Finally, receptors of the NR3 subfamily (i.e.; ERR, ER, SR) bind ERE (Estrogen Response Element). Thus, ERR, which is expressed as two isoforms (a short and a long isoform), binds ERE as homo- or heterodimers formed by the two isoforms, or to SFRE (Steroidogenic Factor Binding Element) as a homodimer for the short isoform, and as monomer for the long isoform (Horard *et al.*, 2004). ER and SR also bind to ERE as homodimers (Bridgham *et al.*, 2008, Paris *et al.*, 2008a). These results show that, globally, amphioxus NRs function in a similar way as their vertebrate orthologues, except for SR, which can bind ERE, in contrast to its vertebrate orthologues AR (Androgen Receptor), MR (Mineralocorticoid Receptor), PR (Progesterone Receptor) and GR (Glucocorticoid Receptor) (Bridgham *et al.*, 2008).

The ligand binding capacity of different NRs has also been tested either directly (using limited proteolysis assays or mass spectrometry) or indirectly (using a transactivation assay after transient transfection). Thus, RAR binds all-*trans* and 9-*cis* RA (Escriva *et al.*, 2006), similarly to its vertebrate orthologues with similar affinities, but TR does not bind vertebrate thyroid hormones (triiodothyronine (T3) or thyroxine (T4)). However, a new ligand was discovered in amphioxus which is a thyroid hormone derivative, TRIAC, that binds with high affinity to the amphioxus TR (Paris *et al.*, 2008a). Other receptors for which ligand binding has been studied are RXR, which binds several molecules such as 9-*cis* RA, DHA (DocosaHexaenoic Acid) or oleic acid (with lower affinities than vertebrate RXRs) (Tocchini-Valentini *et al.*, 2009). TR2/4 does not bind any ligand but is able to transactivate expression of a reporter gene controlled by an IR7 element and also to inhibit the transactivation of the RAR-RXR heterodimer by competing for the DR5 response element (Escriva *et al.*, 2002).

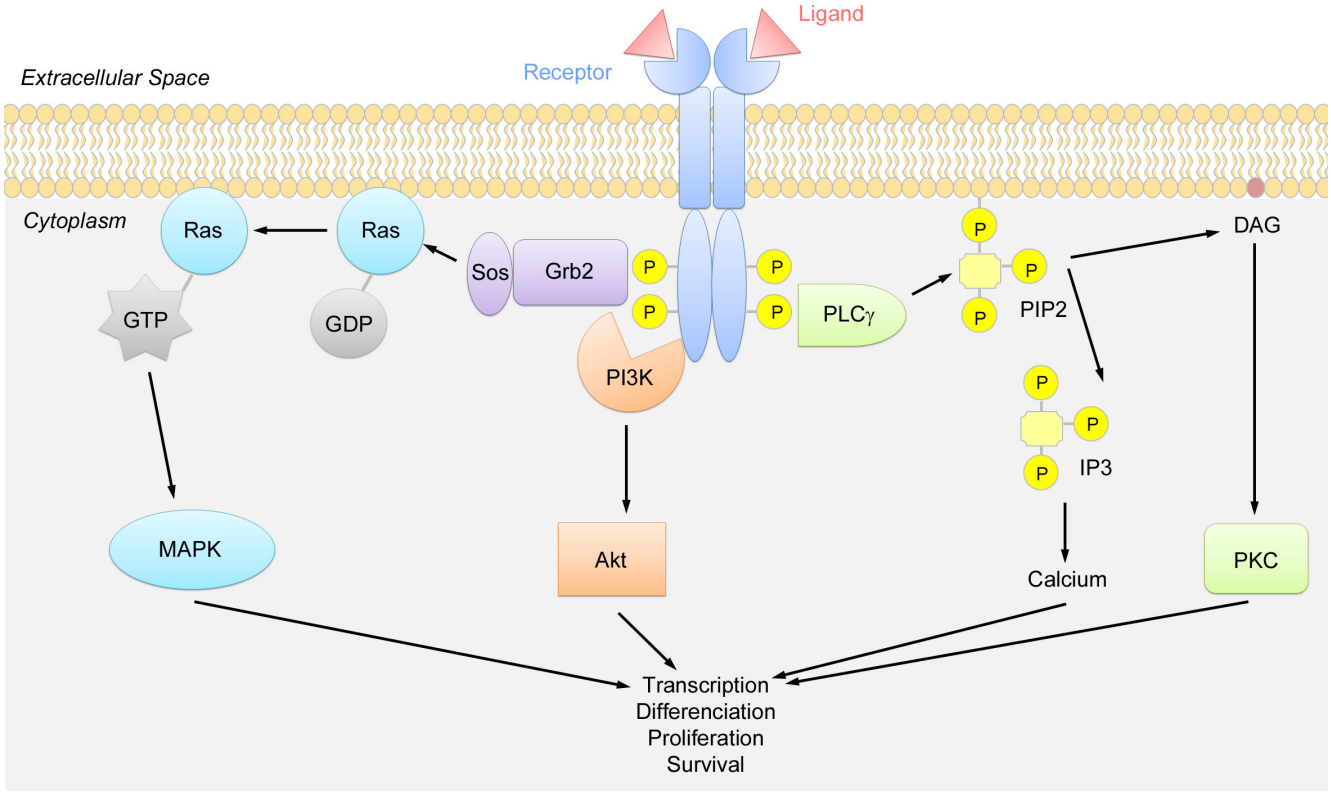

Fig. 5. Simplified schematic view of the receptor tyrosine kinase (RTK) canonical signalling pathway. *RTK are transmembrane receptors and interaction of dimers with their ligands induces a transphosphorylation on tyrosine residues of the intracellular domain. The phosphorylated cytoplasmic domain interacts with the adaptor protein Grb2 (Growth factor receptor-bound protein 2), which is associated with the Ras-guanine exchange factor Sos (Son of sevenless) that transforms Ras-GDP into Ras-GTP. This is the first step of activation of the MAPK (Mitogen-activated protein kinase) cascade, which ends with the activation of target gene transcription. Phosphorylated receptors also interact with PI3K (phosphatidylinositide 3-kinase) and PLCγ. Active PI3K then activates Akt whereas PLCγ (Phospholipase C-γ) catalyzes the formation of two secondary messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), from phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 diffuses in the cytoplasm and, through interaction with its receptor, induces the release of Ca^{2+} from the endoplasmic reticulum resulting in an increase of Ca^{2+} cytoplasmic concentration. DAG activates PKC (Protein Kinase C), which phosphorylates several substrates. The integration of the activation of the three intracellular cascades leads to many different outputs at the cellular level.*

COUP-TF is also able to inhibit receptor-mediated activation of transcription of different mouse receptors bound to different response elements from DR1 to DR5 and ERE (Langlois *et al.*, 2000). The orphan receptor ERR can also transactivate through an ERE (both long and short isoforms); however, on an SFRE, only the short isoform can transactivate, while the long isoform has no effect (Horard *et al.*, 2004). The other steroid receptors, ER and SR, do not behave as classical vertebrate steroid receptors in amphioxus. Thus, amphioxus ER does not bind estrogens and it has only been shown to bind BPA (bisphenol A). In contrast, SR binds estrogens with low affinity (Bridgham *et al.*, 2008, Paris *et al.*, 2008b). Thus, amphioxus ER behaves as a dominant negative estrogen receptor in mammalian cells, even in the presence of BPA (Paris *et al.*, 2008b), while SR transactivates in the presence of estrogens (Bridgham *et al.*, 2008). Finally, the last group of receptors for which ligand-binding capacities have been studied is the NR1H subfamily (LXR and FXR). Amphioxus possesses 10 paralogues of the FXR/LXR (Farnesoid X Receptor/Liver X Receptor) subfamily due to a lineage-specific duplication event (Lecroisey *et al.*, 2012, Schubert *et al.*, 2008), with one of them being a clear orthologue of vertebrate LXR. Phylogenetic approaches only clarified the orthology of 5 of the other receptors with vertebrate FXRs (Fonseca *et al.*, 2017). Ligand binding has been characterized using transient transactivation assays for the amphioxus LXR with four different ligands (the synthetic molecule T0901317, and the natural ligands 24(S)-hydroxycholesterol, 25-hydroxycholesterol and 24(S),25-epoxycholesterol). While the amphioxus LXR is able to bind and transactivate a reporter gene in the presence of these ligands, it shows a low binding affinity for them compared with its vertebrate orthologues (Fonseca *et al.*, 2017).

Receptor tyrosine kinase pathway

How does it work?

Receptor Tyrosine Kinases (RTKs) consist of a superfamily of transmembrane proteins presenting a variety of extracellular domain combinations and a tyrosine kinase cytoplasmic domain. The RTKs are receptors for several growth factors, as well as for some cytokines and hormones. The interaction between the receptors and their ligands usually induces a stabilization of a dimer of receptors that can transphosphorylate each other on tyrosine residues present in the intracellular domain (Lemmon and Schlessinger, 2010) (Fig. 5). This phosphorylation then allows interaction with several proteins that in turn are able to activate different cytoplasmic signalling cascades such as the MAPK (mitogen activated protein kinase) pathway, the PLCγ (Phospholipase C-γ) pathway or the PI3K (phosphatidylinositide 3-kinase) pathway (Fig. 5).

The analysis of RTK gene content in amphioxus showed that it possesses at least one member of each of the subfamilies that are widespread in bilaterians (19 families) (D'Aniello *et al.*, 2008). Amphioxus has generally one member of each of these subfamilies, but also some lineage-specific duplications, giving rise to 22 genes coding for proteins of the NOK family, 8 genes that are related to MET and AXL families, and 47 genes coding for receptors related to TIE, PD/VEGFR, RET, and FGFR families, which were named EXTKs (D'Aniello *et al.*, 2008). However, analyses of gene expression have only been undertaken for a few of the receptors or their ligands. We will detail below expression and functional data for these specific RTKs.

Erythropoietin-producing human hepatocellular receptors

The EPH (erythropoietin-producing human hepatocellular receptors) gene family can be separated into two groups in vertebrates: the EPHA group, which contains up to 10 members; and the EPHB group, with 6 members. In amphioxus, two genes, EPH1 and EPH2, resulting from a specific duplication in the cephalochordate lineage, were discovered in the *B. floridae* genome (D'Aniello *et al.*, 2008, Mellott and Burke, 2008). They are both ohnologous to all the EPH genes from vertebrates, and they code for receptors with an EPH ligand binding domain, a TNFR (Tumor Necrosis Factor Receptor) domain, and two fibronectin III domains in the extracellular region (D'Aniello *et al.*, 2008). In *B. lanceolatum*, *EPH1* is expressed in the blastopore of gastrula stage embryos (Bosne, 2010). During neurulation, expression is detected in the cerebral vesicle and in the paraxial mesoderm. At the late neurula stage, before the mouth opens, *EPH1* transcripts are detected in the anterior and posterior notochord, in the somites, the neural tube and in the pharyngeal endoderm. In the larva, expression is observed in the anterior and posterior notochord, in the cerebral vesicle as well as in the mouth, the club-shaped gland, the endostyle and the pre-oral pit. The other receptor gene, *EPH2*, shows ubiquitous expression until the late neurula stage (Bosne, 2010). Prior to mouth opening, expression is stronger in the pharyngeal endoderm and in anterior and posterior notochord. In the larva, *EPH2* transcripts are detected in the mouth and in the pharynx.

Ephrins (Eph receptor-interacting proteins), the ligands for EPHs, are membrane-bound proteins, implying that activation of the EPH signalling pathway is only possible through direct cell-cell interaction. Two families of ligands are known in vertebrates, the EfnA and the EfnB groups. In amphioxus, there are three *Efn* genes (*Efn1*, *2* and *3*) that result from a lineage-specific duplication in cephalochordates (Bosne, 2010). It is, up to now, not clear whether they are more closely related to EfnA or EfnB groups from vertebrates, although it has been proposed that group A may only be present in Olfactores (the clade containing vertebrates and urochordates) (Mellott and Burke, 2008). The expression patterns of *Efn1* and *Efn2* have been described in *B. lanceolatum* (Bosne, 2010). *Efn1* is expressed in the mesendoderm of the blastopore region of gastrulating embryos. At the neurula stage, expression is observed in the paraxial mesoderm and in some neurons in a segmented manner. At the late neurula stage, before mouth opening, *Efn1* transcripts are detected in the pharynx, in the posterior presumptive gut and in the tailbud. In the larva, expression is restricted to the mouth, the pharynx and the cerebral vesicle. At the gastrula stage *Efn2* is ubiquitously expressed, and in neurulae expression is widespread in the mesendoderm, although not in the most posterior region. Before the mouth opens, *Efn2* transcripts are observed in the ventral pharyngeal endoderm, in the presumptive endostyle region and in the posterior part of the future gut. In larvae, *Efn2* is expressed mainly in the pharynx. No functional data on the role of the EPH signalling pathway during amphioxus embryogenesis have been described so far.

Fibroblast growth factor receptor

Amphioxus has a unique *FGFR* (Fibroblast Growth Factor Receptor) gene, coding for a receptor with three Immunoglobulin extracellular domains, similarly to the four receptors found in vertebrates (D'Aniello *et al.*, 2008, Suga *et al.*, 1999). During embryogenesis, *FGFR* is first expressed at the gastrula stage in

the anterior mesendoderm (Bertrand *et al.*, 2011). During neurulation, expression is detected in the presomitic mesoderm and later on in presumptive notochord and somitic regions. At the late neurula stage, before the mouth opens, *FGFR* is expressed in the notochord, the anterior endoderm and the somites forming from the tailbud. In the larva, labelling is detected in the anterior pharyngeal endoderm, in the notochord, and at a lower level in the gut, except in the ilio-colonic region (Bertrand *et al.*, 2011).

FGFs are small proteins that are usually secreted. Eight FGF genes have been found in the *B. floridae* genome, but the orthology relationships with vertebrate FGFs are well supported using phylogenetic reconstruction for only three of them, namely *FGF1/2*, *FGF8/17/18* and *FGF9/16/20* (Bertrand *et al.*, 2011). However, synteny conservation analyses suggests that *FGFA* belongs to the *FGF3/7/10/22* paralogy group, *FGFB* to the *FGF4/5/6* group and *FGFC* to the *FGF19/21/23* group. In contrast, no orthology relationships with vertebrate FGFs could be proposed for *FGFD* and *FGFE*. Here we will keep this nomenclature of amphioxus FGFs even if other studies suggested different orthology relationships for some of the 8 amphioxus FGFs (Oulion *et al.*, 2012). The expression of these ligands is very diverse and dynamic (Bertrand *et al.*, 2011). *FGF1/2* is ubiquitously expressed during embryogenesis, whereas *FGFB* and *FGFD* expression could not be detected using *in situ* hybridization. *FGF8/17/18* and *FGF9/16/20* are the first FGFs to show a regionalized expression at the gastrula stage. They are expressed complementarily in the dorsal part of the gastrula with *FGF9/16/20* labelling being observed in the presumptive neural plate and *FGF8/17/18* being expressed in the dorsal mesendoderm. At the neurula stage, expression of *FGF8/17/18* completely fades in the mesendoderm and appears in the future cerebral vesicle, whereas *FGF9/16/20* is expressed in the neural tube and in the pharyngeal endoderm. At this stage *FGFA* expression starts in the cerebral vesicle and in the pharyngeal endoderm as well as in the mesoderm with an antero-posterior gradient. Later on, *FGF8/17/18* expression disappears in the cerebral vesicle and is observed in the most anterior tip of the epidermis, and in two regions of the pharyngeal endoderm corresponding to the future mouth and first gill slit territories in *B. lanceolatum* and *B. floridae* (Bertrand *et al.*, 2011, Meulemans and Bronner-Fraser, 2007). This pattern is observed until the larva stage. At the late neurula stage *FGF9/16/20* is expressed in a broad region of the ventral pharyngeal endoderm as well as in the neural tube and, in the larva, labelling is detected in the club-shaped gland, the first gill slit, the midgut as well as in the anus. *FGFA* is expressed in the cerebral vesicle and anterior ventral pharyngeal endoderm during late neurula stages, and in the endostyle, the club-shaped gland, the first gill slit, the mouth and the anus in larvae. *FGFE* expression starts in mid-neurula stage embryos in the Hatschek's nephridium anlage. Later on, expression is observed in some neurons and, in the larva, *FGFE* expression is restricted to several neural tube neurons, to the club-shaped gland and to the gut, except the ilio-colonic region. *FGFC* expression starts at the late neurula stage before the mouth opens in the most anterior endoderm and in the future gut just posterior to the pharyngeal region. In the larva, *FGFC* is expressed in the pre-oral pit, the endostyle and in the club-shaped gland.

FGF signalling function was assessed using pharmacological treatments with an inhibitor of FGFR (SU5402) at different time points (Bertrand *et al.*, 2011). Inhibiting this pathway before gastrulation induces the loss of the most anterior somites, whereas the posterior ones form normally. Treatment after the gastrula stage leads to a milder phenotype and all the somites form, although the correct morphogenesis of the notochord is affected. The function of FGF signalling at these stages is likely mediated by the MAPK pathway, as treatments with the MEK1/2 inhibitor U0126 induce the same phenotypes (Bertrand *et al.*, 2011). On the other hand, when embryos are treated at the 8-cell stage with SU5402, gastrulation is strongly affected, while treatment with U0126 has no effect. This result suggests that the early function of the FGF signalling pathway is mediated by the activation of a cytoplasmic cascade that does not involve MAPK. Interestingly, it was shown that FGF signalling does not interact with the retinoic acid pathway during somitogenesis, in contrast to what is known in vertebrates (Bertrand *et al.*, 2015).

Insulin receptor

Three receptors of the INSR (insulin receptor) subfamily are known in vertebrates: IGF1R (insulin-like growth factor 1 receptor), INSR (insulin receptor) and INSRR (insulin receptor-related receptor) (Brunet *et al.*, 2016). These receptors are characterized by the presence in their ectodomain of two L-domains separated by a furin-like cysteine rich region and followed by three fibronectin type III domains (Adams *et al.*, 2000). Their ligands are the insulin and insulin-like growth factors (IGF) 1 and 2, which are secreted proteins of the insulin-relaxin family that are characterized by the presence of a cystine knot motif (Jin Chan and Steiner, 2000).

In amphioxus, a unique receptor presenting the classical domain organization detailed above has been described (D'Aniello *et al.*, 2008, Pashmforoush *et al.*, 1996). The expression of *INSR* has so far only been assessed by Northern blot in *B. californiensis* adults transversally cut into four parts (Pashmforoush *et al.*, 1996). The authors showed that transcripts could be detected in all of these four regions.

Two genes, coding for Insulin-like peptides (ILP) were described in the genome of *B. floridae* (Lecroisey *et al.*, 2015) long after the first description of an *Ilp* gene in *B. californiensis* (Chan *et al.*, 1990). Both are ohnologues of vertebrate insulin genes IGF1 and IGF2. The embryonic expression pattern of one of them, *Ilp*, was studied in *B. lanceolatum* (Lecroisey *et al.*, 2015) and in neurulae of *B. floridae* (Holland *et al.*, 1997). The authors showed that expression starts at the gastrula stage in the dorsal paraxial mesendoderm. Then, at the beginning of neurulation, *Ilp* is expressed in the most anterior forming somites and in the posterior endoderm, with a pattern reminiscent of the one observed for *Delta* at this stage. Expression then becomes restricted to the central endoderm in both species (Holland *et al.*, 1997, Lecroisey *et al.*, 2015). In late larvae prior to mouth opening, *Ilp* transcripts are detected in the dorsal pharyngeal endoderm and in the future gut, just posterior to the pharyngeal region. Finally, in larvae, *Ilp* is expressed in the pharyngeal anterior endoderm and in the club-shaped gland. In adults, *Ilp* (also known as *IGF*) is expressed in the hindgut and in the hepatic caecum (Guo *et al.*, 2009).

There are no functional data concerning the role of the INSR signalling pathway during embryogenesis. However, the mode of action of INSR and its interaction with ILP or vertebrate ligands has been assessed by several laboratories *in vitro* or in mammalian cells (Guo *et al.*, 2009, Liu and Zhang, 2011, Pashmforoush *et al.*, 1996).

Platelet-derived- and vascular endothelial- growth factor receptors

In vertebrates, there are five receptors of the PDGFR (Platelet-Derived Growth Factor Receptor) family, and three of the VEGFR (Vascular Endothelial Growth Factor Receptor) family. Amphioxus possesses a unique receptor related to these two groups of RTKs, named PD/VEGFR. Phylogenetic and synteny conservation analyses suggest that the ancestral *VEGFR* and *PDGFR* found in vertebrates arose by tandem duplication of a unique *PD/VEGFR* gene before the two rounds of whole genome duplication (D'Aniello *et al.*, 2008). The amphioxus PD/VEGFR protein shows a domain organization similar to vertebrate VEGFR receptors with seven extracellular immunoglobulin domains (D'Aniello *et al.*, 2008, Suga *et al.*, 1999). During embryonic development, *PD/VEGFR* is first expressed at the neurula stage in two populations of cells of mesodermal origin on both sides of the embryo in the anterior region (Pascual-Anaya *et al.*, 2013). The cells on the left side were proposed to belong to the Hatschek's nephridium anlage, which will form the excretory organ of amphioxus. Before the mouth opens, PD/VEGFR expression is still detected in the forming Hatschek's nephridium as well as in single cells on the right side of the embryo (Pascual-Anaya *et al.*, 2013). At the larval stage, *PD/VEGFR* expression is detected in lateral cells located between the notochord and the gut and below the somites. Labelling is also observed in the anlagen of both the two dorsal aorta branches and the subintestinal vessels, and in the club-shaped gland (Pascual-Anaya *et al.*, 2013). There are currently no data on genes coding for putative ligands of the amphioxus PD/VEGFR. The embryonic function of the PD/VEGFR signalling pathway was assessed using an inhibitor of the receptor (SU5416) (Pascual-Anaya *et al.*, 2013). Continuous treatment starting at the neurula stage induces a mild but penetrant phenotype with late neurula embryos and larvae presenting a curved posterior region (Pascual-Anaya *et al.*, 2013). The authors showed that this phenotype is associated with a reduction in Laminin immunostaining in the regions of the dorsal aorta and subintestinal vessels, suggesting that the PD/VEGFR pathway is implicated in the formation of the circulatory system in amphioxus (Pascual-Anaya *et al.*, 2013).

Tropomyosin receptor kinase

Trk (tropomyosin receptor kinase) family members are neurotrophin receptors implicated in neuronal development, plasticity and survival (Lu *et al.*, 2005). Amphioxus possesses a unique receptor ohnologue of vertebrate NTRK1, NTRK2 and NTRK3, and the protein presents a comparable domain organization to its vertebrate counterparts with cysteine-rich clusters, several leucine-rich repeats, and two IgC2 domains in its extracellular region (Benito-Gutierrez *et al.*, 2005). Expression of *B. floridae Trk* is first observed in several epidermal cells in the ventral midline at the early neurula stage. Subsequently, labelling is detected in additional cells located mediolaterally. It has been proposed that these cells may be epidermal sensory neurons of the peripheral nervous system that migrate during embryonic development. At the larva stage, *Trk* expression is detected in the pre-oral pit and transiently in some anterior cells.

Trk receptors have several ligands in vertebrates, including NGF (Nerve Growth Factor), BDNF (Brain-derived neurotrophic factor), NT-3 (Neurotrophin-3) and NT-4 (Neurotrophin-4), which are secreted proteins. A unique ligand gene has been found in amphioxus (Hallbook *et al.*, 2006) but there is currently no information on its expression pattern. Interestingly, it has been shown that the amphioxus Trk receptor is activated by vertebrate neurotrophins and drives phosphorylation of both Akt and ERK1/2 in mammalian cells (Benito-Gutierrez *et al.*, 2005). However, the cephalochordate Trk is not able to induce PLCγ phosphorylation after NGF stimulation in the same context, highlighting possible differences with the vertebrate neurotrophin pathway mode of action (Benito-Gutierrez *et al.*, 2005).

Other actors

Amphioxus possesses at least one member of each of the gene families coding for proteins implicated in the three main cascades activated by RTK, namely the MAPK, the PLCγ and the PI3K pathways (Bertrand *et al.*, 2009). The embryonic expression patterns of two genes from each cascade have so far been assessed in *B. lanceolatum* (Bertrand *et al.*, 2009). *H/K/NRASa,* coding for a RAS GTPase implicated in the MAPK cascade, is expressed ubiquitously during early development (Bertrand *et al.*, 2009). Expression then becomes restricted to the notochord and the club-shaped gland anlage. In the larva, expression is detected in the posterior tip of the notochord and in the club-shaped gland. *RAF*, which codes for a MAPKKK, shows a ubiquitous expression pattern at early developmental stages (Bertrand *et al.*, 2009). Later on, expression is observed in the whole mesoderm and endoderm as well as in the cerebral vesicle before the mouth opens. In the larva, similar expression is observed with high levels in the club-shaped gland. *PDK* and *AKT*, coding for proteins of the PI3K cascade, are ubiquitously expressed, except in the epidermis, at almost all developmental stages (Bertrand *et al.*, 2009). Within the PLCγ cascade, *PKCα/β/γ* is expressed ubiquitously at early stages (Bertrand *et al.*, 2009). During neurulation, expression becomes restricted to ventral neurons of the cerebral vesicle and neural tube. In the larva, neural expression is still observed and *PKCα/β/γ* transcripts are also detected in the gut, except in the ilio-colonic region, in the club-shaped gland and in the pre-oral pit. Finally, *PKCγ* expression is ubiquitous, although stronger expression is evident at the larva stage in the club-shaped gland and in the gut, again with the exception of the ilio-colonic region (Bertrand *et al.*, 2009). Altogether, these data suggest that RTKs are able to activate only some specific intracellular cascades in particular embryonic regions in which the adequate actors are expressed at a given stage during amphioxus development.

Transforming growth factor β pathway

How does it work?

The TGF-β (Transforming Growth Factor β) signalling pathway plays crucial roles during embryogenesis as well as in cellular processes in adult animals (Derynck and Akhurst, 2007, Kishigami and Mishina, 2005, Massague *et al.*, 2000). TGF-β family members are generally secreted and are cleaved into their active form, which contains a cystine knot (Weiss and Attisano, 2012). They bind to heterodimeric complexes composed of homodimers of type I and type II serine/threonine kinase transmembrane receptors. Fixation of the ligand induces the phosphorylation of the type I receptors by the type II receptors (Fig. 6). This phosphorylation results in the activation of the type I receptors, which subsequently phosphorylate intracellular molecules called Smads (the name is a contrac-

tion of Sma and Mad (Mothers against decapentaplegic)), which transduce the signal to the nucleus to regulate the transcription of target genes (Weiss and Attisano, 2012) (Fig. 6). Two signalling pathways are distinguished based on which Smad proteins are phophorylated by the activated transmembrane receptors after ligand binding: the BMP (Bone Morphogenetic Protein) signalling pathway, which is mediated by Smad1, -5 and -8; and the TGF-β/Nodal/Activin signalling pathway, which utilises Smad2 and 3 in vertebrates (Bragdon *et al.*, 2011, Chin *et al.*, 2004, Massague *et al.*, 2000). Interestingly, besides Smad signalling, it is now well established that other cascades are also activated downstream of TGF-β receptors, such as the MAPK cascade, the Rho-like GTPase signalling pathway and the PI3K pathway, indicating that both Smad and non-Smad signalling participate in the final outcome of the cellular response to TGF-β proteins (Bragdon *et al.*, 2011, Zhang, 2009).

In addition to the diversity of TGF-β ligands and receptors, both BMP and TGF-β signalling pathways are regulated at the extracellular level by a cocktail of proteins, including pseudoreceptors and extracellular inhibitors as well as metalloproteinases (Bier and De Robertis, 2015). Pseudoreceptors can interact with TGF-β receptors and prevent the formation of active ligand/receptor complexes, whereas extracellular antagonists are able to bind to the ligands and decrease their affinity for their receptors (Balemans and Van Hul, 2002, Chin *et al.*, 2004, Gazzerro and Canalis, 2006). Here we will focus our attention on extracellular regulation of TGF-β signalling in amphioxus.

Receptors and extracellular actors in amphioxus

In the *B. floridae* genome, three type I (*Alk1/2*, *Alk3/6* and *Alk4/5/7*) and three type II (*TGF-βRII*, *ActRII* and *BMPRII*) receptor genes have been identified (Satou *et al.*, 2008), but no expression pattern has been reported yet. However, the expression of the pseudoreceptor *BAMBI* (BMP and Activin Membrane-Bound Inhibitor) has been analyzed during early developmental stages. *BAMBI* transcripts are detected at the gastrula stage in the ventral mesendoderm and, later on during neurulation, in the endoderm (Yu *et al.*, 2007).

Eighteen TGF-β genes are present in the *B. floridae* genome (Satou *et al.*, 2008). Among these extracellular ligands, the orthology relationships with vertebrate TGF-β family members have been clearly defined by phylogenetic analysis for thirteen of them (Satou *et al.*, 2008). Six genes coding for ligands of the *BMP* subfamily were identified as *ADMP, BMP2/4*, *BMP3/3b, BMP5-8*, *BMP9/10* and *GDF5/6/7*, and seven of the TGF-β/Nodal/Activin subfamily, namely *Lefty, Myostatin, Nodal*, *TGF-β*, *Vg1*, and two *Activin/Inhibin* genes (Satou *et al.*, 2008). Developmental expression patterns have been described in amphioxus for *ADMP* (Kozmikova *et al.*, 2013, Yu *et al.*, 2007), *BMP2/4* (Kozmikova *et al.*, 2013, Panopoulou *et al.*, 1998, Yong *et al.*, 2017, Yu *et al.*, 2007), *BMP3/3b* (Sun *et al.*, 2010), *BMP5-8* (Kozmikova *et al.*, 2013, Yu *et al.*, 2007), *Lefty* (Morov *et al.*, 2016, Onai *et al.*, 2010, Yu *et al.*, 2007), *Nodal* (Morov *et al.*, 2016, Onai *et al.*, 2010, Yu *et al.*, 2002, Yu *et al.*, 2007) and *Vg1* (Onai *et al.*, 2010).

ADMP is first expressed in the dorsal half of the mesendoderm from the early gastrula stage until the end of gastrulation in *B. floridae*. At the mid-gastrula stage, *ADMP* starts to be expressed in the neural plate; this expression is maintained until the neurula stage, when transcript detection becomes restricted to the medial part of the plate. At this stage, paraxial mesoderm expression of *ADMP* is progressively lost but is maintained in the axial mesoderm (Kozmikova *et al.*, 2013, Yu *et al.*, 2007).

From the onset of gastrulation, *BMP2/4* and *BMP5-8* are co-expressed in the entire mesendoderm until the late gastrula stage, when their expression in the axial mesoderm begins to disappear, concomitantly with the appearance of expression of *BMP2/4* in lateral and ventral ectoderm (Kozmikova *et al.*, 2013, Panopoulou *et al.*, 1998, Yu *et al.*, 2007). *BMP2/4* expression is then lost in the

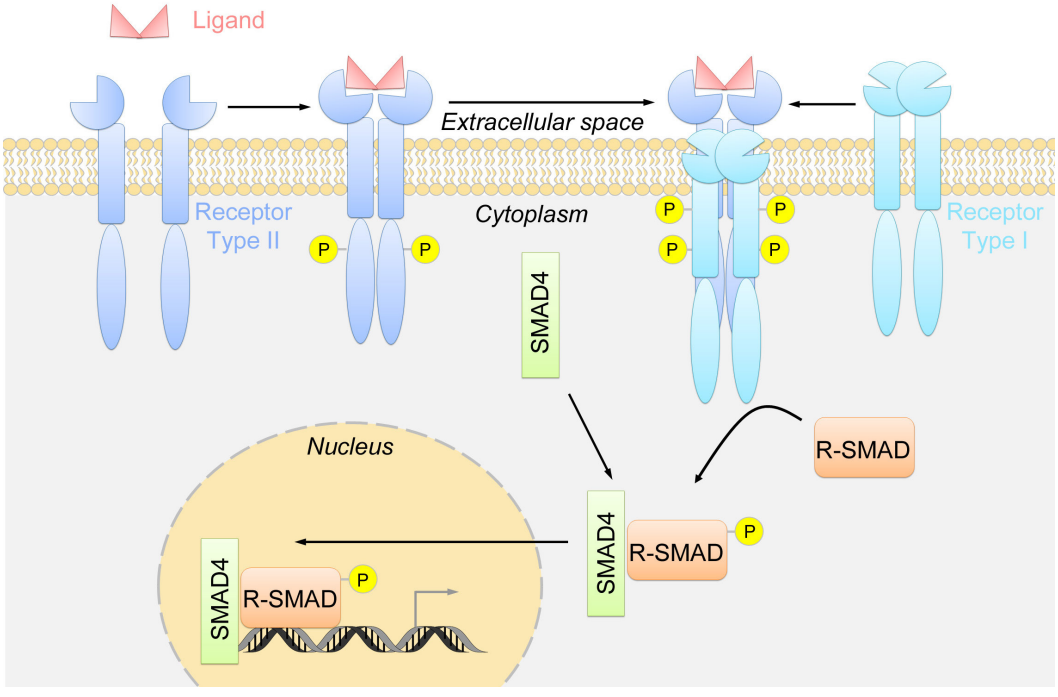

Fig. 6. Simplified schematic view of the canonical transforming growth factor (TGF)-β signalling pathway. *The binding of ligands to a dimer of type II receptors leads to their phosphorylation. In turn, this dimer interacts with, and phosphorylates, a dimer of type I receptors. Activated type I dimers phosphorylate a regulatory SMAD (R-SMAD), which interacts with SMAD4. The dimer enters the nucleus and activates the transcription of target genes after DNA binding.*

somites during neurulation, except in the tailbud. Expression is also observed in some epidermal cells in front of the neuropore. Within the endodermal derivatives, *BMP2/4* is expressed in the pharyngeal region and in the posterior presumptive gut; no expression is detected in the midgut from the neurula stage onwards. At the late neurula stage, *BMP2/4* transcripts are detected in the forming club-shaped gland and in the Hatschek's right diverticulum as well as in a row of mesothelial cells on the right along the "ventral midline". In the larva, *BMP2/4* expression pattern is quite similar (Panopoulou *et al.*, 1998). No expression data are available for *BMP5-8* after the early neurula stage.

BMP3/3b is expressed during gastrulation in the whole mesendoderm of *B. japonicum*. At the neurula stage, *BMP3/3b* is first expressed in the border of the neural plate and in the paraxial mesoderm. Later on, expression is detected in part of the neural plate and in median somites. At the larva stage, *BMP3/3b* is expressed in the anterior part of the closed neural tube, in the pharynx, the Hatschek's left diverticulum, the pre-oral ciliated pit, the club-shaped gland and the gill slits (Sun *et al.*, 2010). Expression of *BMP3/3b* has also been reported in adult amphioxus, where the *BMP3/3b* transcripts are detected in the nerve cord, the sheath surrounding the notochord, the metapleural fold and in the oocytes (Sun *et al.*, 2010).

Nodal, *Vg1* and *Lefty* possess very similar expression pattern during embryonic development in amphioxus. Both *Nodal* and *Vg1* are expressed maternally. *Vg1* is expressed ubiquitously until the end of the blastula stage, whereas *Nodal* expression is restricted to the two-thirds of the embryo towards the animal pole. During gastrulation, *Nodal*, *Vg1* and *Lefty* are expressed in the dorsal ectoderm and mesendoderm, with *Lefty* expressed in a more axially restricted region (Morov *et al.*, 2016, Onai *et al.*, 2010, Yu *et al.*, 2002, Yu *et al.*, 2007). At the neurula stage, these three genes begin to be expressed only on the left part of the embryo, first in the three most anterior somites and later on in all of the left somites, with stronger expression in the most anterior somites and the posterior forming somites (Onai *et al.*, 2010, Yu *et al.*, 2002, Yu *et al.*, 2007). *Nodal* is also expressed in the Hatschek's left diverticulum, the lateral ectoderm and endoderm corresponding to the future region where the mouth will open at the late neurula stage (Yu *et al.*, 2002). More detailed expression patterns for *Vg1* and *Lefty* are not available after neurula stage. At the larval stage, *Nodal* expression is restricted to the most posterior part of the left somitic tissue, expression which disappears in one week old larvae (Yu *et al.*, 2002).

Other actors

In amphioxus, seven extracellular proteins orthologous to proteins known to exert an antagonist or modulator activity on BMP or TGF-β/Nodal/Activin ligands have been identified: Cerberus, Chordin, Gremlin, and NBL1 (Neuroblastoma suppressor of tumorigenicity 1, also called DAN), Noggin, Tsg (Twisted gastrulation) and Tolloid (also called Xolloid in *Xenopus* and BMP1 in human). The expression pattern has been described for all the corresponding genes except *Noggin*.

Cerberus, Gremlin and NBL1 belong to the DAN (Differential screening-selected gene Aberrative in Neuroblastoma) family and present a typical cystine knot structure. *Cerberus* is first expressed at the gastrula stage in the anterior mesendoderm underlying the presumptive neural plate. At the early neurula stage, *Cerberus* expression is observed in the first anterior somites before becoming asymmetrical after the loss of expression on the left side. At the mid-neurula stage, *Cerberus* is no longer expressed in the somites and transcripts are only detected in the ventral part of the closing neural tube. No expression could be detected by *in situ* hybridization at later stages (Le Petillon *et al.*, 2013, Onai *et al.*, 2010).

Gremlin is first expressed in a small, restricted region of the ventral mesendoderm, expression which remains at the early neurula stage when *Gremlin* also begins to be expressed in cells along the borders of the neural plate. At the mid-neurula stage, *Gremlin* is expressed in cells lateral to the neural tube and at the level of the midline of the ventral endoderm. *Gremlin* expression is also detected at the level of the first left somite in the presumptive Hatschek's nephridium. At the late neurula stage these territories still express *Gremlin,* which begins to be expressed in the cerebral vesicle. At the larval stage, *Gremlin* is expressed in some cells of the cerebral vesicle and in cells of the pre-oral pit and endostyle (Le Petillon *et al.*, 2013).

In amphioxus, *NBL1* expression has only been detected at the larval stage in the somites and in the club-shaped gland (Le Petillon *et al.*, 2013).

Chordin is one of the first BMP modulator proteins to have been characterized in amphioxus. Interestingly, expression of *Chordin* is similar to the expression of *ADMP* in amphioxus. *Chordin* is first expressed in the dorsal ectoderm and mesendoderm during gastrulation. At the neurula stage, expression is observed in the neural plate and in the axial and paraxial mesoderm before being restricted to the medial part of the closing neural plate and to the axial mesoderm (Somorjai *et al.*, 2008, Yu *et al.*, 2007). Then, before mouth opening, *Chordin* is expressed in the posterior region of the embryo and in particular neurons. Finally, in the larva, *Chordin* is expressed in the posterior region, in the club-shaped gland, and in the cerebral vesicle (Somorjai *et al.*, 2008).

Tsg and Tolloid are two proteins known to modulate BMP signalling (Troilo *et al.*, 2016). Tolloid is a zinc metalloproteinase able to cleave Chordin, making it unable to antagonize BMP ligands. Tsg is able to bind either BMP or the BMP/Chordin complex, thereby having antagonistic or agonistic activity. It can also enhance Tolloid cleavage activity on Chordin, decreasing its anti-BMP action. In amphioxus, *Tsg* begins to be expressed at the early gastrula stage in the dorsal mesendoderm and is expressed during neurula stage in the whole mesoderm (Yu *et al.*, 2007). *Tolloid-like* is first expressed at the mid-gastrula stage in the ventral mesendoderm and then in the ventral endoderm during neurulation (Yu *et al.*, 2007).

Functional studies

The main strategy used to modify TGF-β signalling during amphioxus development is the use of small molecule inhibitors and recombinant proteins to inhibit or activate TGF-β pathways, respectively. Dorsomorphin is used to inhibit BMP type I receptor (Alk3/6) and has been shown to disrupt the nuclear localization of phosphorylated Smad1/5/8 (Kaji *et al.*, 2016, Kozmikova *et al.*, 2013, Lu *et al.*, 2012). SB505124 and SB431542 are two inhibitors of TGF-β type I receptors (Alk4/5/7) used to inhibit TGF-β/Nodal/Activin pathway (Bertrand *et al.*, 2015, Kaji *et al.*, 2016, Kozmikova *et al.*, 2013, Li *et al.*, 2017, Morov *et al.*, 2016, Onai *et al.*, 2010, Soukup *et al.*, 2015). In contrast, recombinant proteins such as zBMP4 (Lu *et al.*, 2012, Onai *et al.*, 2010, Yu *et al.*, 2008, Yu *et al.*, 2007), hBMP2 (Kozmikova *et al.*, 2013), hActivin (Onai

et al., 2010) and mNodal (Li *et al.*, 2017) (h=human, m=mouse, z=zebrafish) have been used to activate TGF-β pathways in amphioxus. Due to the technical difficulties involved in microinjecting amphioxus eggs, the use of other tools is still scarce. Nevertheless, injections of mRNA, morpholinos, plasmids or TALENs have also been used to assess the embryonic function of TGF-β pathways, as detailed below.

It was first shown using pharmacological treatments that ectopic BMP activation induces the ventralization of the amphioxus embryo, with a loss of expression of dorsal genes and an expansion of ventral gene expression (Kozmikova *et al.*, 2013, Onai *et al.*, 2010, Yu *et al.*, 2008, Yu *et al.*, 2007). In contrast, inhibiting BMP signalling at the blastula stage induces a dorsalization of the embryo with an expansion of the expression of dorsal markers (Kozmikova *et al.*, 2013). On the other hand, earlier inhibition induces the dorsalization of the mesendoderm whereas the ectoderm stays in an uncommitted fate and expresses the pan-ectodermal gene *SoxB1a* but fails to express epidermal or neural markers. These results strongly suggest that BMP signal inhibition is insufficient for neural induction in amphioxus, but that it is required for epidermal fate acquisition by ectodermal cells (Le Petillon *et al.*, 2017).

Interestingly, the TGF-β/Nodal/Activin pathway appears to act in opposition to the BMP signal for axis formation during early development. Indeed, activation of this pathway using recombinant hActivin treatment induces an expansion of dorsal ectodermal and mesodermal gene expression, and a loss of expression of ventral genes (Onai *et al.*, 2010), whereas its repression before gastrulation induces the ventralization of the embryo (Morov *et al.*, 2016, Onai *et al.*, 2010). Moreover, it has been shown that BMP and TGF-β/Nodal/Activin signals concomitantly control antero-posterior patterning with an anteriorizing role for the TGF-β/Nodal/Activin signal and a posteriorizing role for the BMP signal (Onai *et al.*, 2010). The authors also analyzed the function of *Chordin* by injecting corresponding morpholinos. They showed that *Chordin* knock-down induces a mild phenotype with a loss of anterior marker expression, but to a lesser extent than when the BMP signal was ectopically activated, suggesting that other BMP antagonists might be playing a parallel role in inhibiting BMP signal in the dorsal part of the amphioxus embryo (Onai *et al.*, 2010). In contrast, injection of *Cerberus* mRNA induces a ventralization and posteriorization similarly to BMP signal activation and the authors therefore suggest that Cerberus might be a TGF-β/Nodal/Activin signal antagonist in amphioxus (Onai *et al.*, 2010). It was also recently shown that Nodal/Activin is playing a crucial role for neural induction in amphioxus independently of BMP signal inhibition and that the interactions between both pathways might be tightly regulated in time and space (Le Petillon *et al.*, 2017).

In addition to the role of BMP during antero-posterior and dorso-ventral patterning, it has been proposed that the BMP signal might be involved in the formation of epidermal sensory neurons (ESNs) in amphioxus, since manipulating BMP signalling levels affects the distribution of ESNs (Lu *et al.*, 2012). Interestingly, as previously discussed, Delta/notch signalling is implicated in the specification of ESN, and Lu and colleagues showed that BMP signal functions upstream of Delta/Notch to induce/maintain *in vivo* a ventral neurogenic domain from which the ESNs will derive (Lu *et al.*, 2012). Based on comparative observations with vertebrates, the authors proposed that differences in BMP signal regulation could explain differences in peripheral nervous system formation in the different chordate lineages (Lu *et al.*, 2012).

Nodal signalling is also involved in left/right axis establishment. Based on chemical inhibition of TGF-β type I receptors after gastrulation, Soukup and colleagues showed that after inhibition of the TGF-β/Nodal/Activin signalling pathway, the left-sided genes are mostly downregulated during early neurula stage and that their expression is later lost. On the other hand, the right-sided genes become symmetrically expressed after such inhibition (Soukup *et al.*, 2015). Consequently, asymmetry of some structures like the pharynx, the somites and the associated neural system, is lost in embryos deprived of Nodal signalling, which present two right sides (Bertrand *et al.*, 2015, Soukup *et al.*, 2015). Recently, these observations were confirmed using transgenic lines of amphioxus with a mutated version of *Cerberus*, created using the TALEN technique, and using heat shock promoter driving overexpression of several actors of the Nodal pathway such as *Cerberus*, *Nodal*, *Lefty* and *Pitx* (Li *et al.*, 2017).

A consequence of the loss of asymmetry due to Nodal signal inhibition is the absence of the mouth, which normally forms on the left side of the amphioxus embryo. Indeed, Kaji and colleagues observed that at the level of the left-most anterior somite, a mesovesicle they suggested to be crucial for mouth opening is absent from embryos depleted of Nodal signal after gastrulation (Kaji *et al.*, 2016). The same authors showed that inhibiting the BMP pathway using dorsomorphin impedes mouth opening, suggesting that both Nodal and BMP signals are crucial for this developmental process (Kaji *et al.*, 2016). Conversely, deregulation of endogenous Nodal activity in Cerberus mutants induces the formation of a mouth on both sides of the embryo (Li *et al.*, 2017).

Wnt/β-catenin pathway

How does it work?

Wnt ligands are secreted glycoproteins mediating cell fate decisions, proliferation, and cell movements during embryogenesis across metazoans (Loh *et al.*, 2016). The first Wnt discovered in mouse, named *int1* (integration 1) for its oncogenic role (Nusse and Varmus, 1982), was later shown to be homologous to the *Wingless (Wg)* segment polarity gene in *Drosophila*. In its simplest form, during β-catenin dependent or "canonical" signalling, Wnt proteins bind the Frizzled 7-transmembrane domain receptors at the membrane along with LRP5/6 (Low-density lipoprotein receptor-related protein), thereby recruiting Dishevelled (Fig. 7) (Clevers, 2006). This prevents the scaffolding proteins including APC (Adenomatous polyposis coli) and Axin from assembling the β-catenin "destruction complex", a large multi-protein assemblage including GSK3β (Glycogen synthase kinase 3β) and CK1 (Caseine kinase 1) kinases; β-catenin is then able to accumulate in the cytoplasm, and free to enter the nucleus, binds TCF (T-cell factor) and other transcriptional partners such as Groucho to mediate downstream signalling events (Fig. 7) (Clevers, 2006). However, in the absence of Wnt ligand, β-catenin is targeted by the β-TrCP E3 ubiquitin ligase for ubiquitination, and is degraded by the proteasome (Baron and Kneissel, 2013). This is mediated by assembly of the destruction complex at β-catenin, and CK1α and GSK3β phosphorylation at key residues in its N-terminus (Clevers, 2006). In the absence of signalling, Axin may also undertake an alternate conformation that blocks its interaction with LRP5/6, but leaves it free to bind β-catenin to mediate its degradation (Song *et al.*, 2014).

The Wnt/β-catenin pathway is also modulated by a number of co-receptors and extracellular antagonists, including secreted frizzled related proteins (sFRPs), Dickkopf (Dkk) proteins, and Kremens. sFRPs may antagonize signalling in a variety of ways, including *via* direct interaction with Wnt proteins through their CRD and Netrin domains, or through heterodimerization with Frizzleds, in each case effectively preventing Wnt/Frizzled coupling. Instances in which sFRPs promote pathway activation have also been reported, however, highlighting the importance of cellular context in mediating interactions between possible binding partners (Bovolenta *et al.*, 2008). Dkks in the Dkk1/2/4 class, in contrast, are thought to inhibit Wnt/β-catenin by binding directly to the ectodomains of LRP5/6, and thus interfering with Wnt ligand binding (Bao *et al.*, 2012). Kremens are co-receptors for Dkks, and can also bind LRP5/6 in a ternary complex (Zebisch *et al.*, 2016); interestingly, in the presence of Dkk, Kremen proteins enhance Wnt inhibition, while in the absence of Dkk they potentiate LRP5/6 mediated signalling, thus acting as a bi-modal switch during Wnt signalling.

Alternative, "non-canonical" β-catenin-independent pathways have also been identified, but are poorly understood and much less well characterized in most systems. Some are mediated by Wnt ligands, Frizzled receptors and Dishevelled at the membrane, in addition to other G-protein coupled receptors. Indeed, Dishevelled has been considered to be a branching point between β-catenin dependent and independent pathways (Gao and Chen, 2010), and it is becoming apparent that such cross-functionality is common (Angers and Moon, 2009). Others utilize alternative receptors, including receptor tyrosine kinases such as ROR or RYK, and do not act positively on β-catenin mediated transcription in the nucleus, but rather on the actin cytoskeleton, calcium signaling or other nuclear targets (Van Amerongen, 2012). These are collectively known as "Wnt polarity" pathways due to their roles in cellular behavior such as convergence extension or axon guidance (Loh *et al.*, 2016). Given our still limited understanding of how these different pathways are structured, particularly in amphioxus, we focus on a review of members in the context of Wnt/β-catenin signalling, with the caveat that many of the upstream components (ligands, receptors *etc*) described here may be functionally involved in more than one Wnt pathway.

Receptors and ligands in amphioxus

Wnt ligands are characterized by an N-terminal signal peptide, and C-terminal cysteine residues responsible for the physical interaction with Frizzled receptors. In amphioxus, 12 Wnt genes have been identified corresponding to each of the subfamilies thought to have been present in the bilaterian last common ancestor, with the exception of *WntA* (reviewed in Albalat and Canestro, 2016). In vertebrates, a number of Wnt duplicates have been retained following the 2 whole genome duplications in the vertebrate ancestor within these subfamilies. Eight amphioxus Wnt ligands were first characterized in *B. floridae*, including *Wnt1, -3, -4, -5, -6, -7, -*8 and *Wnt11* (Holland *et al.*, 2000, Schubert *et al.*, 2000a, Schubert *et al.*, 2000c, Schubert *et al.*, 2000d, Schubert *et al.*, 2001). Five of these have been partially characterized in *B. lanceolatum* (*Wnt3-Wnt8*), and one in *B. japonicum* (*Wnt8*); their expression shows broad conservation with that seen in the American species (Albuixech-Crespo *et al.*, 2017, Morov *et al.*, 2016, Somorjai *et al.*, 2008).

Wnt1 is first detected around the lip of the blastopore in gastrulae, and is the most posterior expressed ligand, with expression in the posterior wall of the neurenteric canal in late neurulae (Holland *et al.*, 2000). Qian and colleagues also report that it is maternally expressed by RT-qPCR (Qian *et al.*, 2013).

Wnt3 shows similar blastoporal expression in early gastrulae, but is expressed along the edges of the neural plate by the early

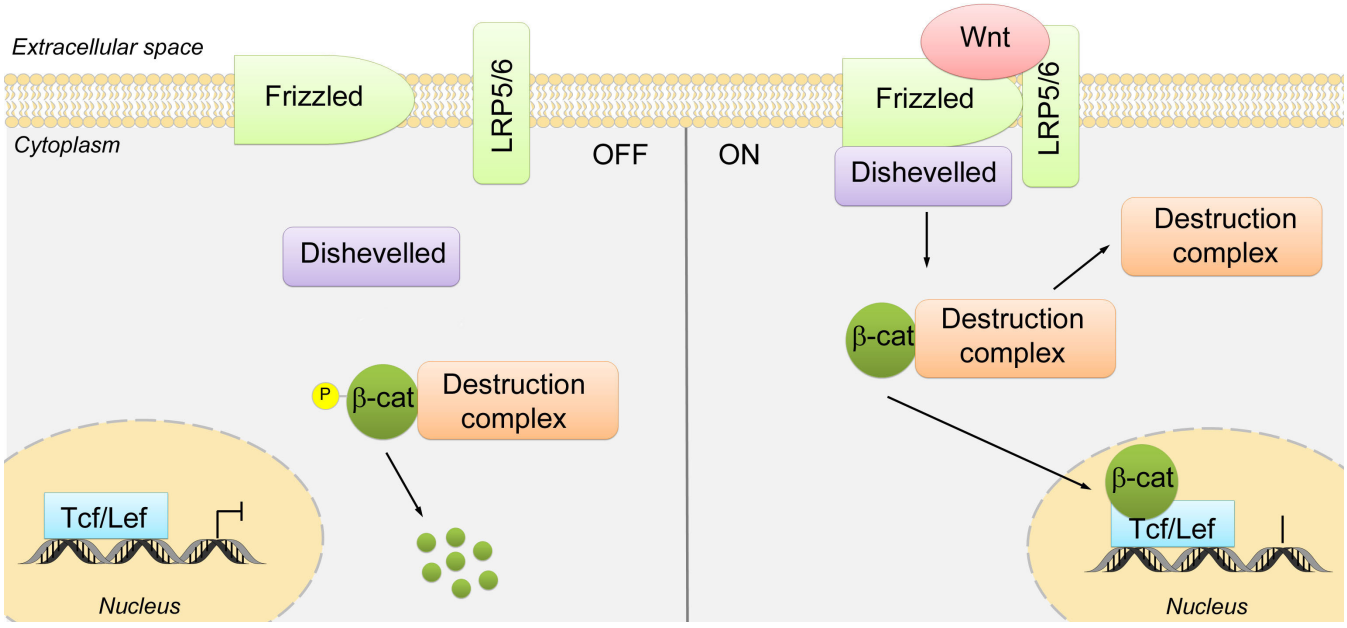

Fig. 7. Simplified schematic view of the Wnt/β-catenin signalling pathway. *In the absence of Wnt (OFF), β-catenin (β-cat) is phosphorylated by a multi-protein "destruction complex", then ubiquitinated, and, finally, degraded by the proteasome. When a Wnt ligand binds to the receptor Frizzled along with LRP5/6 (ON), Dishevelled is recruited at the membrane. This prevents the formation of the "destruction complex". β-catenin then accumulates in the cytoplasm and enters the nucleus, where it binds partners such as Tcf/Lef to mediate transcriptional activation of target genes.*

neurula stage except most anteriorly (Schubert *et al.*, 2001). Similar expression is seen in mid-neurulae of *B. floridae* and *B. lanceolatum* (Albuixech-Crespo *et al.*, 2017, Schubert *et al.*, 2001). As neurulation proceeds, this expression domain resolves to the posterior neural tube and ectoderm, and the posterior wall of the neurenteric canal. In early larvae, *Wnt3* can also be detected in the cerebral vesicle, in addition to these domains (Schubert *et al.*, 2001).

In contrast to *Wnt3*, *Wnt4* is expressed throughout the mesendoderm in gastrulae, but most strongly around the blastopore excluding the ectoderm (Schubert *et al.*, 2000b). In neurulae, transcripts are detected in the posterior paraxial mesoderm and hindgut endoderm, but not in the nascent notochord or neural plate; later expression is most notable in the posterior at tailbud level, in the neural tube and cerebral vesicle. In larvae, expression persists in somites, neural tube, hindgut endoderm and posterior cerebral vesicle, in addition to appearing in endostylar endoderm and mesothelial cells near the forming second gill slit (Schubert *et al.*, 2000b).

Wnt5 is expressed in the mesendoderm of the gastrula, but only and very conspicuously around the blastopore, more similarly to *Wnt3* (Schubert *et al.*, 2001). By early to mid-neurula stages, expression is strongest in posterior paraxial mesoderm and ventral endoderm in both American and European species (Albuixech-Crespo *et al.*, 2017, Schubert *et al.*, 2001, Somorjai *et al.*, 2008), clearly corresponding to the tailbud by premouth larval stages. *Wnt5* is also expressed in the cerebral vesicle and pharynx, resolving to the club-shaped gland in late larvae, as well as the anterior notochord and nascent somite of the tailbud (Schubert *et al.*, 2001).

Wnt6 is maternally expressed (Qian *et al.*, 2013), and as with many other *Wnt* genes, *Wnt6* is expressed around the blastopore in gastrulae. However, *Wnt6* is most conspicuously expressed in the CNS in pairs of spots on either edge of the neural plate in early neurulae, as well as in posterior mesoderm (Schubert *et al.*, 2001, Somorjai *et al.*, 2008). In late neurulae, transcripts are detected in the last pair of forming somites, along the length of the neural tube but not in the cerebral vesicle, and in the posterior wall of the neurenteric canal; both the posterior-most neural tube and neurenteric canal expression domains persist in 7-day larvae in *B. floridae* (Schubert *et al.*, 2001).

Wnt7 has so far not been detected in any stage prior to or including the blastula. In gastrulae, *Wnt7* is expressed throughout the mesendoderm, but by early neurula stages is almost entirely restricted to a small central region at the edges of the closing neural tube (Schubert *et al.*, 2000b, Somorjai *et al.*, 2008). In *B. lanceolatum* mid-neurulae, the anteriormost extension of *Wnt7* abuts the posterior boundary of the DiMes (Di-Mesencephalic primordium), thought to be equivalent to the vertebrate Midbrain/Hindbrain boundary, similarly to *Wnt3* (Albuixech-Crespo *et al.*, 2017). As neurulation proceeds, transcripts are detected throughout the neural tube, with the exception of the most posterior parts, but including posterior parts of the cerebral vesicle (Schubert *et al.*, 2000b). In 90hpf *B. floridae* larvae, roughly the anterior two-third of the neural tube expresses *Wnt7*, and expression in the posterior cerebral vesicle persists, with a new domain appearing consisting of a few endodermal cells of the endostyle (Schubert *et al.*, 2000b).

Wnt8 expression in amphioxus is highly dynamic, and is first detected in the early gastrula dorso-laterally in prospective paraxial mesoderm on either side of the archenteron in *B.floridae* and *B.japonicum* (Morov *et al.*, 2016, Schubert *et al.*, 2000d). In early neurulae, posterior paraxial mesoderm is strongly labelled, later defining all somites except the first pair, and in a restricted posterior domain of the endoderm that will give rise to the hindgut. In late neurulae of both *B. floridae* and *B. lanceolatum*, transcript expression remains strongest in the posterior mesoderm and endoderm of the growth zone anterior to the tailbud and in several anterior somites posterior to the first pair, but is reduced in intervening somites (Albuixech-Crespo *et al.*, 2017, Schubert *et al.*, 2000d). In 3-day *B. floridae* larvae, a subset of ventral cells in the cerebral vesicle continues to express *Wnt8*, but otherwise the gene is downregulated (Schubert *et al.*, 2000d).

In cephalochordates, *Wnt11* has only been characterized in *B. floridae* and, besides being maternally expressed (Qian *et al.*, 2013), shows the earliest zygotic expression of the Wnt ligands, with expression in a few cells of the presumptive dorsal side in late blastulae beginning at gastrulation (Schubert *et al.*, 2000a). In neurulae, *Wnt11* is expressed in mesodermal myotomes as well as in posterior endodermal and ectodermal domains. By larval stages, most mesodermal expression is lost except in posterior somites, and transcripts can be detected in antero-ventral ectoderm, some ventral mesothelial cells and posterior ectoderm possibly giving rise to the tail fin (Schubert *et al.*, 2000a).

Of the remaining ligands, expression data are still lacking for *Wnt2, Wnt9, Wnt10* and *Wnt16*, but *Wnt9* and *Wnt10* at least are maternally expressed (Qian *et al.*, 2013). Nothing is known about the role of any *Wnt* in later larval development, but *Wnt5* is also expressed in the undifferentiated blastema during tail regeneration in adult *B. lanceolatum* (Somorjai *et al.*, 2012a).

The Frizzled proteins are G-protein coupled receptors (GPCRs) containing a cysteine-rich domain (CRD). Four Frizzled receptors, Fz1/2/7, Fz4, Fz5/8 and Fz9/10, have been described in the Asian species *B. belcheri* (Qian *et al.*, 2013), corresponding to the four subfamilies present in the bilaterian plus cnidarian common ancestor (Schenkelaars *et al.,* 2015). The fifth, Fz3/6, appears to be an innovation of the Olfactores, most closely related to the Fz1/2/7 clade (Schenkelaars *et al.*, 2015). RT-qPCR suggests that all four *Frizzled* genes are expressed maternally and ubiquitously in the 4-cell stage (Qian *et al.*, 2013). In the early neurula *Fz1/2/7* is expressed in the neural plate as well as in somites. The authors suggest that in the larva *Fz1/2/7* is expressed in the mouth and the pre-oral pit. Early *Fz1/2/7* domains partially overlap with those of *Fz4*. *Fz4* is the only gene clearly expressed in the cerebral vesicle from mid-neurula to larval stages; otherwise it is broadly expressed in mesendoderm, as well as in the pre-oral pit and in components of the pharynx (Qian *et al.*, 2013). *Fz5/8* is distinctive in maintaining expression from late gastrula to late larva in the anterior-most ectoderm and endoderm of the embryo (Qian *et al.*, 2013). Finally *Fz9/10* has the most posterior expression in the early neurula of all *Frizzled* genes analysed, showing expression in the posterior neural plate of the mid-neurula stage embryo, and in the last-formed somites (Qian *et al.*, 2013).

Inhibitors/antagonists

sFRPs are Frizzled Related Proteins that can be secreted due to the replacement of the transmembrane domains with a Netrin domain, and can compete with Frizzled for binding to Wnt ligands. Two have been identified in cephalochordates, and their expression patterns characterized in *B. floridae* (Yu *et al.*, 2007) and *B. belcheri* (Kong *et al.*, 2012), where they are broadly consistent. *sFRP1/2/5,* otherwise named *sfrp2-like,* is expressed in the

mesendoderm in gastrulating embryos, later becoming restricted to the anterior mesoderm and endoderm in neurula stages. Kong and colleagues further report that expression becomes undetectable from the late neurula stage onwards in *B. belcheri* (Kong *et al.*, 2012). The second, *sFRP3/4,* has only been described for early stages in *B. floridae*, where like *sFRP1/2/5* it is expressed in mesendoderm in mid-gastrulae. By late gastrula/early neurula, expression is restricted to a domain around the blastopore, and weakly in the mesoderm.

The first Dickkopf protein, Dkk1, was described in *Xenopus*, where it was shown to be a potent antagonist of Wnt signalling, and to have head-inducing activity (Glinka *et al.*, 1998). Two antagonists of the Dickkopf family have been described in cephalochordates, corresponding to the two paralogy groups found in vertebrates: Dkk1, -2 and -4 and Dkk3. While Dkk1 -2 and -4 show Wnt antagonizing activity, the role of Dkk3 in Wnt signalling is unclear, but it may potentiate Wnt signalling in some contexts (Nakamura *et al.*, 2007). In *B. floridae*, *Dkk1/2/4* is expressed vegetally in early gastrulae, and most strongly in a mesendodermal ring around the blastopore (Yu *et al.*, 2007). By neurula stages, it is expressed not only at the site of blastopore closure, but also in two clear anterior stripes on either side of the midline in the first two pairs of somites. This expression is similar in *B. belcheri* until mid-neurula stages, when the posterior domain further resolves into a pair of expression spots in what appear to represent the last formed somites (Zhang and Mao, 2010). Later, expression becomes asymmetric on the left side in tailbud mesoderm, where by premouth larval stages it has disappeared entirely. In larvae, *Dkk1/2/4* is only expressed in two anterior left patches in the pharyngeal region, and finally only in the cerebral vesicle of late larvae (Zhang and Mao, 2010).

In amphioxus, Dkk3 possesses an additional TGF-β receptor 2 domain in the N-terminus, which is absent in other species (Onai *et al.*, 2012). *Dkk3* expression in *B. floridae* is virtually a mirror image of *Dkk1/2/4* at early stages, when it is expressed most strongly in the animal pole, until resolving into a strong anterior ectodermal and an anterior-ventral endodermal domain in neurulae (Yu *et al.*, 2007). Careful sectioning shows that the expression of *Dkk3* in the anterior endoderm is restricted to the left side, while that in the ectoderm is limited to the anterior end of the neural plate (Onai *et al.*, 2012). Surprisingly, in *B. belcheri Dkk3* expression has only been detected in a central patch of the larval gut (Zhang and Mao, 2010), which is difficult to corroborate as data are absent in other cephalochordates for late stages. Taken together with dorsal/ventral expression of BMP ligands and antagonists, the expression of Wnt antagonists such as *Dkk1/2/4* and *sFRPs* anteriorly, along with multiple *Wnt* ligands posteriorly, suggested the existence of a dorsal organizer in amphioxus as found in vertebrates (Yu *et al.*, 2007). The presence of such an organizer in amphioxus has indeed recently been confirmed (Le Petillon *et al.,* 2017).

Co-regulators

In addition to the receptors and antagonists/inhibitors described above, the Wnt/β-catenin pathway is regulated by a number of co-receptors extracellularly, including low-density lipoprotein receptor-related proteins (LRP) and Kremen proteins, many of which act through binding Dkks with high affinity. In vertebrates, LRP5 and -6 are orthologous to the single LRP5/6 ("Arrow" in *Drosophila*) found in amphioxus. *LRP5/6* expression has been described only in *B. floridae*, and perhaps unsurprisingly given its function, is expressed almost ubiquitously in the mesoderm and endoderm of neurula stage embryos, with the exception of the tailbud (Wang *et al.*, 2016).

In vertebrates, two Kremens have been identified, Kremen1 and Kremen2, with clear roles in potentiating the Wnt inhibitory effects of Dkk1 (Cruciat and Niehrs, 2013) *via* direct binding in a ternary complex with LRP6 (Zebisch *et al.*, 2016). In the *B. floridae* genome, four Kremen genes (*Krm1, -2, -3, -4*) with close affinity and similar domain structure content to vertebrate Kremens 1 and -2 have been localized to the same scaffold, and thus appear to have evolved *via* tandem duplication (Zhang and Mao, 2010). The authors further identify at least three additional Kremen-like (Krl) proteins, all with alternate domain structures lacking the Kringle domain (Zhang and Mao, 2010). Developmental expression of *B. belcheri Krm1* and *Krm2* has been described (Zhang and Mao, 2010). *Krm1* is reportedly expressed throughout the mesendoderm and axial somitic and notochordal mesoderm and dorsal endoderm in neurulae and larvae, respectively. In contrast, *Krm2* shows more localized expression in three spots in the pharyngeal region on the left and right sides of larvae. *Krm4, Krl1* or *Krl3* are reported to lack clear developmental expression (Zhang and Mao, 2010).

Cytoplasmic regulators

In the *B. floridae* genome, a single Dishevelled (*Dvl)* has been identified, and is unusual in being one of the only known Wnt pathway members to be expressed asymmetrically in the animal pole in cleavage stages (Wang *et al.*, 2016). This translates to stronger dorsal expression in gastrula stages. Although broadly expressed in the mesendoderm of neurula stage embryos, at least in early stages there is an apparent antero-posterior gradient. From late neurula to early larval stages, *Dvl* is broadly expressed, but most strongly in the paraxial mesoderm forming the somites (Wang *et al.*, 2016).

Adenomatous polyposis coli (APC) is a tumour suppressor associated with colorectal cancers, and is a key protein of the "destruction complex". *Apc* expression has been documented in *B. floridae*, where it was found to be ubiquitous in early cleavage stages and blastulae (Wang *et al.*, 2016). Particularly in larval stages, *Apc* expression is similar to that of *LRP5/6* in concentrating in anterior neurons of the CNS as well as in the pre-oral pit (Wang *et al.*, 2016).

Three kinases associated with β-catenin destruction in the absence of Wnt/Fz/LRP signalling have been characterized: GSK3β, CK1α and CK1δ (Wang *et al.*, 2016). *GSK3β* gene expression in *B. floridae* is broad, occurring almost ubiquitously from early cleavage to larval stages in mesendoderm (Wang *et al.*, 2016). *CK1α* and *CK1δ* show similar expression until gastrulation, when *CK1δ* appears to be more strongly expressed dorsally. Asymmetry in dorsal mesoderm and the neural plate continues for both transcripts throughout neurulation, with an absence of expression most anteriorly. Expression of *CK1α* and *CK1δ* diverge somewhat in late neurulae, with the most distinct patterns found in larvae (Wang *et al.*, 2016); expression continues to be absent in anterior structures such as the cerebral vesicle, and *CK1δ* is downregulated except in some pharyngeal structures, possibly representing the club-shaped gland or endostyle (Wang *et al.*, 2016).

Two Axin proteins have been identified in vertebrates, Axin and Axin2/Conductin, both of which may act as scaffolds for the degradation of β-catenin (Chia and Costantini, 2005). The expression

of *B. floridae Axin* has been reported (Beaster-Jones *et al.*, 2008, Wang *et al.*, 2016), and is particularly interesting as vertebrate *Axin2* is not only a negative regulator but also a target of Wnt/β-catenin signalling (Jho *et al.*, 2002). As for other members of the destruction complex, early expression of *Axin* is broadly uniform until after the blastula stage (Wang *et al.*, 2016). In gastrulae, *Axin* is expressed throughout the mesendoderm, but most strongly around forming lips of the blastopore excluding the ectoderm (Beaster-Jones *et al.*, 2008, Wang *et al.*, 2016). By late gastrula/early neurula stages however, *Axin* is strongly expressed in the forming posterior growth zone, with no detectable expression in the anterior-ventral mesendoderm (Beaster-Jones *et al.*, 2008, Wang *et al.*, 2016). In late neurulae and larvae, transcripts are detected in the growth zone/forming tailbud (Beaster-Jones *et al.*, 2008, Wang *et al.*, 2016), consistent with expression of many Wnt ligands, as well as possibly in patches in the pharyngeal endoderm (Wang *et al.*, 2016).

Nuclear signalling

The key effector of Wnt/β-catenin in the nucleus is β-catenin itself. β-catenin, known by the name Armadillo due to its mutant "naked" cuticle phenotype in *Drosophila* embryos, consists of an intrinsically disordered N-terminal region, a central Armadillo domain consisting of repeating alpha-helix coiled coils, and a C-terminus containing the transactivation domain. In vertebrates, 9 Armadillo containing proteins within three subfamilies have been identified, of which two β-catenin orthologues exist: β-catenin itself, and Plakoglobin (γ-catenin) (McCrea and Gottardi, 2016). Surprisingly, the developmental expression of amphioxus' unique *β-catenin* has still not been reported. However, the protein has been localized using two different antibodies, one in *B. floridae* (Holland *et al.*, 2005) and the other in *B. belcheri* (Oda *et al.*, 2004, Yasui *et al.*, 2002). The first was generated against the N-terminal 173 amino acids of the sea urchin *Lytechinus variegatus* protein, while the second recognises the C-terminus of vertebrate β-catenin (Holland *et al.*, 2005, Oda *et al.*, 2004, Yasui *et al.*, 2002). Interestingly, the antibodies have together revealed the transcriptional and adhesive functions predicted for the protein. In particular, in *B. floridae*, the N-terminal antibody localises to the animal pole in early cleavage stages, with clear nuclear localisation in all blastomeres until gastrulation. In gastrulae, nuclear β-catenin is most evident around the blastopore and then in the ectodermal lips of the neural plate. Nuclear localization is lost in the neural plate itself and dorsal ectoderm, remaining ventro-posteriorly until late neurula stages (Holland *et al.*, 2005). In contrast, the C-terminal antibody clearly shows that β-catenin is also found in the membranes (Oda *et al.*, 2004), where it colocalizes with amphioxus Cadherin-like proteins at the adherens junction (Oda *et al.*, 2004), but only localizes to nuclei clearly in some early stages (Yasui *et al.*, 2002), and in a distribution that differs somewhat from that seen by Holland and colleagues (Holland *et al.*, 2005). While the antibody used in *B. floridae* is no longer available, that used in *B. belcheri* is commercially available and has been used to assess the cellular structure in *B. lanceolatum* adult regenerating tails (Somorjai *et al.*, 2012b). Interestingly, β-catenin appears to be upregulated at the membranes and adherens junctions in the blastema (Somorjai *et al.*, 2012b), similarly to the larval tailbud (Oda *et al.*, 2004), but is also not appreciably detectable in the nucleus. This may either reflect the very low (non-detectable) levels of β-catenin actually required to mediate nuclear signalling at steady states, or a conformational change in the protein that might mask the epitope when it shuttles into the nucleus.

β-catenin binds T-cell factor/Lymphoid enhancer-binding factors (TCF/LEF) in the nucleus, in addition to a number of other proteins including CtBP (C-terminal binding protein), to mediate target gene activation or repression in a cell-context specific fashion. In amphioxus, a single *Tcf/Lef* has been identified; in mammals, four TCF genes exist, *TCF1* (or *TCF7*), *TCF2* (or *LEF1*), *TCF3* (or *TCF7L1*) and *TCF4* (and *TCF7L2*), which have complex spatio-temporal regulation, and which differ in their mode of action. A complete developmental expression profile has been characterized in *B. floridae* (Lin *et al.*, 2006). Cytoplasmic maternal *Tcf/Lef* localises to the animal pole until gastrulation, at which stage zygotic expression is seen in mesendoderm. Expression is detectable in the anterior end of the neural plate and anterior notochord, as well as in the pharynx, hindgut, and somites in neurulae. The notochord, forming somites and pharynx continue to express *Tcf/Lef* in early larvae, as well as the tailbud, the ciliated pit, and the cerebral vesicle (Lin *et al.*, 2006). Careful mapping in *B. lanceolatum* at the 7 somite neurula stage suggests this region marks the basal domain within the rostral and intermediate compartments of a hypothalamo-prethalamic primordium (HyPTh), and thus anterior to the diencephalon *sensu stricto* (Albuixech-Crespo *et al.*, 2017).

Groucho acts as co-repressor with TCF/LEF, but is directly displaced by β-catenin upon Wnt/β-catenin pathway activation (Daniels and Weis, 2005). *Groucho* is expressed in *B. floridae* ubiquitously until the blastula/early gastrula, when it is expressed throughout tissues except the ectoderm. This becomes more predominant on the dorsal side, with an apparent gradient of expression strongest in anterior and dorsal mesendoderm and the neural plate by neurula stages. By the late neurula, expression is absent in the midline, and more prominent in the posterior growth zone, the anterior endoderm, and in the paraxial mesoderm of the somites. By larval stages, expression is reported to decrease, remaining only near the tailbud and in parts of pharynx (Wang *et al.*, 2016).

Functional studies

Studies of Wnt/β-catenin signalling in cephalochordates have predominantly focused on its role in polarity establishment and A/P axis formation. The primary means of addressing this in amphioxus, as in many other organisms, has been through the use of small molecules that target the GSK3β kinase. Inhibition of GSK3β through lithium treatment, the application of BIO, CHIR99021, Alsterpaullone or 1-Azakenpaullone result in stabilisation of β-catenin protein, which is then free to enter the nucleus where it binds its transcriptional targets to activate downstream signalling (Kramer *et al.*, 2012). So far, no study has achieved Wnt signalling knockdown by directly targeting ligands or receptors during embryogenesis.

The earliest studies of Wnt/β-catenin signalling in amphioxus addressed the role of early polarity establishment using lithium treatments in *B. belcheri* (Yasui *et al.*, 2002) and *B. floridae* (Holland *et al.*, 2005). In *B. belcheri*, treatment after fertilisation results in defects in cleavage and the generation of masses of cells lacking obvious polarity, with "escapers" developing into radially symmetric embryos, but which maintain some dorso-ventral polarity (Yasui *et al.*, 2002). Short pulses of lithium starting at the early blastula stage in *B. floridae* generate exogastrulae, but a slight delay in treatment permits gastrulation and some elongation to occur to

produce truncated but largely recognizable larvae (Holland *et al.*, 2005). Treatment in both cases results in modified distribution of β-catenin to the nucleus (Holland *et al.*, 2005, Yasui *et al.*, 2002), and suggests an important conserved role for Wnt/β-catenin in A/P polarity establishment in chordates, but not dorsal determination as found in vertebrates (Holland *et al.*, 2005, Yasui *et al.*, 2002).

In addition to its role in early polarity establishment, Wnt/β-catenin signalling plays a number of roles during antero-posterior (A/P) axis extension and patterning, possibly in combination with other pathways. Holland and colleagues (Holland *et al.*, 2005) demonstrated that following the pulse of lithium treatment at the late blastula the treated embryos are significantly more elongated than controls by mid-neurula stages. Although morphologically somewhat amorphous, lithium treatment clearly caused a lack of anterior and neural plate identity (Holland *et al.*, 2005). Combining similar treatments with retinoic acid (RA) or BMS009 (an RA antagonist), Onai and colleagues (Onai *et al.*, 2009) show that Wnt/β-catenin and RA have differential effects on A/P patterning, even though phenotypes may appear similar. As expected, lithium caused anterior truncation and posteriorization, with concomitant changes in anterior, neural and posterior marker expression, while RA treatments often showed more subtle effects, at least until larval stages. In contrast, modulating RA results in dramatic changes in *Hox1* and *Hox3* domains of gene expression, while lithium does not. Interestingly, treatments of 0.75-1.5 μM with the more specific GSK3β inhibitor Alsterpaullone are reported to show similar effects to lithium treatment, but with more severe elongation defects (Onai *et al.*, 2009). Nevertheless, taken together, the results indicate a clear effect of Wnt/β-catenin on determining posterior identity in amphioxus, but highlight a parallel function of both Wnt and RA signalling in specifying neuronal identity and the hindbrain territory, respectively. Moreover, the results suggest that there may be only limited cross-talk between RA and Wnt/beta-catenin in amphioxus, in contrast to the situation in vertebrates (Onai *et al.*, 2009). Chemical inhibition using two specific GSK3β inhibitors has identified possible downstream targets of Wnt/β-catenin signalling both during gastrulation and axis extension (Dailey *et al.*, 2017). Continuous treatment with 1-Azakenpaullone at 10μM from late gastrula onwards results in expansion of the posterior *Brachyury2* (*Bra2*) domain by mid-neurula, as expected if the latter were a *bona fide* target of Wnt/β-catenin signalling, as well as a lack of anterior structures including a pharynx in larvae. This is accompanied by a downregulation of *Sp5* in the same domain, an unexpected result given that *Sp5* was shown to be activated downstream of canonical Wnt/β-catenin signalling (Fujimura *et al.*, 2007, Weidinger *et al.*, 2005). The authors propose that this may be the result of a negative auto-regulatory feedback loop, as other pharmacological treatments result in *Sp5* upregulation (Dailey *et al.*, 2017), highlighting the importance of developmental stage and duration of signal modulation in evaluating the role of any given signalling pathway.

In one of few studies in which direct targeting of Wnt pathway components has been achieved, Onai and colleagues show that Dkk3 plays a role in head formation in *B. floridae*, similarly to Dkk1/2/4 members in other systems (Onai *et al.*, 2012). Injection of morpholinos (MO) directed against *Dkk3* resulted in severe truncations by larval stages, with specific loss of anterior structures including the head and pharynx, concomitant with a reduction in anterior markers. Treatment with 0.5μM of the GSK3β inhibitor BIO has similar, albeit stronger effects, particularly in posteriorizing embryos. Evidence that *Dkk3* knockdown potentiates the BIO phenotype, combined with experiments in *Xenopus* animal caps, suggests that Dkk3 plays dual roles in amphioxus: it can inhibit both Wnt/β-catenin and Nodal/Vg1 signalling, although the latter is probably not its dominant function and does not appear to be mediated through amphioxus Dkk3's novel TGF-β R2 domain (Onai *et al.*, 2012). This study highlights some fundamental differences in the roles of signalling pathways in axis specification between cephalochordates and vertebrates.

The only other study to functionally address components of the Wnt pathway showed that amphioxus sFRP1/2/5 is a likely antagonist of a subset of Wnt proteins using *Xenopus* luciferase and secondary axis formation assays (Kong *et al.*, 2012). Knockdown and overexpression in amphioxus embryos will be crucial to validate these results.

Conclusions

Although our knowledge concerning the embryonic functions of different signalling pathways in amphioxus is still incomplete, the analysis of gene expression patterns and the use of pharmacological treatments and other functional techniques already give some insights into the evolution of their function in the chordate lineage. While some signals seem to play a conserved role with those in vertebrates, like RA in antero-posterior patterning, others seem to act differently in each chordate clade in the control of several developmental processes, such as for FGF during somitogenesis or Wnt/β-catenin in dorsal determination. More detailed comparisons should help us understand how vertebrates acquired their specific morphological traits, and we believe the development of new genome editing techniques such as TALEN (Transcription Activator-Like Effector Nuclease) or CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 nuclease) (Chen *et al.*, 2014) will open new avenues to better decipher how cells communicate during embryogenesis in amphioxus.

Acknowledgements

The laboratory of H.E. was supported by the CNRS and the ANR-16-CE12-0008-01 and S.B. by the Institut Universitaire de France. The laboratory of I.M.L.S. is currently supported by Wellcome Trust ISSF grant 204821/Z/16/Z.

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