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RESEARCH PAPER

Diversification of amphioxus and vertebrate Cerberus protein function in modulating Nodal, BMP and Wnt signals

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Abstract

Cerberus is a multifunctional antagonist implicated in embryonic patterning through modulation of Nodal, BMP and Wnt signals. Although its function is largely conserved in chordates, certain activities have diverged, even among vertebrates. Moreover, the antagonistic action of Cerberus from the basal chordate amphioxus toward Nodal, BMP and Wnt signals remains elusive. Here, we compared the activity of amphioxus and *Xenopus* Cerberus proteins using cross-species assays. We found that amphioxus and *Xenopus* Cerberus proteins display similar activities in antagonizing Nodal-induced events, but they exhibit both shared and distinct activities in modulating BMP and Wnt signals. Amphioxus Cerberus has reduced neuralizing activity that is dependent on inhibition of BMP signaling, and it modulates the signals of a restricted subset of Wnt proteins. Furthermore, we revealed that *Xenopus* Cerberus interacts with Wnt4 and Wnt11 to activate canonical Wnt signaling, whereas amphioxus Cerberus lacks this activity. These differences may be correlated with the divergence in the N-terminal region of Cerberus proteins between amphioxus and *Xenopus*. Our results indicate that chordate Cerberus proteins have evolved sub-functionalities that depend not only on their concentrations, but also on the properties of BMP and Wnt signals. This may account for their evolutionary distinct functions in different patterning processes.

Keywords Amphioxus · BMP · Cerberus · Chordate · Nodal · Wnt · *Xenopus*

Introduction

The fundamental patterning mechanism of embryonic axes is largely conserved among chordates, with certain degrees of modification (Coolen et al. 2007; De Robertis 2008; Holland and Onai 2012). The formation of embryonic axes, including those of dorsoventral, anteroposterior, and left–right, relies essentially on the opposing activities of different signaling pathways, such as Nodal, bone morphogenetic protein (BMP) or Wnt, and on the interactions between these factors with their extracellular antagonists (Carron and Shi 2016). In vertebrates, it is well established that the Spemann organizer coordinates the formation of all three axes (De Robertis et al. 2000), and dorsoanterior development requires the inhibition of Nodal, BMP, and Wnt signaling (Glinka et al. 1997; Piccolo et al. 1999). Thus, the Spemann organizer is a source of secreted antagonists for BMP, Wnt, and Nodal. They act in combination and establish signaling gradients to determine the pattern of the dorsoventral and anteroposterior axes (Carron and Shi 2016; Niehrs 2004).

Cerberus belongs to the cystine knot superfamily of secreted proteins and represents a multifunctional antagonist for Nodal, BMP, and Wnt. It is expressed in the mesendoderm at the onset of gastrulation in *Xenopus*, and is sufficient and necessary for head formation (Bouwmeester et al. 1996; Piccolo et al. 1999; Silva et al. 2003). Nevertheless, although Cerberus proteins share structural, expression, and functional characteristics among different animal lineages, these are only conserved to a limited extent. Mouse Cerberus-like protein does not bind Wnt (Belo et al. 2000) and it is not required for head development (Belo et al. 2000; Shawlot et al. 2000; Simpson et al. 1999; Stanley et al. 2000). However, it is necessary to restrict the formation of primitive streaks by antagonizing Nodal activity (Perea-Gomez et al.

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2002). Similarly, Cerberus-related genes in zebrafish and chicks seem to be only implicated in the establishment of the left–right asymmetry by limiting Nodal expression domain (Hashimoto et al. 2004; Tavares et al. 2007). In addition, chick Cerberus may function as an agonist for BMP in this process (Yu et al. 2008). Thus, the shared and distinct functions of Cerberus proteins may be due to their activity to differentially modulate Nodal, BMP, and Wnt signaling, which is consistent both with their differential temporal and spatial expression patterns, and their divergent sequence identity among chordates.

The cephalochordate amphioxus is evolutionarily related to vertebrates, sharing many common developmental regulatory genes and patterning mechanisms (Holland 2002; Kozmikova et al. 2011; Petersen and Reddien 2009). Most amphioxus orthologues of vertebrate organizer genes show similar expression patterns to those in vertebrates (Yu et al. 2007). Several studies have suggested that there is significantly conserved function of Cerberus proteins between amphioxus and vertebrates. Expression, gain-of-function and knockout analyses indicate that they could be involved in anteroposterior patterning and left–right asymmetry (Le petillon et al. 2013; Li et al. 2017; Onai et al. 2010). However, their antagonistic actions toward Nodal, BMP, and Wnt signals remain to be investigated. This aspect merits further investigation, because chordate Cerberus proteins seem to exert divergent functions. Thus, understanding the evolutionary conservation and divergence of Cerberus functions could help to elucidate the regulatory mechanism underlying the activation of important signal transduction pathways in embryonic patterning.

In this study, we compared the activity of amphioxus and *Xenopus* Cerberus proteins in regulating Nodal, BMP, and Wnt signaling using *Xenopus* embryos and explant assays, which are best suited for functional analyses. Our results indicate that they display both shared and distinct activities, whereas both amphioxus and *Xenopus* Cerberus potently inhibit Nodal-mediated induction events, amphioxus Cerberus is less effective in triggering neuralization that is dependent on the blockade of BMP signaling. Furthermore, *Xenopus* Cerberus differentially blocks the function of Wnt1, Wnt8, and Wnt3A, but enhances the activity of Wnt4 and Wnt11 in canonical Wnt signaling. Amphioxus Cerberus weakly inhibits the activity of Wnt3A and Wnt8, but has no effect on Wnt1, Wnt4, and Wnt11. These observations reveal that chordate Cerberus proteins have acquired divergent regulatory activity toward important signaling factors, which may account for their evolutionary distinct functions in different patterning processes.

Results

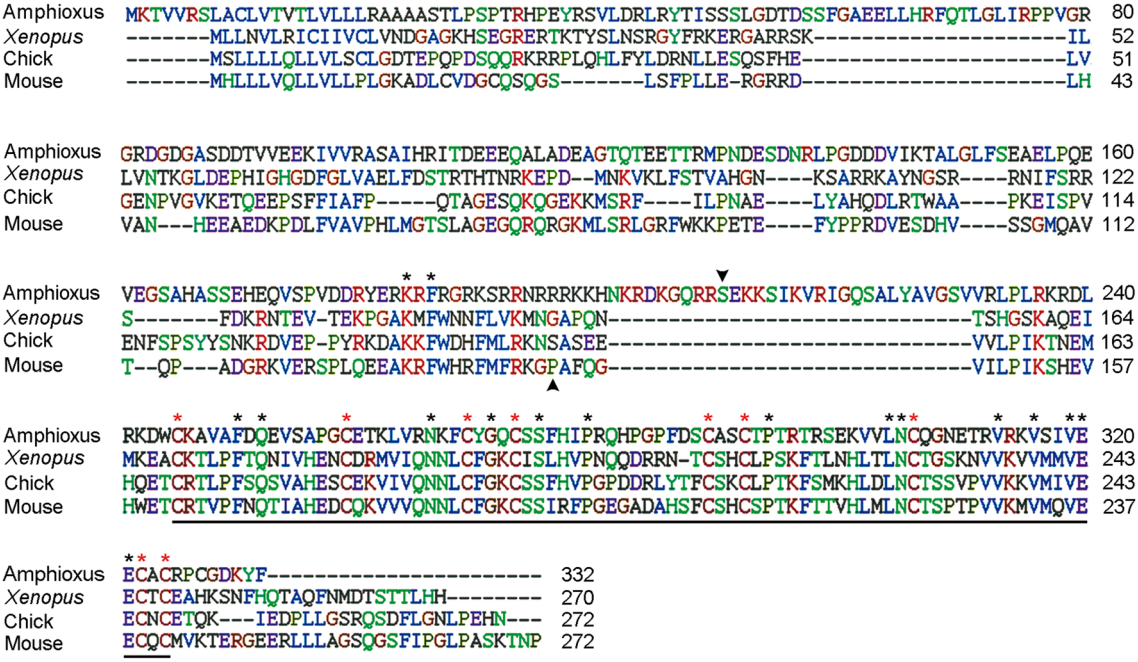
Sequence comparison between amphioxus and vertebrate Cerberus proteins

To provide a structural and functional relationship between chordate Cerberus proteins, we first compared the degree of conservation between amphioxus *B. belcheri* and representative vertebrate Cerberus proteins. Although functional analyses suggest that they are members of the cystine knot superfamily involved in embryonic axis patterning, alignment of these protein sequences indicates that they exhibit surprisingly low levels of conservation (Fig. 1a). The amino-terminal half presents very few identical residues, while the carboxyl-terminal half of amphioxus Cerberus protein shows 24%, 28% and 25% identity, and 53%, 48% and 52% similarity with the corresponding region from *Xenopus*, chick and mouse Cerberus proteins, respectively. The cystine knot region presents the highest level of identity. In particular, the nine cysteine residues within this domain are entirely conserved, and spaced by the same numbers of amino acids (Fig. 1b). The function of *Xenopus* Cerberus protein has been better described, and its amino-terminal and carboxyl-terminal halves have been shown to be responsible for binding to Wnt8 and Nodal proteins, respectively, whereas the full-length protein is necessary for interaction with BMP4 (Piccolo et al. 1999). Along with previous functional analyses (Li et al. 2017; Onai et al. 2010), the conservation of the cystine knot domain suggests that both amphioxus and *Xenopus* Cerberus proteins could potentially antagonize Nodal signaling. However, the divergence of the amino-terminal region raises the question as to how these proteins modulate BMP and Wnt signals.

Amphioxus and *Xenopus* Cerberus proteins potently block Nodal-induced gene expression

Nodal signaling induces the expression of mesoderm genes in *Xenopus* early gastrula. To compare the function of amphioxus and *Xenopus* Cerberus proteins in Nodal signaling, we injected the corresponding synthetic mRNAs (200 pg) into the dorsal region of four-cell stage *Xenopus* embryos and performed in situ hybridization at the early gastrula stage. The analysis showed that both amphioxus and *Xenopus* Cerberus proteins potently blocked the expression of Spemann organizer gene *chordin*, and the pan-mesoderm gene *Xbra*, in the cells derived from injected dorsal blastomeres (Fig. 2a–f). These genes are either direct or indirect targets of Nodal signaling, and BMP signaling does not activate their expression (Carron

A



B

	Xenopus		Chick		Mouse	
	Cerb-N	Cerb-C	Cerb-N	Cerb-C	Cerb-N	Cerb-C
Amphioxus	12 (27)	24 (53)	12 (33)	28 (48)	14 (26)	25 (52)
Xenopus			17 (40)	46 (68)	12 (37)	39 (56)
Chick					35 (57)	54 (69)

Fig. 1 Comparison of chordate Cerberus proteins. **a** Sequence alignment of representative chordate Cerberus proteins using the Clustal X 1.83 program. Numbers on the right indicate the positions of amino acids. Dashes are introduced to optimize sequence alignment. Identical amino acids are indicated by asterisks above the sequences; red asterisks show the nine conserved cysteine residues within the cystine knot domain (underlined). **b** Percentage of identity of the amino-terminal and carboxyl-terminal halves (separated by arrowheads above the amphioxus sequence and below the vertebrate sequences) between chordate Cerberus proteins. Numbers in brackets represent the percentage similarity. The protein sequences are from amphioxus *B. belcheri* (XP_019632388.1), *Xenopus laevis* (NP_001081800.1), *Gallus gallus* (NP_990154.1), and *Mus musculus* (NP_034017.1)

and Shi 2016). Thus, this result is consistent with a conserved function of Cerberus proteins in inhibiting Nodal signaling in chordates. It suggests that the cystine knot domain of amphioxus Cerberus may be sufficient to bind Nodal protein and that amphioxus Cerberus in the cross-species assay inhibits Nodal-induced gene expression as efficiently as its *Xenopus* counterpart, thus confirming the feasibility of this assay. Nevertheless, there may be also the possibility that overexpressed Cerberus proteins antagonize Wnt signals, which contributes in part to the inhibition of *chordin* and *Xbra* expression in the early gastrula.

Amphioxus Cerberus protein displays weak neuralizing activity

Xenopus Cerberus strongly neuralizes ectoderm by antagonizing the BMP signal (Piccolo et al. 1999). To compare the neuralizing activity between *Xenopus* and amphioxus Cerberus proteins, we injected the same amount of the corresponding mRNAs (200 pg) in the animal pole region of two-cell stage *Xenopus* embryos. Animal cap explants dissected at the late blastula stage were cultured to early neurula stage equivalent. In situ hybridization analysis indicated that uninjected explants differentiated into epidermis that did not express the anterior neuroectoderm gene *XCG-1* (Fig. 2g). However, *Xenopus* Cerberus potently induced the expression of *XCG-1* in all injected explants (Fig. 2h), indicating a strong BMP-inhibiting and neuralizing activity. Amphioxus Cerberus weakly induced *XCG1* expression in a small number of injected explants (Fig. 2i), suggesting that it lacks or displays very weak neuralizing activity and thus does not efficiently inhibit BMP signal. Nevertheless, it has previously been shown that amphioxus Cerberus is able to suppress *Xenopus* BMP4-activated reporter gene at the early

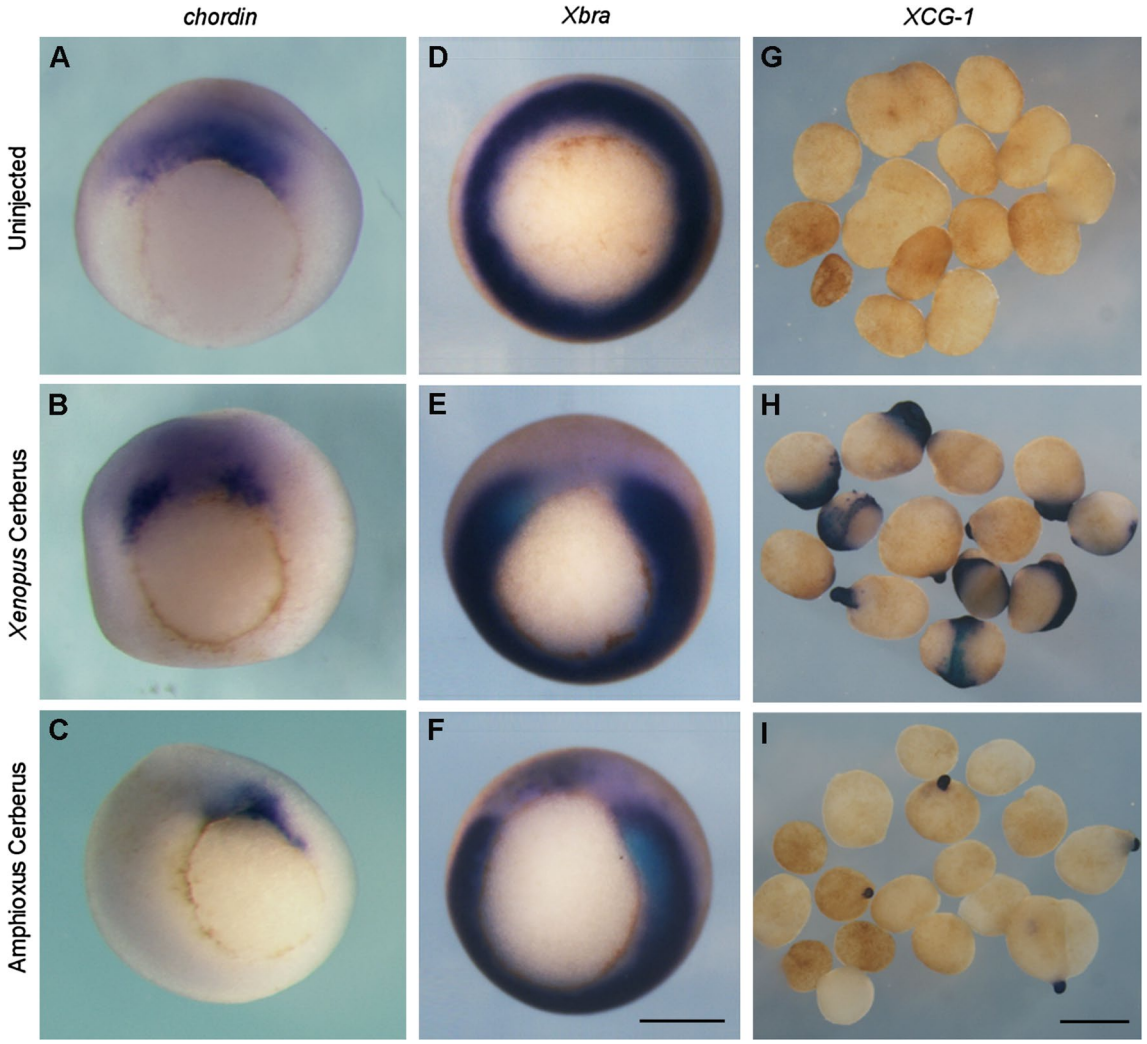


Fig. 2 The antagonizing activity of amphioxus and *Xenopus* Cerberus proteins on Nodal and BMP signals. In situ hybridization of *Xenopus* whole embryos and animal cap explants to analyze the expression of indicated genes. The expression of *chordin* and *Xbra* represents a Nodal-induced event, and the expression of the anterior neuroectoderm gene *XCG-1* in animal cap explants is a result of BMP inhibition. **a** Expression of *chordin* in an uninjected early gastrula. **b**, **c** Both *Xenopus* and amphioxus Cerberus proteins inhibit the expression of *chordin* at the injection sites. **d** Expression of *Xbra* in an uninjected early gastrula. **e**, **f** *Xenopus* and amphioxus Cerberus proteins similarly block *Xbra* expression. All embryos are vegetal view with dorsal region on the top. **g** Uninjected *Xenopus* animal cap explants differentiate into epidermis, with the absence of *XCG-1* expression. **h** Overexpression of *Xenopus* Cerberus results in strong neuralizing activity by inducing the expression of *XCG-1* in all injected explants. **i** Amphioxus Cerberus leads to weak neuralization, with few explants expressing weakly *XCG-1* gene. Scale bars: (**a**–**f**) 500 μm; (**g**–**i**) 100 μm

gastrula stage (Onai et al. 2010). This discrepancy may be explained by the assays used. Although amphioxus Cerberus could block reporter gene activation induced by exogenous BMP4, it may be not sufficient to block endogenous BMPs to trigger neuralization.

Amphioxus and *Xenopus* Cerberus proteins differentially modulate Wnt signals

Many members of the so-called canonical and non-canonical Wnts are implicated in regulating diverse developmental processes. Different orthologous genes for vertebrate Wnts are also present in amphioxus (Holland et al. 2000; Schubert et al. 2000a, b, c, d, 2001). To assay how amphioxus and *Xenopus* Cerberus proteins interact with these Wnts, we chose to use the *siamois* reporter assay in animal cap explants, which fully recapitulates the activation of canonical Wnt signaling (Brannon et al. 1997). Synthetic mRNA (5 pg) encoding canonical Wnt1, Wnt3A, or Wnt8 was coinjected with different amounts of amphioxus or *Xenopus Cerberus* mRNA (50 pg, 100 pg, and 200 pg), and luciferase assays were performed at the early gastrula stage. The

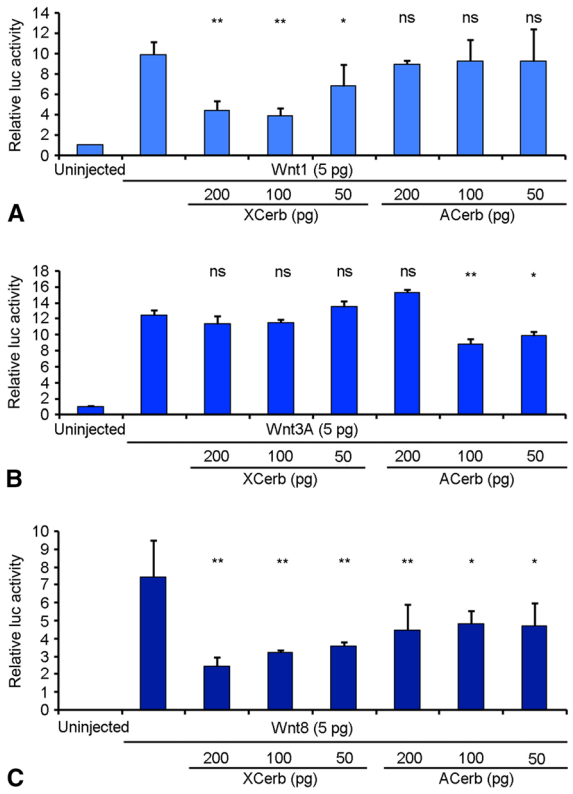


Fig. 3 Distinct antagonizing actions of amphioxus and *Xenopus* Cerberus proteins on canonical Wnt signals. Luciferase reporter assays in *Xenopus* animal cap explants. **a** *Xenopus* Cerberus (XCerb) inhibits Wnt1 signal in a dose-dependent manner, while amphioxus Cerberus (ACerb) shows no effect. **b** *Xenopus* Cerberus has no effect on Wnt3A, while low concentrations of amphioxus Cerberus inhibit Wnt3A signal. **c** Both *Xenopus* and amphioxus Cerberus proteins inhibit Wnt8 signal. Bars represent the mean ± SD from at least three independent experiments. The data were normalized to the uninjected condition, which was set as 1. The statistical significance in coinjected conditions was determined against Wnt-injected conditions (*$P < 0.05$; **$P < 0.01$; *ns* not significant; Student's *t* test)

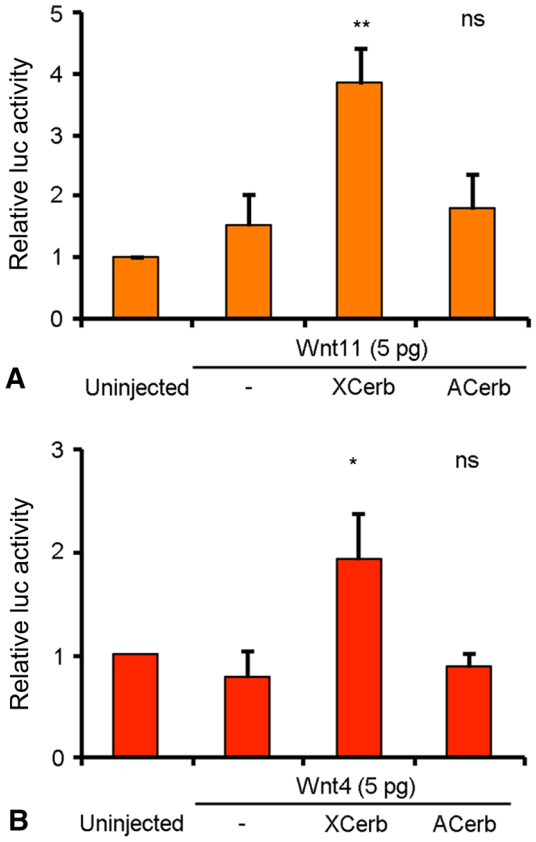


Fig. 4 Distinct activities of *Xenopus* and amphioxus Cerberus proteins on non-canonical Wnts. Luciferase reporter assays in *Xenopus* animal cap explants. **a** Wnt11 weakly activates canonical Wnt signaling, which is enhanced by *Xenopus* Cerberus (XCerb), but not by amphioxus Cerberus (ACerb). **b** *Xenopus* Cerberus, but not amphioxus Cerberus, is an agonist of Wnt4. Bars represent the mean ± SD from at least three independent experiments. The data were normalized to the uninjected condition, which was set as 1, and the statistical significance in coinjected conditions was determined against Wnt-injected conditions (*$P < 0.05$; **$P < 0.01$; *ns* not significant; Student's *t* test)

results indicated that *Xenopus* Cerberus, but not amphioxus Cerberus, inhibited Wnt1-induced reporter activity (Fig. 3a). However, *Xenopus* Cerberus did not inhibit Wnt3A signal at different concentrations, whereas amphioxus Cerberus was able to antagonize Wnt3A at low concentrations (Fig. 3b). In addition, we found that both *Xenopus* and amphioxus Cerberus proteins antagonized Wnt8 signal, although amphioxus Cerberus seemed to be less active than *Xenopus* Cerberus in this assay (Fig. 3c).

Since non-canonical Wnts such as Wnt 4 and Wnt11 have been shown capable of activating canonical Wnt signaling depending on the context (Umbhauer et al. 2000), we examined how *Xenopus* and amphioxus Cerberus proteins modulate these Wnt signals. Injection of *Wnt11* mRNA (5 pg) weakly activated the *siamois* reporter. Unexpectedly, this was significantly enhanced by coinjection of *Xenopus* Cerberus mRNA (200 pg), but not by the amphioxus counterpart (Fig. 4a). Overexpression of Wnt4 by injecting the corresponding mRNA (5 pg) was not able to activate canonical Wnt signaling, but it activated the *siamois* promoter activity when provided with *Xenopus* Cerberus, whereas amphioxus Cerberus had no effect (Fig. 4b). This reveals that *Xenopus* Cerberus, but not the amphioxus counterpart, interacts with non-canonical Wnts and promotes their activity in canonical Wnt signaling. Together, these observations indicate that *Xenopus* and amphioxus Cerberus proteins display both shared and distinct activity in modulating canonical Wnt signaling. Moreover, they suggest that the regulation of different Wnt ligands by chordate Cerberus is complex, depending not only on the concentration of Cerberus protein, but also on the properties of the Wnts. These sub-functionalities may account for the divergent functions of various chordate Cerberus proteins in embryonic patterning.

Discussion

In the basal chordate amphioxus, Cerberus is first expressed in the dorsoanterior mesoderm, a site that is comparable to the anterior mesendoderm in *Xenopus* embryos (Onai et al. 2010), and is then restricted to the anterior-most right somites. In contrast to the phenotype obtained following overexpression of *Xenopus* Cerberus protein in *Xenopus* early embryos, which suppresses the formation of the posterior mesoderm, overexpression of amphioxus Cerberus protein in amphioxus embryos strongly ventralizes and posteriorizes dorsal and anterior structures, with the loss of the central nervous system, notochord and anterior identity (Onai et al. 2010). In addition, overexpression of amphioxus Cerberus protein in the ventral region of the *Xenopus* embryo induces a complete secondary axis with head and trunk regions (Onai et al. 2010). However, ventral overexpression of *Xenopus* Cerberus protein only induces head formation (Bouwmeester et al. 1996). These differences clearly suggest that amphioxus and *Xenopus* Cerberus proteins display both shared and distinct activities in embryonic patterning, which is consistent with the present finding showing that they are differentially involved in regulating the activity of different growth factors.

Dorsoventral identity in *Xenopus* is mediated by maternal β-catenin that preferentially accumulates in the nuclei of the dorsal region following fertilization (Carron and Shi 2016). In amphioxus, however, maternal nuclear β-catenin is not required for the specification of dorsal fate, although Wnt signaling is important for establishing posterior identity during gastrulation (Holland et al. 2005). Amphioxus Cerberus protein has been characterized as an inhibitor of both Nodal and BMP signaling (Onai et al. 2010). Our present results obtained in cross-species assays extend previous observations by showing that amphioxus Cerberus protein displays only weak BMP-inhibiting and neuralizing activity. We also find that amphioxus and *Xenopus* Cerberus proteins differentially interact with canonical and non-canonical Wnts. Consistent with the diversification of chordate Cerberus proteins in modulating Wnt signals, *Xenopus* and mouse Cerberus proteins have been shown to exhibit distinct activities on Wnt ligands, indicating that the function of vertebrate Cerberus proteins to antagonize Wnt signals may not be fully conserved. This highlights the interest in understanding the specificity of Cerberus on Wnt signaling in species across wide phylogenetic distances. Moreover, because of the functional modification of Wnt signals in patterning the early amphioxus embryos, investigation of the interaction between Wnts and the multifunctional antagonist Cerberus in amphioxus should also help to elucidate the evolutionary conservation and modification of molecular mechanisms that control embryonic induction and patterning.

We demonstrate that amphioxus Cerberus protein is not a potent antagonist for canonical or non-canonical Wnts in heterologous system, namely by reporter gene assay in *Xenopus* embryos. This activity of amphioxus Cerberus protein closely resembles that of its mouse homologue, but differs significantly from its *Xenopus* counterpart. The amino-terminal region of *Xenopus* Cerberus protein binds to Wnt8, whereas the carboxyl-terminal cystine knot domain binds to Nodal, and the full-length protein is necessary to interact with BMP (Piccolo et al. 1999). The fact that amphioxus and *Xenopus* Cerberus proteins differentially modulate Wnt and BMP signaling is consistent with the divergence of the amino-terminal region. This implies that the modification of the Wnt and BMP antagonistic activities might arise from variation of the amino-terminal region during evolution. However, both amphioxus and *Xenopus* Cerberus proteins potently antagonize Nodal signaling, which may be explained by the presence of a relatively conserved cystine knot domain. It is noteworthy that Cerberus-related proteins in amphioxus and vertebrates have consistently been shown to play an essential role in the establishment of normal left–right asymmetry by controlling the Nodal expression domain (Hashimoto et al. 2004; Li et al. 2017; Tavares et al. 2007). These shared and distinct activities suggest that chordate Cerberus proteins have evolved sub-functionalities. The diversification of Cerberus protein functions might not only arise from the divergence time, but should also reflect the developmental changes and morphological differences that occur in the embryos of different animal lineages. For example, the amphioxus embryo presents some unusual characteristics of morphology, including the asymmetrical positioning of the mouth and the primary and secondary gill slits in the larva. Thus, our findings provide insights into the evolutionary conservation and modification of Cerberus proteins with respect to their specificity and mode of regulation on Wnt, BMP and Nodal signaling in different patterning processes.

Materials and methods

Xenopus embryos, explants and microinjections

A *Xenopus* colony was maintained in the laboratory. Females were stimulated by 500 IU of human chorionic gonadotropin (Sigma), and laid eggs were artificially fertilized with minced testes. Whole embryos were cultured in 0.1 × Modified Barth's Medium (MBS) at 20 °C. Animal cap explants were dissected at the late blastula stage and cultured in 1 × MBS for an appropriate period.

Microinjections were done at the two-cell stage in the animal pole region, or at the four-cell stage in the two dorsal

blastomeres, using a PLI-100A Picoliter Microinjector (Harvard Apparatus).

Plasmid constructs

Adult amphioxus *B. belcheri* was collected from Shazikou in Qingdao (China). Ripe adults were spawned and the eggs were fertilized in the laboratory. The amphioxus *Cerberus* coding region was amplified by PCR according to a published sequence (XP_019632388.1) from embryonic cDNA using specific primers (5′-GAGAAACGTGCGGTGAAG CATGAAG-3′ and 5′-GTCAAACTGTGTCCGTGAAAT CAGAAG-3′), cloned in a pCS2 vector, and sequenced before use. *Xenopus Cerberus* construct was previously described (Bouwmeester et al. 1996). *Xenopus Wnt1*, *Wnt3A*, *Wnt4*, *Wnt8*, and *Wnt11* in pSP64T or pCS2 vector were reported previously (Kong et al. 2012; Umbhauer et al. 2000). In vitro transcription of capped mRNA used for microinjection was performed according to Li et al. (2006).

In situ hybridization

Whole embryos and animal cap explants were fixed in 4% paraformaldehyde and stored in methanol. Whole-mount in situ hybridization using digoxigenin-labeled *chordin*, *Xbra* and *XCG-1* probes was performed as previously reported (Li et al. 2013). BM purple (Roche Diagnostics) was used as a substrate for the alkaline phosphatase. Images were acquired under a stereomicroscope (Leica M165C).

Luciferase reporter assays

Siamois promoter-driven luciferase reporter assays were used to examine canonical Wnt activity. The reporter DNA (200 pg) was injected alone, or coinjected with synthetic mRNAs, into the animal pole region of *Xenopus* embryos at the two-cell stage. Ten animal cap explants dissected at the early gastrula stage were lysed and processed for luciferase assays according to the manufacturer's recommendation (Promega). All experiments were performed at least in triplicate using different batches of embryos and the mean value was calculated using Student's *t* test. *P* values less than 0.05 were considered as statistically significant.

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Author contributions YJZ and DLS performed the experiments and analyzed the data; DLS designed the experiments and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Animal and human rights statement The experiments using *Xenopus* adults and embryos were performed by following the regulations and guidelines issued by the French Ministère de l'Enseignement Supérieur et de la Recherche (permit number 04845.04).

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