Published in final edited form as:
Nature. 1999 February 25; 397(6721): 707–710.

The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals

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Abstract

Embryological and genetic evidence indicates that the vertebrate head is induced by a different set of signals from those that organize trunk–tail development[1–6]. The gene *cerberus* encodes a secreted protein that is expressed in anterior endoderm and has the unique property of inducing ectopic heads in the absence of trunk structures[1]. Here we show that the *cerberus* protein functions as a multivalent growth-factor antagonist in the extracellular space: it binds to Nodal, BMP and Wnt proteins via independent sites. The expression of *cerberus* during gastrulation is activated by earlier nodal-related signals in endoderm and by Spemann-organizer factors that repress signalling by BMP and Wnt. In order for the head territory to form, we propose that signals involved in trunk development, such as those involving BMP, Wnt and Nodal proteins, must be inhibited in rostral regions.

The principal activities of injected *cerberus* (*cer*) messenger RNA in *Xenopus* embryos are the inhibition of trunk mesoderm and neuralization of the ectoderm[1]. *Xenopus cerberus* protein (Cer) is structurally related to a family of cystine-knot secreted proteins that behave as antagonists of members of the transforming growth factor-β (TGF-β) family[7,8]. To determine the molecular mechanism of Cer function, we prepared soluble *Xenopus* Cer protein tagged with the Flag epitope. In animal cap cells expressing *cer* mRNA, Cer–Flag was secreted as a soluble protein of relative molecular mass (M_r) 46,000, designated Cer-long (Cer-L); a minor form of M_r 33,000, designated Cer-short (Cer-S), was also produced (Fig. 1a, lane 1). Transfected human 293T cells (Fig. 1a, lane 2) produced almost exclusively the proteolytically processed Cer-S form (Fig. 1b). To test whether Cerberus binds to any of the three main mesoderm-inducing candidate factors, we produced biologically active preparations of activin, Vg1 and Xnr-1 (*Xenopus* nodal-related-1, ref. [9]), tagged with haemagglutinin (HA) at the amino terminus (Fig. 1c). In immunoprecipitation experiments Cer–Flag proteins bound Xnr-1 but not activin or Vg1 proteins, displaying a remarkable degree of specificity (Fig. 1d). In the presence of either Cer-L or Cer-S protein, the *Xbra*-inducing activity of Xnr-1 was blocked, but that of activin or Vg1 proteins was not (Fig. 1e). In animal cap assays, we found that at 10^{-9} M, Cer-L and Cer-S proteins titrated Xnr-1 signalling stoichiometrically, indicating that the affinity of the interaction must be in the subnanomolar range (Fig. 1f).

The neuralizing activity of *cer* mRNA can be antagonized by *BMP-4* (refs 1,[7]). We performed co-immunoprecipitation experiments to test whether *Xenopus* Cerberus proteins bind BMPs directly. Cer-L–Flag protein bound BMP-4, with a dissociation constant (K_D) of 0.6 nM (Fig.

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1g). This interaction was highly specific, as it could be displaced by a 10-fold excess of the related molecule BMP-2, but not by TGF-β-1, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF; Fig. 1h, lanes 3−6). In biological assays, Cer-L protein had neutralizing activity (Fig. 1i, lane 3), whereas the proteolytically processed Cer-S protein did not bind BMP-4 (even in 10-fold excess, Fig. 1h, lane 9) and did not induce neural markers (Fig. 1i, lane 4).

An additional property of *cer* mRNA is that it blocks the induction of secondary axes by *Xwnt-8* mRNA[4]. We found that in animal caps, full-length *cer* mRNA inhibited Xwnt-8 but not β-catenin signalling (Fig. 1j), suggesting that Cer-L might antagonize Xwnt-8 extracellularly. To test this directly, we incubated Xwnt-8–HA protein produced in oocytes with Cer-L–Flag or Cer-S–Flag and immunoprecipitated with anti-Flag antibody. We found that Xwnt-8 bound to Cer-L, but not to the Cer-S protein (Fig. 1k, lanes 3−5). Thus, the Cer-L protein is a trifunctional antagonist that can bind Xnr-1, BMP and Xwnt-8 in solution.

The binding of Xnr-1 to Cer-L–Flag could not be displaced by excess BMP (Fig. 1l) and Xwnt-8 binding was not displaced by excess BMP or Xnr-1 (Fig. 1m). This suggests that Cer-L binds each ligand at independent binding sites (Fig. 1n). In addition, the biochemical studies show that Cer-L is proteolytically processed into the Cer-S protein containing the cystine-knot (Fig. 1b), which retains only the anti-Xnr-1 activity. This proteolytic cleavage could be important in regulating Cerberus function *in vivo*; a regulatory proteolytic step has been described in the case of chordin (Chd), in which active BMP ligands are released from inactive Chd/BMP complexes[10].

Xenopus Cerberus has three distinct inhibitory activities and, as shown below, each one is required for the induction of ectopic head-like structures. To study the phenotypic effects of specifically inhibiting nodal-related signals in the context of the *Xenopus* embryo, we generated a construct encoding a secreted form of Cer-S, designated *cer-S*. Injection of *cer-S* mRNA into single blastomeres did not induce ectopic heads but instead gave rise to anterior defects in the endogenous head, including cyclopia (not shown). Embryos in which each blastomere was injected at the four-cell stage with *cer-S* mRNA lacked axial structures and the expression of the trunk mesoderm markers *Xbra* and *Xwnt-8* was blocked (Fig. 2a–d). In addition to maintaining a mesodermal fate, nodal-related signals are required for the formation of anterior endoderm: expression of endogenous *cerberus* was blocked by *cer-S* mRNA (Fig. 2e, lane 2) and induced by *Xnr-1* mRNA (Fig. 2f). Interestingly, endodermal patterning by *Xnr-1* mRNA appears to function early in development, as injection of an *Xnr-1* DNA construct expressed after mid-blastula was unable to induce *cerberus* (Fig. 2f, lanes 6−8). Taken together, these gain- and loss-of-function studies support the view that nodal-related signals have a central role in the patterning of anterior endoderm and in trunk mesoderm formation in *Xenopus*, in agreement with genetic studies in other vertebrates[11–17].

Since the anti-nodal activity of *cer-S* was not sufficient to induce ectopic head-like structures, we next tested it in combination with anti-BMP reagents in explants of ventral marginal zone (VMZ), which normally develops into postero-ventral mesoderm containing blood (Fig. 2g, k). Microinjection of *cer-S* or of *tBR*, a dominant-negative BMP receptor mRNA[6], did not cause head formation (Fig. 2h, i). However, injection of combined *cer-S* and *tBR* produced a striking change in VMZ fate into head-like structures (Fig. 2j, m). The explants contained a large cyclopic eye (often with crystalline lens), brain, cement gland and endoderm, but no notochord or somites, indicating the generation of head organizer activity. Indistinguishable head-like structures were formed by injection of full-length *cer* mRNA (Fig. 2l). Co-injection of a dominant-negative putative nodal receptor[18], *tALK4*, with *tBR* also induced head-like structures (Fig. 2n), showing that the *cer* constructs do not have an instructive role. Although these experiments in VMZs show that simultaneous inhibition of nodal-related and BMP

signalling is sufficient for head specification, they do not indicate whether the anti-Wnt activity of *cerberus* is required for head formation. Indeed, in VMZs the main Wnt, *Xwnt-8*, is inhibited at the gene-expression level in embryos injected with *cer-S* (Fig. 2d) or *tBR* mRNA[19].

To test whether triple inhibition of Wnt, nodal-related and BMP signalling is required for ectopic head formation, we carried out epistatic combinatorial experiments in the context of the whole embryo. Co-injection of *chd* (anti-BMP) and *cer-S* (anti-nodal) into a ventral blastomere resulted in ectopic heads containing a cyclopic eye (58%, $n = 115$); this induction was blocked by co-injection of *Xwnt-8* DNA (Fig. 3a, b). Similarly, selective inactivation of the anti-nodal activity of full-length *cer* mRNA by co-injection of a constitutively active ALK-4 receptor[18], even at doses that only weakly promote trunk mesoderm formation, abolished ectopic head induction by *cer* (Fig. 3c, d). Mimicking BMP signalling by co-injecting a constitutively active BMP receptor construct also antagonized ectopic head induction (Fig. 3e). These epistatic experiments indicate that all three inhibitory activities of *cerberus* are required for the formation of ectopic head structures.

Niehrs and co-workers have shown that simultaneous inhibition of BMP and Wnt signalling is sufficient to induce secondary axes containing heads with cyclopic eyes[4]. This is puzzling, for this head induction would occur without apparent inhibition of nodal-related signals. We confirmed these results using co-injection of *chd*[10] and *Frzb-1* (ref. 20) mRNAs, which encode secreted inhibitors specific for BMP and Wnt proteins, respectively (Fig. 3g). Head formation by *chd* and *Frzb-1* was suppressed by co-injection of *Xnr-1* DNA (Fig. 3h and 3f), suggesting that inhibition of *nodal* signalling could also be involved in this experimental situation, perhaps by activating transcription of *cerberus*. As shown in Fig. 3i–l, co-injection of *Frzb-1* and *tBR* mRNA led to strong ectopic expression of *cerberus* in ventral endoderm at levels comparable to those seen with endogenous *cerberus* mRNA (Fig. 3l, arrow), with concomitant inhibition of the trunk mesodermal marker *Xbra* near the site of injection (Fig. 3m, n).

In mice, the anterior visceral endoderm (AVE) has a fundamental role in head formation[2,14,16,21] and is the topological equivalent of the anterior endoderm that expresses *cerberus* in *Xenopus*[2,22]. Robertson and colleagues have shown that *nodal* activity in the AVE is required for head formation in chimaeric embryos[14], whereas we report here that inhibition of nodal-related signalling by *cerberus* is required for head formation. These paradoxical observations can be reconciled by the proposal that, in mice, *nodal* signalling[14–17] may be required in the AVE to express secreted factors that antagonize the formation of mesoderm in the overlying epiblast[16,17], in which the head forms. In *Xenopus*, we have found evidence that *cerberus* expression requires an early nodal-related function mimicked by injection of *Xnr-1* mRNA, but not DNA (Fig. 2e, f). This early signal, which correlates with the expression of *Xnr-1* in endoderm at late blastula[9], leads to the expression of a multivalent antagonist, Cerberus, which inhibits the function of nodal-related signals in rostral regions at later stages, preventing trunk mesoderm from forming in the head field. As shown in Fig. 4, the expression of *cerberus* in anterior endoderm at the mid-gastrula stage[1] results from earlier signals provided by TGF-β proteins expressed in the endoderm[9,23] as well as by organizer-specific secreted inhibitors of the Wnt[5,20,21] and BMP[6] pathways. In this view, the secretion of Cerberus into the extracellular space would be an important downstream event that would lock the head-organizer programme in place by simultaneously antagonizing three signalling pathways involved in trunk formation, thereby restricting the trunk territory to the posterior part of the body.

Methods

DNA constructs

To generate HA-tagged soluble Xnr-1, Vg1, activin and Xwnt-8, we designed a secreted protein-expression cassette vector that contains the pre-pro region and proteolytic cleavage site of activin-βB[24] followed by the sequence RGL(YPYDVPDYA)LE (pCS2–*proAct–HA*). The two amino acids (LE) after the HA tag were inserted to provide an *Xho*I cloning site. The mature regions of signalling proteins were generated by the PCR reaction using primers flanked by *Xho*I and *Xba*I sequences and subcloned into the HA expression cassette. Mature signalling sequences started at residue C259 of activin-βB, S247 of Vg1, N286 of Xnr-1 and V26 of Xwnt-8. pCS2–*cer-S* was generated by PCR, deleting residues R26 to A116 of *cer*.

Protein binding

Soluble Xwnt-8, Xnr-1, Vg1 and activin were secreted by manually defolliculated *Xenopus* oocytes injected with 50 ng of mRNA and incubated in 5−10 μl per oocyte of OR2 medium for 2 days[24]. Cer-S was prepared by transfection of 293T cells[10]; Cer-L was prepared from animal caps of embryos injected with 1 ng *cer–Flag* mRNA. Typically, 10 caps were dissociated at stage 9 and incubated in 100 μl of Ca–Mg-free medium for 3 h. For immunoprecipitations, protein mixtures were incubated on ice (2 h) in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM CaCl, 1 mM MgCl, 2% glycerol, 0.1% BSA and 0.1% each of the detergents Triton X-100, CHAPS and octylglucoside (Pierce). Protein-A beads pre-bound with anti-Flag polyclonal antibody (Santa Cruz Inc.) were then added and samples incubated with end-over-end rotation for 1 h at 4 °C. After three 5-min washings in the same buffer, protein complexes were analysed by electrophoresis.

Acknowledgements

We thank L. S. Mathews, N. Ueno, R. Moon, D. Melton and K. Cho for gifts of plasmids, K. Masuhara for anti-BMP4 mAb, Genetics Institute for BMPs, and members of the De Robertis laboratory for comments. S.P., E.A., S.B. and H.G were supported by Telethon, ARC, MSTP and DFG, respectively. This work was supported by a grant from the NIH. E.M.D.R. is a Howard Hughes Medical Institute investigator.

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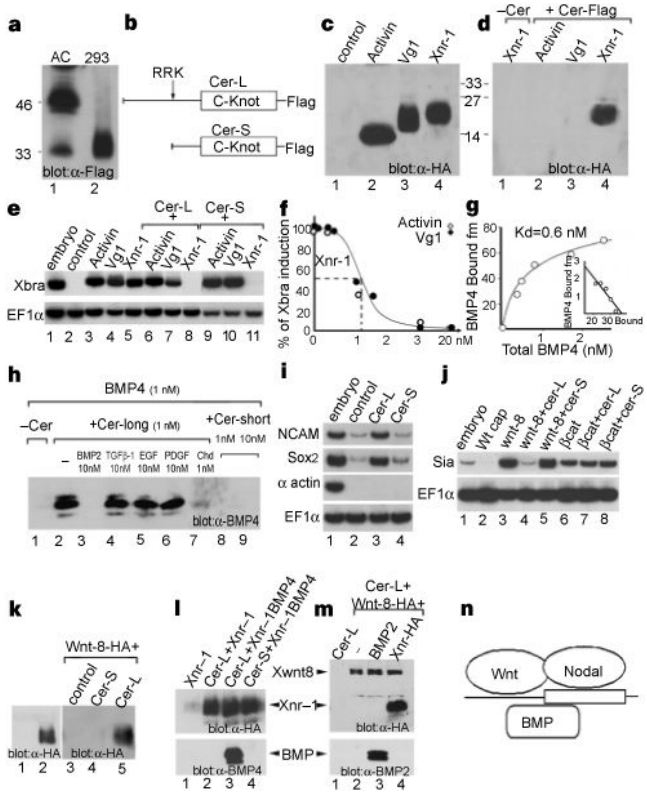

Figure 1.

Cerberus protein binds to Xnr-1, BMP-4 and Xwnt-8. **a**, Cer–Flag secreted by *Xenopus* animal cap (AC) and cultured 293T cells. **b**, The two Cer protein products. **c**, HA-tagged activin, Vg1 and Xnr-1 secreted by *Xenopus* oocytes. **d**, Xnr-1 is bound specifically by Cer-S–Flag (lanes 2−4) or Cer-L–Flag (not shown). **e**, Cer binding inactivates Xnr-1 signalling. Animal cap explants were treated with oocyte control medium, activin (15 pM), Vg1 (0.3 nM) or Xnr-1 (1 nM) proteins, either alone (lanes 2−5) or together with 5 nM Cer-S (lanes 6−8) or Cer-L (lanes 9−11). **f**, Cer inhibits Xnr-1 with high affinity; 2 nM Xnr-1 was incubated with increasing concentrations of Cer-S (closed circles) or Cer-L (open circles). **g**, Cer-L–Flag binds BMP-4 with a K_D of 0.6 nM. **h**, Binding of Cer-L–Flag to BMP-4 is competed for by BMP-2 but not by TGF-β-1, EGF or PDGF; Cer-S does not bind BMP-4. **i**, Cer-L (10 nM), but not Cer-S (20 nM), is a direct neural inducer of animal cap cells sensitized by brief dissociation–re-aggregation (but not of intact caps). **j**, *Cer inhibits Xwnt8* but not *β-catenin* mRNA; induction of *Siamois*, a target of Wnt signalling, was assayed at stage 10+. **k**, Lanes 1, 2, soluble Xwnt-8–HA protein is secreted by mRNA-injected oocytes. Lanes 3−5, binding of Xwnt-8-HA (5 nM) to Cer–Flag proteins (10 nM). **l**, **m**, The Xwnt-8–HA, Xnr-1–HA and BMP binding sites in Cer-L are independent. Cer-L–Flag (2 nM) was bound to Xnr-1 (1 nM) or Xwnt-8 (2 nM) and competed with BMP-4 (10 nM), BMP-2 (27 nM) or Xnr-1 (8 nM). **n**, Multiple ligand-binding sites on Cerberus.

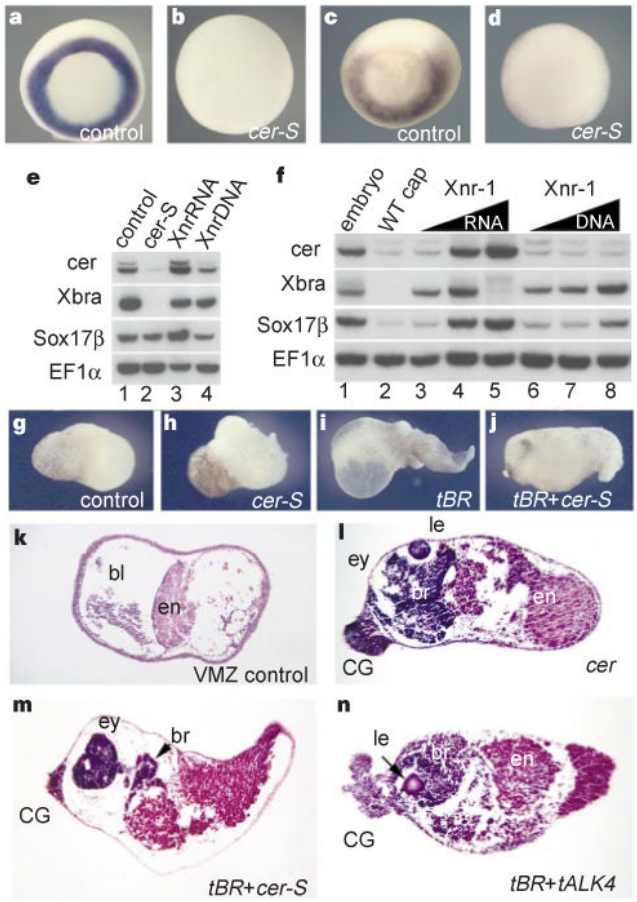

Figure 2.

Phenotypic effects of *cer-S* mRNA, an inhibitor of nodal-related signals. Embryos were injected radially at the 4-cell stage. **a–d**, *In situ* hybridization for *Xbra* (**a**,**b**) and *Xwnt-8* (**c**,**d**) in wild type and in embryos injected with 120 pg of *cer-S* mRNA per blastomere. **e**, Anterior endoderm formation requires early nodal-related signals in injected embryos: endogenous *cerberus* transcripts are inhibited by *cer-S* mRNA (200 pg total) and induced by *Xnr-1* mRNA (50 pg). **f**, Dual function for Xnr-1; *Xnr-1* mRNA (6,12, 24 pg), but not pCS2–*Xnr-1* DNA (20, 40, 80 pg), can induce *cer* expression in animal caps. **g–n**, Head induction by simultaneous repression of BMP and nodal signals in explants of ventral marginal zone (VMZ). **g**, **k**, Wild-type VMZs consisting histologically of endoderm (en) and blood (bl). **h**, *cer-S* mRNA (150 pg); **i**, *tBR* mRNA (600 pg); **j**, **m**, co-injection of *tBR* mRNA (600 pg) and *cer-S* mRNA (150 pg) generates head-like structures (76%, $n = 17$), similar to those produced by *cerberus* mRNA (**l**, 50%, $n = 10$), consisting only of anterior structures with cement gland (CG), brain (br) and eye (ey) containing a lens (le). **n**, Blocking BMP and Nodal signalling by *tBR* (600 pg) and *tALK4* (1.5 ng) RNAs is sufficient to induce head-like structures.

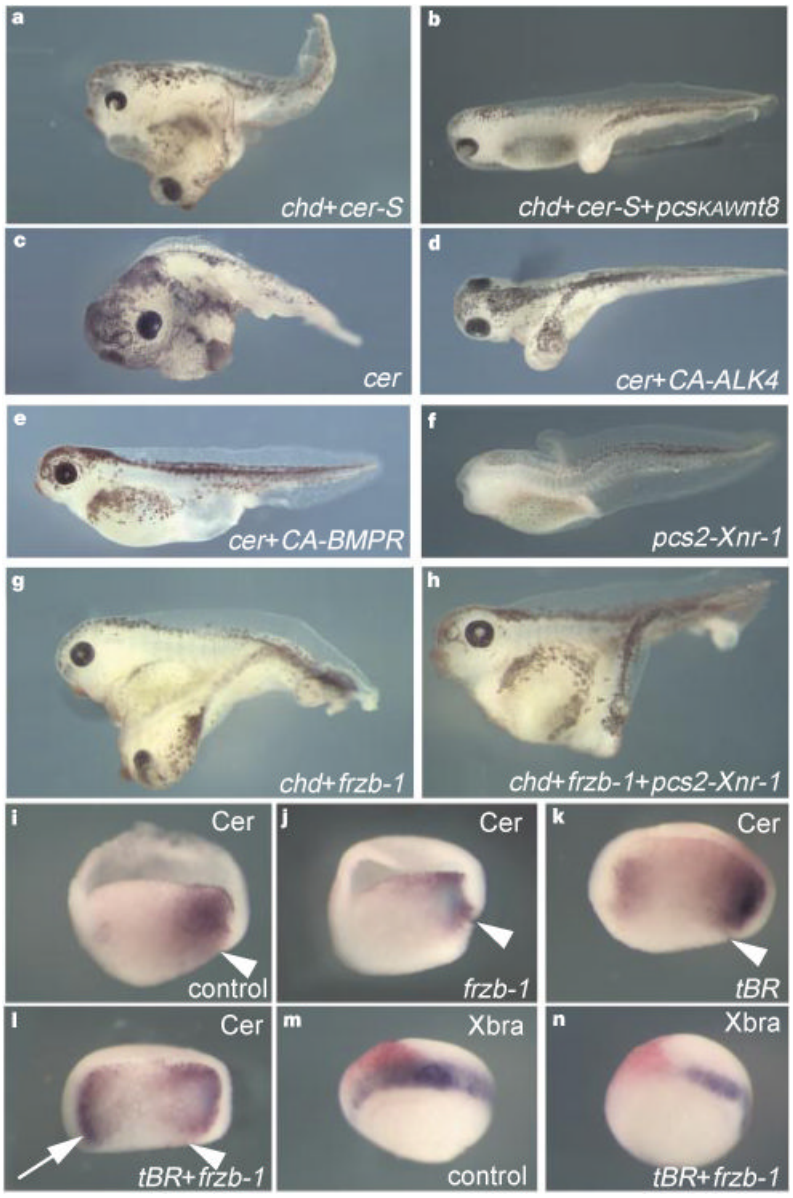

Figure 3.

Head induction requires the triple inhibition of nodal-related, Wnt and BMP signals. Embryos were injected into one ventral blastomere with the indicated mRNAs or DNAs at the 4-cell stage. **a**, Ectopic head induction by a combination of *chd* (50 pg) and *cer-S* (200 pg) mRNA (58%, *n* = 115). **b**, pCSKA–Xwnt-8 DNA (50 pg) antagonizes head induction by *chd/cer-S* (1% ectopic heads, *n* = 111). **c**, Ectopic heads formed by injection of 150 pg *cer* mRNA (57%, *n* = 66) are abolished in **d** and **e** by co-injection of 1 ng *CA–ALK4* mRNA (0%, *n* = 36) or 400 pg of CA–BMPR mRNA (0%, n = 21). **f**, Dorsal injection of pCS2-*Xnr-1* (32 pg) causes head reduction (53%, *n* = 60). **g**, Co-injection of *chd* (25 pg) and *Frzb-1* (200 pg) mRNAs mediates formation of a complete secondary axis with cyclopic head (49%, *n* = 175). **h**, Co-injection of 16 pg of pCS2–*Xnr-1* DNA inhibits head induction by *chd/Frzb-1* (5%, *n* = 133). **i–n**, Simultaneous repression of BMP and Wnt signalling leads to ectopic activation of *cer* in endoderm and concomitant downregulation of *Xbra* in mesoderm. Embryos were cut sagittally in two halves after fixation and before whole-mount hybridization. Arrowhead indicates the

dorsal blastopore lip. *Frzb-1 mRNA* (300 pg) synergizes with *tBR* mRNA (800 pg) in upregulating *cer* in the ventral endoderm (arrow). Note in **n** the inhibition of *Xbra* in the injected area, correlating with the activation of *cer* (an anti-Nodal agent) observed in **l**.

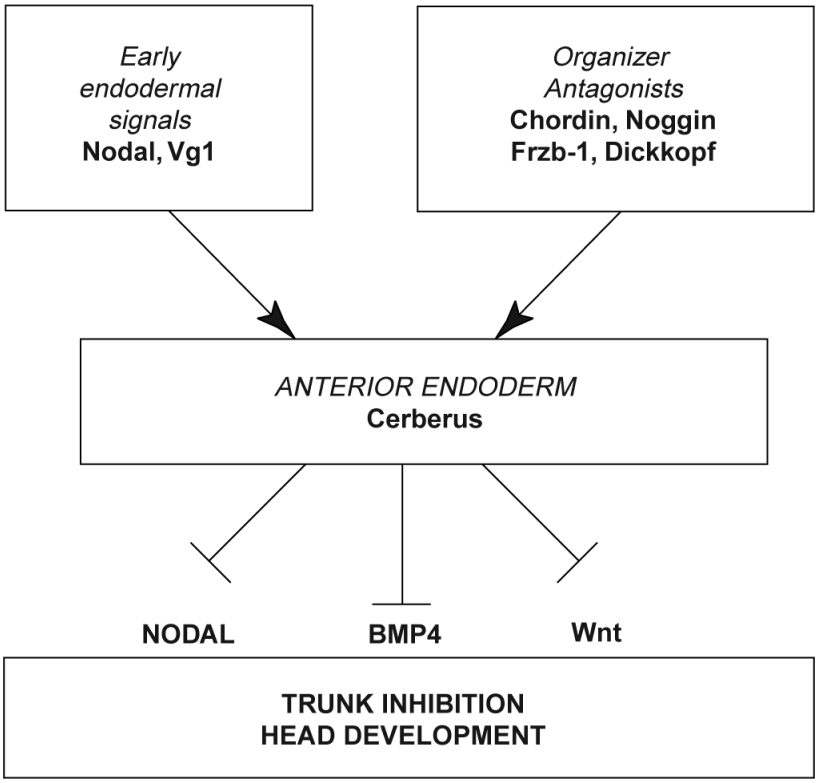

Figure 4.
Model of the formation and function of anterior endoderm in *Xenopus* head development.