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Transient downregulation of Bmp signalling induces extra limbs in vertebrates

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SUMMARY

Bone morphogenetic protein (Bmp) signalling has been implicated in setting up dorsoventral patterning of the vertebrate limb and in its outgrowth. Here, we present evidence that Bmp signalling or, more precisely, its inhibition also plays a role in limb and fin bud initiation. Temporary inhibition of Bmp signalling either by overexpression of *noggin* or using a synthetic Bmp inhibitor is sufficient to induce extra limbs in the *Xenopus* tadpole or exogenous fins in the *Danio rerio* embryo, respectively. We further show that Bmp signalling acts in parallel with retinoic acid signalling, possibly by inhibiting the known limb-inducing gene *wnt2ba*.

KEY WORDS: Bmp signalling, Appendage development, Vertebrate, *Xenopus*, Zebrafish

INTRODUCTION

Limb bud patterning has been extensively studied in mouse and chick with a good understanding of the molecular pathways involved. Limb developmental studies on other classes of vertebrates seem to validate these models, as many of the transcription factors and signalling pathways responsible for limb bud outgrowth are conserved (Carlson et al., 2001; Christen and Slack, 1998; Christensen et al., 2002; Grandel and Brand, 2011; Kawakami et al., 2004; Koshiba et al., 1998). Less is known, though, about how the limb field is defined and the limb bud initiated. The current limb field initiation model places retinoic acid (RA) at the top of the cascade, indirectly inducing a member of the WNT family (*wnt2b* and *wnt8c* in forelimb and hindlimb regions, respectively) in the intermediate mesoderm (Mercader et al., 2006). These WNT family members subsequently induce *fgf10* in the lateral plate mesoderm at specific axial levels where limbs are formed. *fgf10* then signals to the overlaying ectoderm to induce the apical ectodermal ridge (AER), the signalling centre for proximal distal limb outgrowth, by starting a feedback loop involving *wnt3a* and *fgf8* (Kawakami et al., 2001; Mercader, 2007). The present model of limb initiation does not include a role for Bmp signalling. The Bmp pathway, however, has been assigned a prominent role in setting up the dorsoventral (DV) axis of the limb by inducing *en1* in the ventral ectoderm. En1 represses the dorsal inducers *wnt7a* and *lmx1* on the ventral side, therefore allowing for ventral mesoderm patterning (Ahn et al., 2001; Maatouk et al., 2009; Pizette et al., 2001). Furthermore, En1 helps to position the AER along the DV boundary (Kimmel et al., 2000).

Bmps are a subgroup of the transforming growth factor β (Tgfβ) superfamily that signal through one of the two arms of the Tgfβ pathway. Bmps bind to the type I receptors Alk1, Alk2, Alk3 and Alk6, and signal through Smad1, Smad5 and Smad8 (referred to as the Bmp pathway), whereas Tgfβ, Activin, Nodal and some GDFs bind to type I receptors Alk4, Alk5 and Alk7, and activate Smad2 and Smad3 by phosphorylation (from now on referred to as the Tgfβ pathway) (Whitman and Raftery, 2005). The family of Bmp ligands comprises many members, of which only three are implicated in limb development: Bmp2, Bmp4 and Bmp7 (Robert, 2007). Distinct developmental processes require different amounts of Bmp protein for a correct outcome (De Robertis and Kuroda, 2004; Liu et al., 2005; Murali et al., 2005). To achieve the appropriate levels, endogenous extracellular Bmp inhibitors such as Noggin, Chordin and Gremlin are involved in fine-tuning activation of the Bmp pathway. In particular Noggin has been shown to bind preferentially to Bmp2 and Bmp4, and (with a lesser affinity) to Bmp7, thereby directly blocking the binding site for association with the type I and II receptors (Groppe et al., 2002).

Given the role for Bmps in patterning of the limb bud, we were surprised to notice an extra set of hindlimbs in some *Xenopus laevis* tadpoles that were exposed to ectopic *noggin* expression during tail regeneration. Induction of ectopic limbs in *Xenopus* was only possible during a limited period and was accompanied by a dorsalisation of the endogenous limb bud. In addition, we proved that Bmp inhibition is a more universal mechanism of appendage initiation in vertebrates, as downregulation of Bmp signalling in the zebrafish was also sufficient for ectopic pectoral fins to develop. We further present evidence that Bmp signalling acts in parallel with RA, the earliest known fin inducer. Furthermore we established that two of the early limb inducing genes, *wnt2ba* and *sp8*, are both upregulated immediately after Bmp inhibitor treatment, which might provide an explanation for the development of supernumerary fins in these zebrafish embryos. Last, we suggest an expansion of the existing appendage induction model, based on our results.

MATERIALS AND METHODS

Animal maintenance

Xenopus laevis tadpoles (wild type and N1 line) were kept in static tanks at room temperature until the required stage (Nieuwkoop and Faber, 1967). Zebrafish lines [AB Salk, *Tg(hsp70:noggin/clmc2a:GFP)*, *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])*] were maintained under standard conditions (Westerfield, 1995). Embryos were cultured in embryo medium (EM, 13.7 mM NaCl, 0.54 mM KCl, 0.025 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) at 28°C until the required stage or hours post fertilisation (hpf).

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Constructs and transgenesis

Constructs were generated using the multisite gateway system Tol2 kit (Kwan et al., 2007). For *Hsp70:noggin/clmc2a:GFP*, 5′ entry clone was Hsp70 promoter from zebrafish; middle entry clone was *Xenopus* noggin sequence (same as N1 line); and 3′ entry clone was a cmlc2a: GFP reporter. Tol2_Hsp70:noggin/clmc2a:GFP DNA (100 ng) was mixed with Tol2 transposase RNA (80 ng) and injected into wild-type (AB Salk) one-cell-stage embryos. For *xCar:ERCreER/eab2:[EGFP-TmCherry]*, 5′ entry clone was *Xenopus* cardiac actin promoter; middle entry clone was ERCreER from pCAG-ER^{T2}CreER^{T2} (Addgene plasmid 13777) (Matsuda and Crepko, 2007); and 3′ entry clone was rabbit β-globin poly(A). A mixture of Tol2_Car-ERCreER DNA and Tol2 transposase RNA was injected into one-cell stage embryos derived from cross between AB Salk and *Tg(eab2:[EGFP-TmCherry])* (Boniface et al., 2009).

Signalling pathway analysis: Bmp inhibition, Tgfβ inhibition and RA signalling

For Noggin induction, *Xenopus* tadpoles were heat shocked at the appropriate stage in water bath at 34°C for 20 minutes. To induce extra limbs, tadpoles at stage 49 were heat shocked once a day for 7 days. To induce the transgene in zebrafish, embryos were heat shocked in a 37°C incubator at various stages for 8, 12 or 24 hours per day.

For the small-molecule inhibitor experiments, wild-type or (*Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])*) eggs were collected and incubated at 28°C in EM until the desired stages. Staging was according to Kimmel et al. (Kimmel et al., 1995) and treatments were started at 4, 5, 6, 7 or 8 hpf. At the end of treatment period, Bmp inhibitor was washed off. Bmp inhibitor (Stemolecule Bmp inhibitor LDN-193189 from Stemgent) was dissolved in chloroform (10 mM) before being diluted to 1 mM in DMSO. Concentrations tested were 0.5, 1, 2 and 4 μM Bmp inhibitor. Tgfβ RI kinase inhibitor III (Calbiochem, Cat. No. 616453) (1 mM stock in water) used at 1, 2, 4, 5, 10 and 20 μM. Controls were chloroform:DMSO 1:9 at 6-7 hpf.

Morpholinos used were GDF8/myostatin (CTGTGTAAAATGCATG-TTCCAAGGC) and GDF11 (TCAGGAAACAATGGTTTTCTCTTGA).

For RA and DEAB treatments, retinoic acid (10 μM stock in DMSO) was used at 10-50nM in EM, while diethylaminobenzaldehyde (DEAB, 10 mM stock in DMSO) was used at 10 μM in EM. RA and DEAB were added together with Bmp inhibitor for just 1 hour (6-7 hpf) unless otherwise indicated.

RNA extraction and cDNA synthesis

Zebrafish embryos were treated with 2 μM Bmp inhibitor between 6 and 7 hpf. Total RNA was extracted from embryos at indicated time points using TRIZOL according to manufacturer's guidelines (Invitrogen) and purified using RNAeasy columns with DNaseI on-column treatment. Total RNA (1 μg) was used in standard reverse transcription reaction using random hexameres. A pool of 50-100 embryos was used for each replicate.

Real-time quantitative PCR

Real-time PCR was carried out as described previously (Sleep et al., 2010). Relative expression of PCR products was determined by comparative Ct method with highest relative quantity set at one while normalising against three genes. Results are accumulated from two (Fgfs) or three (rest of genes) experiments and one technical replica. Primer sets are in supplementary material Table S1.

In situ hybridisation

In situ hybridisation was carried out as described by Pownall et al. (Pownall et al., 1996). To make in situ probes, a partial sequence for *lmx1b* was obtained (lmx1bs), cut with *Not*I and transcribed with T7. En1sp72 was cut with *Xba*I and transcribed with Sp6, and fgf8bs 5.2 was cut with *Xba*I and transcribed with T3 (Christen and Slack, 1997).

Skeletal preparation

Standard protocols for bone and/or cartilage visualisation in tadpoles, froglets and zebrafish embryos were used.

Visualisation of muscle

Muscle was visualised by antibody staining with MF20 antibody (MF20-s1, from Hybridoma Bank, Iowa, USA; 1:10) using secondary antibody α-mouse AP (Jackson Laboratory 715055151; 1:200). Embryos (4-5 dpf) with extra fins were used in a standard protocol.

For in vivo muscle visualisation in *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])*, the Bmp inhibitor was added (6-8 hpf) followed by treatment with 1 μM 4-hydroxytamoxifen for the next 2-3 days with daily changes. *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])* strain substitutes GFP expression in muscle with mCherry expression upon addition of tamoxifen (Boniface et al., 2009). Presence of muscle was scored at 5 dpf.

Western blot

A standard protocol was followed in which 25 μg total protein was loaded per lane. Antibodies used were p-Smad 1, p-Smad5 and p-Smad 8 (1:1000, Cell Signaling #9511); p-Smad 2 and p-Smad 3 (1:1000, Cell Signaling #3101); α-tubulin (1:5000, Sigma #6074); anti-rabbit HRP (1:10,000, GE Healthcare, NA9340); and anti-mouse HRP (1:10,000, GE Healthcare, NA9310).

Animal welfare

All animal experiments were carried out with approval of institutional (CEEA-PRBB; http://portal.prbb.org/ciencia/comite_etic) and governmental (Generalitat de Catalunya) ethics committees.

RESULTS

Extra limb phenotype

We have previously characterised the N1 *Xenopus laevis* line, which carries an inducible transgene comprising the Bmp inhibitor *noggin* under the control of the heat shock promoter hsp70 (Beck et al., 2006; Beck et al., 2003). We noticed that a few N1 tadpoles, which were heat shocked during tail regeneration, had an extra pair of hindlimbs. Intrigued, we looked at limb initiation in *Xenopus*. At stage 48 (Nieuwkoop and Faber, 1967), hindlimb buds become first apparent as tiny outgrowths that grow rounder and bigger by stage 49 until their width and length become equal at stage 50. By stage 51, the limb buds are conical and start to show pigmentation. To determine the period during which *noggin* overexpression was able to induce extra hindlimbs, we activated *noggin* expression systematically for a week at different developmental stages. We noticed that ectopic limbs were induced in only a narrow time window. If *noggin* induction was initiated at stage 48, secondary hindlimbs were frequently induced, positioned slightly more ventral than endogenous limbs and developmentally retarded when compared with forelimbs (Fig. 1A); however, as outgrowth of the endogenous limbs was suppressed, just one pair of hindlimbs developed. By contrast, starting the heat shocks at stage 49 often resulted in the formation of two pairs of normal looking hindlimbs (Fig. 1B). Induction of *noggin* at stage 50 or later only lead to abnormal limb development (Fig. 1C), a phenotype that has been described by Pizette and colleagues during chick limb development (Pizette et al., 2001).

Within the stage 49 time window, the phenotype itself varied, with a maximal penetration of one-third of tadpoles showing some duplication of skeletal limb elements. If an entire new limb field was created, the limbs were fully separated at the base with a full set of skeletal elements, autopod, zeugopod and stylopod. In these cases, the additional ectopic limbs were always developmentally younger than the endogenous limbs (Fig. 1D,G, arrows). If the split happened further distally in the zeugopod, the duplications were the same size and shape. Duplications could occur in either the anterior-posterior (Fig. 1E) or the dorsal-ventral axis (Fig. 1F). During tadpole stages, the endogenous and the extra limbs looked fairly normal, with the ectopic limbs growing out ventrally from

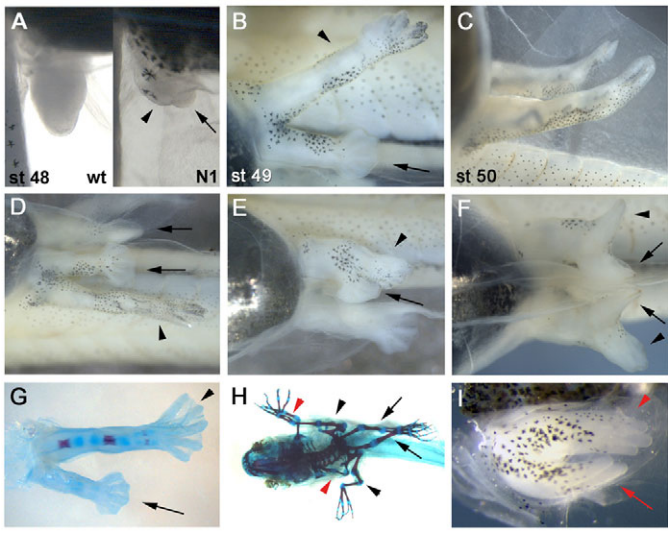

Fig. 1. Extra limb phenotype in the *Xenopus* N1 line. (**A**) Overexpression of *noggin* at stage 48 leads to inhibition of limb outgrowth (arrowhead) with occasional formation of a secondary limb. (**B**) Overexpression of *noggin* at stage 49 leads to induction of extra limbs in about 30% of the tadpoles. Endogenous limb is developmentally more advanced than the extra limb. (**C**) Overexpression of *noggin* at stage 50 or later leads to malformation of the limb. (**D-F**) Variability of the extra limb phenotype. Induction of full extra limbs (D). Partial duplications of distal elements with split in antero-posterior (E) or dorso-ventral (F) axis. (**G**,**H**) Alizarin Red staining for bone and Alcian Blue staining for cartilage in pre-metamorphotic tadpole limbs (G) and a froglet (H). Endogenous limbs look almost normal during development but protrude at different angles after metamorphosis. (**I**) Duplication of a forelimb. Arrowheads indicate endogenous limbs; arrows indicate extra limbs. Red arrowheads and arrows indicate forelimbs.

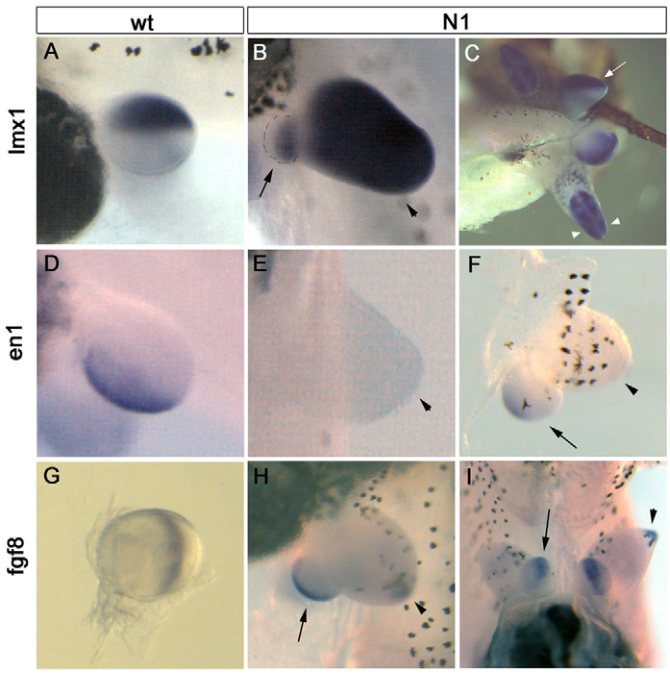

Fig. 2. In situ hybridisation in *Xenopus* wild type and N1 line after induction of extra limbs. (**A-C**) *lmx1* expression in the wild-type limb is restricted to the dorsal mesenchyme (A) but is present in the entire mesenchyme in the endogenous limb of the N1 line (B,C), indicating a dorsalisation of the limb; the extra limb shows normal *lmx1* expression restricted to the dorsal mesenchyme (C). (**D-F**) *en1* expression is restricted to the ventral ectoderm in the wild type (D) but is absent in the N1 endogenous limbs (E), whereas extra limb shows normal expression of *en1* (F). (**G-I**) Expression of *fgf8* is restricted to the AER in wild-type limbs (G) but is expressed in a broader domain in N1 endogenous limbs while normally expressed in the AER of the extra limbs (H,I). Arrowheads indicate endogenous limbs; arrows indicate extra limbs.

the endogenous limbs at all times (Fig. 1G). During metamorphosis, however, the endogenous limbs started to protrude at different angles (Fig. 1H, arrowheads). Forelimb duplications were also observed, but the period for induction was slightly shifted backwards, as forelimb development lags behind hindlimb development in *Xenopus* (Fig. 1H,I). Forelimbs are quite small and develop inside a skin pocket; therefore, we concentrated our study on the more easily visible and accessible hindlimbs.

Abnormal dorsal-ventral axis

As Bmp signalling plays an important role in DV patterning of the limb, we decided to look at the expression of two DV determining genes, *en1* and *lmx1*, in the endogenous and ectopic limbs of N1 transgenics. In the wild-type limb, *lmx1* expression was restricted to the dorsal mesenchyme, as expected (Fig. 2A). In the N1 line, however, 5 days after inducing *noggin* for a week, the dorsal mesenchyme marker *lmx1* had spread ventrally and was expressed in the entire limb bud mesenchyme of the endogenous limb, indicating a dorsalisation of the limb (Fig. 2B). Interestingly, *lmx1* was restricted to the dorsal mesenchyme in the secondary limbs, as is clearly visible in older tadpoles (Fig. 2B,C). Similarly, expression of *en1*, which is restricted to the ventral ectoderm in wild-type limbs (Fig. 2D), was lost in the endogenous limbs after *noggin* induction (Fig. 2E) but normal in the ectopic limb bud (Fig. 2F). Pizette and colleagues (Pizette et al., 2001) have previously reported a dorsalisation of the chick limb bud by widespread infection with a noggin virus. In the same paper, however, they also showed that Bmp signalling plays a role in AER induction in a DV-independent pathway, inducing *fgf8* expression via the Msx genes. We therefore examined *fgf8* expression. In the wild-type limb, *fgf8* is expressed in its normal domain in the AER (Fig. 2G) (Christen and Slack, 1998). As expected, *fgf8* is also correctly expressed in the extra limb of the N1 line, but is somewhat abnormally expressed in the endogenous limb (Fig. 2H,I).

Downregulation of Bmp signalling in zebrafish induces extra pectoral fins

To test whether Bmp inhibition is a general requirement for vertebrate limb initiation, we turned to *Danio rerio* (zebrafish). Pectoral fin development is a process that is homologous to limb development and it has been shown that molecular pathways for the initiation and development of the two appendages are conserved (Ito et al., 2010; Kawakami et al., 2004; Mercader, 2007). Transgenic zebrafish with a similar hsp70 *noggin* construct as used in *Xenopus* were made. Founders, with GFP expression in the heart, were screened for a dorsalisation phenotype of their offspring after heat shocking during gastrula stages. The phenotype indicated that the transgene was active, thus lines were established for positive founders. Further batches of F1 and F2 offspring from positive founders were then subjected to heat shocks of various

lengths at three different time point during fin development [20, 24, 40 hours post fertilisation (hpf)]. In zebrafish, expression of fin induction genes such as *tbx5* and *fgf24* are first detected around 18 hpf, while the pectoral fins themselves are not easily detected until 3 dpf (Albalat et al., 2010; Fischer et al., 2003). Although most lines never produced any extra fins, we noticed a few severely dorsalised transgenic embryos (~5%) with one or more extra pectoral fins in apparent random locations on the belly in line #3 (Fig. 3A,C). The extra fins were located more ventrally and on the same or a more posterior level than the endogenous fins. They were often completely separate from the endogenous fins and unpaired, unlike in *Xenopus*. The majority of the transgenic embryos (95%), though, had a lesser dorsalised phenotype and no extra fins. We suspected, therefore, that extra fins were associated with embryo dorsalisation, which happens only if the Bmp pathway is inhibited during gastrulation (pre 8 hpf) but not at later stages (Pyati et al., 2005). It also indicated that the extra fin phenotype observed in line #3 was due to leakage rather than to heat-shock-mediated induction of *noggin*, which in turn suggested that Bmp signalling needed to be inhibited prior to fin outgrowth in zebrafish for successful induction of extra appendages. In our initial screen for positive founders, the embryos were discarded before fin development was obvious. We re-screened old and new founders, inducing *noggin*, during gastrulation stages and found a single embryo each with extra fins among the F1 offspring of two more founders [founder #28 (*n*=1 from eight transgenics), founder #32 (*n*=1 from 17 transgenics)], proving that Bmp inhibition was also sufficient to induce extra fins in zebrafish. The very low induction frequency is probably due to the cross-species *noggin* that we used or to inefficient induction.

To increase the frequency of extra pectoral fin induction, we resorted to a small inhibitory molecule to block Bmp signalling (LDN193189). The addition of this Bmp inhibitor at 6 hpf for 18 hours increased the frequency of extra fins significantly (>50%). The phenotype was very similar to the one observed in the *noggin* lines, resulting in dorsalised embryos with one or more extra fins on the abdomen (Fig. 3D).

To verify the quality of features, we checked for cartilage and muscle within the extra fins. We determined by Alcian Blue staining that all extra fins contained cartilaginous elements independent of the method of induction (Fig. 3C; data not shown). Visualisation of muscle was achieved with two different methods, either by antibody staining using the muscle specific antibody MF20 (Fig. 3G) or by in vivo expression of a fluorescent protein specifically in the muscle using *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry]*(Fig. 3E,F,H,I). With either method, we found small muscle patches only in a minority of the extra fins, indicating that by 5 days post fertilisation (dpf) not all extra fins are fully developed. As the severely dorsalised embryos with extra fins survive for only a short period, we were unable to check for later muscle development.

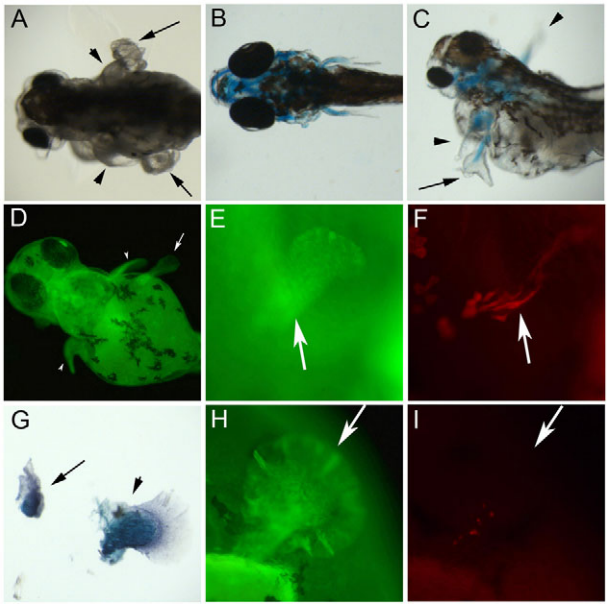

Fig. 3. Extra pectoral fin phenotype in zebrafish. (**A**,**C**,**G**) *noggin*-induced extra fins (line #3) at 5 dpf. (**B**) Wild type at 5 dpf, (**D-F**,**H**,**I**) Bmp inhibitor-induced extra fins in 5 dpf *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])*. The phenotype is very similar in *noggin*- (A) and Bmp inhibitor- (D) induced extra fins, with supernumerary fins located more ventrally and on same or more posterior level on the abdomen than endogenous fins. Cartilage staining in wild type (B) and in *noggin* TG (C) illustrates that endogenous and extra fins possess a well-defined cartilage rod. In the *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])*, we can visualise muscle in red (expressing mCherry). (E,F) A minority of extra fins contain well-defined muscle (F), whereas the majority only have punctual or no muscle development (H,I). (G) Muscle in extra fins was also visualised using the MF20 muscle-specific antibody (blue staining). Arrowheads mark endogenous fins; arrows indicate extra fins.

Brief Bmp downregulation during gastrulation is sufficient for extra fin induction

Next, we wanted to examine the time period when Bmp repression was able to induce supernumerary fins most efficiently. Four different concentrations of inhibitor were added at various time points for different length of time. It became apparent that no treatment was more effective than another, but that different concentrations had different optimal time periods for successful extra fin induction (Table 1) and that 1 hour's exposure to a Bmp inhibitor was sufficient to induce extra fins in over 50% of the embryos. In general terms, low concentrations were better able to induce extra fins when added during early gastrulation (5 or 6 hpf) and for long periods (up to 24 hpf). Higher concentrations were lethal when added during early gastrulation (5 hpf) or for long periods (up to 24 hpf), but were able to induce supernumerary fins in a high percentage when added for only 1 hour at 6 or 7 hpf (Table 1). The higher concentrations were not toxic per se, but caused more severe dorsalisation and hence embryos tended not to survive long enough to be scored for the extra fin phenotype at 3-5 dpf.

No involvement of Tgfβ signalling via Alk4, Alk5 and Alk7

A recent paper described an extra limb phenotype in mouse caused by the double knockout of two Tgfβ ligands, GDF8 (also called myostatin) and GDF11 (McPherron et al., 2009). In these double mutant mice, ectopic forelimbs and finger-like structures appear on the ventral side, resembling what we describe in *Xenopus* and zebrafish. As *noggin* was overexpressed to high levels, as shown by Beck et al. (Beck et al., 2006), we thought that *noggin* might act indiscriminately and inhibit not only the Bmp pathway but also the Tgfβ pathway. Therefore, we checked the activation of the canonical Bmp and Tgfβ signalling pathways by measuring the protein levels of phosphorylated Smad1, Smad5 and Smad8 (Bmp

Table 1. Induction of extra pectoral fins by Bmp inhibition during gastrula stages

	0.5 μM	1 μM	2 μM	4 μM
5-6 hpf	26% (10/38)‡	65% (11/17)*,§	Lethal§	ND
5-7 hpf	38% (3/8)‡,§	57% (4/7)*,§	Lethal§	ND
5-8 hpf	56% (10/18)*,§	Lethal§	Lethal§	ND
5-24 hpf	56% (9/16)*,§	Lethal§	Lethal§	ND
6-7 hpf	3% (2/76)	41% (35/85)‡	51% (68/133)*	27% (3/11)‡,§
6-8 hpf	0% (0/50)	39% (12/31)‡	43% (6/14)‡,§	ND
6-24 hpf	56% (19/34)*	65% (11/17)*,§	ND	ND
7-8 hpf	0% (0/30)	2% (1/54)	28% (20/71)‡	61% (42/69)*
7-24 hpf	4% (3/79)	39% (27/70)‡	ND	ND
8-24 hpf	0% (0/133)	4% (3/80)	4% (1/23)	ND
Chloroform/DMSO control 6-7 hpf	ND	ND	0% (0/60)	ND

Bmp inhibitor at specified concentrations was added at the beginning and washed off at the end of the periods indicated. Percentages of embryos with extra fins are indicated with actual numbers in brackets (embryos with extra fins/total embryos). Although there is no optimum period or optimum concentration for extra fin induction, there is some pattern. Lower concentrations work better if added early and for longer (at 5 hpf and up to 24 hpf), whereas higher concentrations work best at later stages and for short periods (at 6 or 7 hpf and 1 hour treatment). At least 60 embryos were tested in two to five independent experiments and scored between 3 dpf and 5 dpf. In some instances, numbers are small because of high death rate after treatment.
*Most effective in inducing extra pectoral fins.
‡Quite effective in inducing extra pectoral fins.
§High death rate.
ND, not defined.

pathway), and p-Smad 2, p-Smad3 (Tgfβ pathway). Although we could see the expected reduction in p-Smad1, p-Smad5 and p-Smad8 levels in N1 heat-shocked tadpoles, there was no such reduction in p-Smad2 and p-Smad3 levels, or even an increase of these proteins was observed (Fig. 4; supplementary material Fig. S1). This suggested that *noggin* overexpression did not inhibit both arms of the Tgfβ superfamily pathways. To eliminate the possibility that a reduction in p-Smad2 and p-Smad3 protein levels was masked by the approximately two-thirds of limb regions collected that were not going to develop extra limbs, we tried to recreate the extra limb phenotype by treating wild-type stage 49 tadpoles with an Alk4-, Alk5- and Alk7-specific inhibitor (Tgfβ RI kinase inhibitor III) for 7 days. Such a treatment, however, never led to extra limbs (data not shown).

Treating zebrafish embryos with the same Tgfβ RI inhibitor from 6 hpf onwards did not lead to induction of extra fins either. Last, we tried to recreate the double *gdf8/gdf11* knockout extra limb phenotype by knocking down the homologous genes in zebrafish with morpholinos. To achieve this, we designed single morpholinos against each of these genes and injected them together at three different concentrations into the one-cell stage embryo. If injected at 0.35 mM each, it was lethal to the embryo and at 0.2 and 0.1 mM the embryos were fairly normal with only one pair of pectoral fins (data not shown). We concluded that the Tgfβ signalling pathway was not contributing towards the extra limb/fin phenotype.

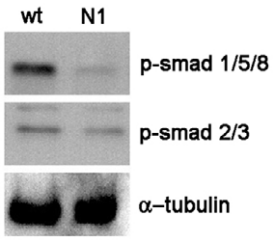

Fig. 4. Western blot. Limb regions were collected immediately after overexpression of *noggin* at stage 49 for 7 days. A less intense phospho-Smad 1/5/8 band is detected in the N1 line, indicating a downregulation of the Bmp pathway as expected. However, no downregulation of the Tgfβ pathway was detected using a phospho-Smad2/3-specific antibody. Loading control was α-tubulin.

Bmp signalling acts in parallel with RA signalling for fin induction

RA is involved in many developmental processes during embryogenesis, like anterior posterior patterning and patterning of the hindbrain and pancreas, and is the earliest factor known to be necessary for limb bud initiation (Duboc and Logan, 2011; Gibert et al., 2006; Grandel and Brand, 2011; Grandel et al., 2002; Niederreither et al., 1996; Rutledge et al., 1994). Mutations in *aldh1a2*, an enzyme involved in RA synthesis, lead to loss of the forelimbs and pectoral fins in mouse and zebrafish, respectively, among other phenotypes (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 2002). A recent study placed pectoral fin induction by RA as occurring during gastrulation (6-8 hpf) (Grandel and Brand, 2011); thus, we were interested in a possible interaction between the Bmp and the RA pathway. For this reason, we treated embryos at gastrula stages with a Bmp inhibitor (LDN193189) and DEAB, a potent RA inhibitor that has been used before to inhibit pectoral fin outgrowth (Gibert et al., 2006; Grandel and Brand, 2011; Mercader et al., 2006). We expected to see loss of endogenous and extra pectoral fins if RA was acting in parallel or downstream of Bmp inhibition, or normal endogenous fins with same number of extra fins if RA signalling was acting upstream of Bmp inhibition. The addition of DEAB for just 1 hour at 6 hpf, together with 2 μM Bmp inhibitor, reduced the number of extra fins drastically but abolished only 45% of endogenous fins (Table 2). However, if embryos were treated with DEAB overnight, as in most published studies (with 2 μM Bmp inhibitor for 1 hour at 6 hpf), all extra and endogenous fins were completely abolished (Fig. 5B; Table 2). Therefore, endogenous and extra fins need an intact RA signalling pathway for their development, indicating that Bmp acts upstream or in parallel with RA signalling. Next, we wondered whether addition of RA could increase the Bmp inhibition phenotype in zebrafish by either increasing frequency or number of extra fins per embryo. Vandersea et al. (Vandersea et al., 1998) reported previously an ectopic fin phenotype in zebrafish by addition of high doses of RA (0.5-1 μM RA) during gastrula stages. They observed that addition of RA led to extra fins that were more anterior to the endogenous pectoral fins, rather than ventral and posterior (as we describe) after Bmp knockdown. We used a low dose of RA (10-50 nM), which is sufficient to rescue the *neckless* mutant (*aldh1a2*) but insufficient for fin duplication (Grandel et al.,

Table 2. Bmp signalling is upstream of the RA pathway

	Extra fins	Distal duplication	Endogenous fins
Bmp inhibitor (2 μM) at 6-7 hpf	70% (171/245)	0%	99%
Bmp inhibitor (2 μM) + DEAB (10 μM) at 6-7 hpf	9% (7/78)	n.d.	55%
Bmp inhibitor (2 μM at 6-7 hpf) + DEAB (10 μM, overnight)	0% (0/69)	n.d.	0%
Bmp inhibitor (2 μM) + RA (10 nM) at 6-7 hpf	65% (104/159)	0%	n.d.
Bmp inhibitor (2 μM) + RA (50 nM) at 6-7 hpf	41% (36/87)	8% (7/87)	n.d.
RA (10 nM) at 6-7 hpf	0% (0/101)	0%	n.d.
RA (50 nM) at 6-7 hpf	0% (0/90)	0%	n.d.
DEAB (10 μM) at 6-7 hpf	0% (0/79)	n.d.	90%
DEAB (10 μM) overnight	0% (0/76)	n.d.	0%

Simultaneous treatment of embryos with Bmp inhibitor and DEAB for 1 hour reduces extra fin development from 70% to 9%, and abolishes both endogenous and extra fin development completely if DEAB treatment is continued overnight, indicating that Bmp signalling is upstream or in parallel with the RA pathway. If embryos were treated with Bmp inhibitor and RA simultaneously, neither frequency nor numbers of extra fins increased. Interestingly, a small percentage of embryos treated with Bmp inhibitor plus a higher dose of RA (50 nM) showed anterior rather then ventral fin duplications, a phenotype previously seen only with a 10 times higher dose of RA (Vandersea et al., 1998). Numbers in brackets are numbers of embryos with extra fins/total numbers of embryos treated.
n.d., not determined.

2002; Vandersea et al., 1998). Treatment with a low dose of RA plus Bmp inhibitor (2 μM Bmp inhibitor and 10 nM RA) between 6 and 7 hpf did not augment either the frequency or number of extra fins (Table 2), whereas a higher dose of RA (2 μM Bmp inhibitor and 50 nM RA) resulted in animals with more severe phenotypes and fewer extra fins (Table 2). Interestingly, treatment with 50 nM RA changed the phenotype of the extra fins in some embryos towards anterior fin duplications, as previously only seen with 10 to 20 times higher RA concentrations in absence of Bmp inhibitor (Fig. 5C) (Vandersea et al., 1998). This change from a ventral to anterior duplication phenotype indicates that Bmp and RA are probably acting in parallel rather than in a linear pathway. However, to rule out a linear component more firmly, we employed real time PCR.

We checked several RA modifier genes for differential expression after Bmp inhibitor treatment. Neither the aldehyde dehydrogenases (Aldh1a2 and Aldh1a3) that are crucial components for the oxidation of retinol to RA nor the two cellular retinoic acid-binding proteins (CrabpI and CrabpII) showed any significant and persistent expression changes after treatment with a Bmp inhibitor (supplementary material Fig. S2). Expression of a fifth factor, Cyp26a, which negatively regulates RA signalling by degrading RA, also did not seem to be influenced by Bmp inhibitor treatment (supplementary material Fig. S2). Therefore, we concluded that the two pathways really act in parallel without a linear component.

Differential expression of limb field inducers

To find which genes are affected by Bmp inhibition, we performed a small-scale gene expression analysis, concentrating on known limb inducer genes (supplementary material Table S1). We covered the time gap between Bmp inhibition (6-7 hpf) and first visible limb field induction (expression of *tbx5* by in situ hybridisation) at around 18-22 hpf, by studying one immediate, two intermediate

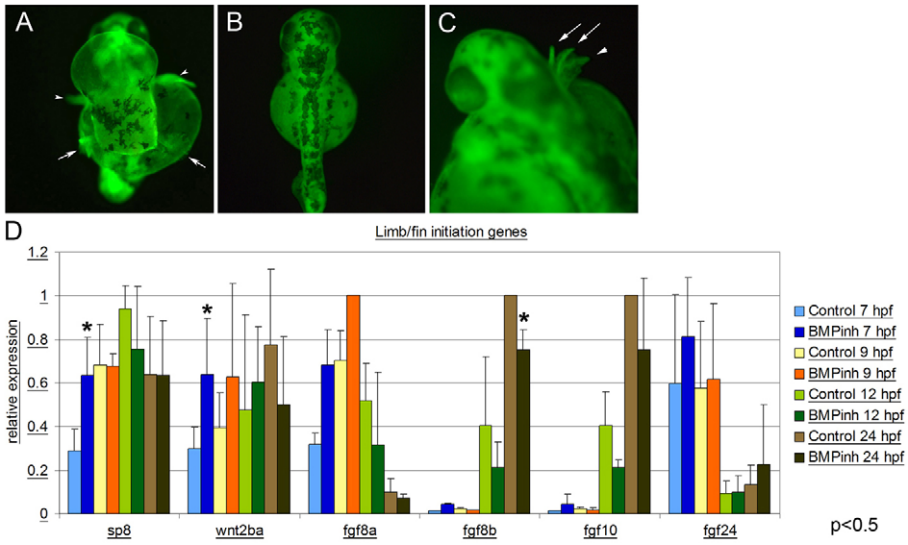

Fig. 5. Bmp pathway and its interaction with RA and limb initiation genes. (A-C) *Tg(xCar:ERCreER/eab2:[EGFP-TmCherry])* embryos (3 dpf) treated with Bmp inhibitor at 6-7 hpf. (**A**) Control embryo with extra fins after treatment with Bmp inhibitor alone. (**B**) Extra and endogenous fins are lost if embryos are treated in addition with DEAB overnight. (**C**) If treated in addition with 50 nM RA, extra fin phenotype changes from ventral to anterior duplication in some embryos. (**D**) Real-time PCR for limb/fin initiation genes of wild-type zebrafish embryos. Embryos were treated with Bmp inhibitor or DMSO (control) at 6-7 hpf and their RNA collected at indicated hpf. *sp8* and *wnt2ba*, two of the early limb/fin initiation genes, are upregulated immediately after Bmp inhibitor treatment; however, this upregulation does not persist. Some of the Fgf genes show the same tendency but the upregulation is not statistically significant at any time point. Data are mean±s.d. **P*<0.5. Expression is relative to highest expression for every gene, which is arbitrarily set as one for each experiment and then accumulated.

and one late timepoint (7, 9, 12 and 24 hpf). As expression of limb field inducers is not restricted to pectoral fin buds and dorsalisation of the embryo might introduce secondary effects, we interpret our results cautiously. Nevertheless, it is worth mentioning that some of the known limb inducer genes are upregulated immediately after Bmp inhibition (Fig. 5D). *wnt2ba* in particular is interesting, as it is one of the earliest inducers and is thought to be regulated by RA indirectly (Mercader et al., 2006; Neto et al., 2012). It is upregulated throughout gastrulation until ~6-somite stage. *sp8* also showed an immediate upregulation at 7 hpf that was lost over the next 2 hours (Fig. 5D). Some of the Fgf genes displayed the same tendency of early upregulation; however, results were not statistically significant.

DISCUSSION

Conservation of limb induction

We have shown that downregulation of Bmp signalling, by either overexpression of the endogenous Bmp inhibitor *noggin* or with a small molecule inhibitor specific against Bmp type I receptors, is sufficient to induce extra limbs in *Xenopus* and supernumerary pectoral fins in zebrafish. Therefore, the induction mechanism for these appendages seems to be conserved, despite the very different timing of bud initiation and outgrowth in these two species. In *Xenopus*, limb bud initiation is delayed compared with other vertebrates. Although in zebrafish, mouse and chick appendage initiation takes place during embryogenesis, *Xenopus* limb initiation occurs several days after embryogenesis has finished (Galis et al., 2003; Satoh et al., 2005). This difference was reflected in the temporal difference of the induction window in these two species. For zebrafish, this time window was during gastrulation stages long before any fin outgrowth became apparent. In *Xenopus*, conversely, competence for limb field induction was delayed and concomitant with limb bud outgrowth (10-15 dpf, stage 48-49). Although competence lasted for several stages and days, supernumerary limbs only developed after Bmp downregulation in a narrow time period at stage 49.

There is evidence that Bmp signalling also plays a role during chick limb induction and outgrowth. Pizette and colleagues (Pizette et al., 2001) have shown that Noggin overexpression during chick limb development caused ectopic AERs to develop on the ventral limb bud analogous to the partial duplications we observed in *Xenopus*. These ectopic AERs, however, were temporary as duplicated elements were never observed later, possibly because continuous Noggin overexpression abolished their outgrowth in chick.

Although the role for Bmp signalling seems to be conserved between limb and pectoral fin induction, its function during outgrowth of these two appendages seems to differ. Continuous blocking of Bmp signalling inhibited endogenous limb outgrowth in *Xenopus*, but fin outgrowth in zebrafish did occur and fins often appeared bigger than in control embryos.

Limb field inducers

Ectopic appendages have been induced previously in chick by application of exogenous factors. Insertion of an Fgf8- or Fgf10-soaked bead into the flank of a chick embryo is sufficient to induce ectopic wings or limbs, depending on the anterior-posterior implantation level (Ohuchi et al., 1997; Isaac et al., 2000). However, only *fgf10* seems to regulate true limb induction, as only knockout of *Fgf10* (but not *Fgf8*) leads to a lack of limb initiation in mice (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999). Similarly, implantation of *wnt2ba* or activated β-catenin-infected cells into the flank region of the chick embryo leads to ectopic wings (Kawakami et al., 2001). Kawakami and colleagues have established that Wnt2b triggers the limb induction programme by inducing *fgf10* in the lateral plate mesoderm.

A further factor shown to be involved in limb field initiation is RA. Treatment of early mouse or zebrafish embryos with high doses of RA leads to induction of ectopic limbs or anterior duplications of the pectoral fins, respectively (Niederreither et al., 1996; Rutledge et al., 1994; Vandersea et al., 1998). In zebrafish, RA has been shown to act upstream of *wnt2b*, the expression of which is lost if RA signalling is blocked with DEAB, resulting in loss of pectoral fins (Mercader et al., 2006; Neto et al., 2012). Moreover, zebrafish and mouse *aldh1a2* mutants are unable to synthesise RA during early development and therefore fail to develop fins or limbs (Begemann et al., 2001; Gibert et al., 2006; Grandel et al., 2002; Niederreither et al., 1999).

As mentioned earlier, ectopic limbs have also been described in the mouse *Gdf8/Gdf11* double knockout mutant (McPherron et al., 2009). In homozygous mutants, finger-like structures are seen on the belly that resemble our zebrafish supernumerary fin phenotype. And at a lower frequency, complete limbs develop immediately ventral to the endogenous limb, resembling our *Xenopus* extra limb phenotype. The similarities between the mouse and *Xenopus*/zebrafish phenotype are striking, and make us believe that there is a connection between them. However, our attempts to induce extra appendages by blocking the Tgfβ pathway or by recreating the double *gdf8/gdf11* knockout in zebrafish were without success and we also failed to find any indication that *noggin* overexpression inhibited both arms of the Tgfβ family pathways. Although currently there are no reports that Gdf8 and Gdf11 signal through any pathway other than the Tgfβ arm via Alk4, Alk5 and Alk7 receptors, we would suggest that they also signal through the Bmp pathway. We were unable to verify this though, owing to our inability to breed double knockout *gdf8/gdf11* pups.

Mechanism of extra appendage induction by Bmp inhibition

Bmp signalling has been implicated in DV patterning and distal outgrowth of the vertebrate limb (Ahn et al., 2001; Ovchinnikov et al., 2006; Pizette et al., 2001). Bmp is involved in positioning the AER by inducing *en1* in the ventral limb ectoderm (Kimmel et al., 2000; Pizette et al., 2001), while stimulation of distal outgrowth by Bmp has been shown to be En1 independent and hence independent of DV patterning (Pizette et al., 2001). Consequently, lowering Bmp signalling in the embryo will induce changes in two axes. During gastrulation, various Bmps are expressed ventrally where they confer ventral identity while they are counteracted dorsally by the secretion of the endogenous Bmp antagonists Noggin and Chordin from the embryonic organiser and other Bmp inhibitory mechanisms (De Robertis, 2009; Furthauer et al., 2004; Neave et al., 1997; Shih and Fraser, 1996; Wagner and Mullins, 2002). It is postulated that low levels of Bmp activity induces genes in the lateral region, whereas higher levels are needed for activation of the ventral genes such as the *vent*, *vox* and *ved* families, which function as inhibitors of dorsal genes (Gilardelli et al., 2004). Downregulation of Bmp signalling by either *noggin* overexpression or with Bmp-specific inhibitors results in a ventral shift of the expression of Bmp target genes. We believe, therefore, that the limb/fin inducer genes, foremost among them *wnt2ba*, which are normally restricted to more dorsal and lateral axial tissues, to be among those genes that expand ventrally where they are then able

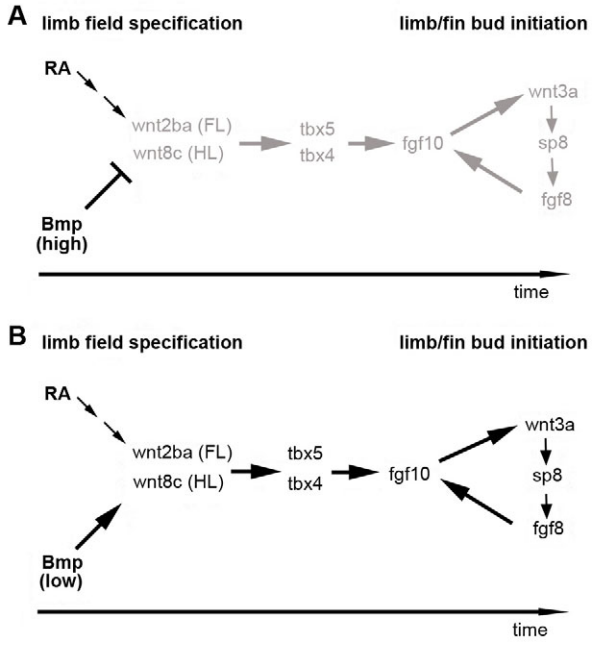

Fig. 6. Limb field initiation scheme. (**A**,**B**) RA signalling from the somites activates *wnt2ba* and *wnt8c* indirectly in the intermediate mesoderm of the forelimb or hindlimb, respectively, while Bmp signalling restricts their expression ventrally. This restriction is either achieved by inhibition of these genes by high levels of Bmp (A) or by activation of the same genes by low levels of Bmp (B). These two Wnt genes subsequently activate *fgf10* via *tbx5* or *tbx4*, respectively, in the lateral plate mesoderm in the limb field region. *fgf10* then signals to the overlaying ectoderm to induce the AER, the signalling centre for proximal distal limb outgrowth, by starting a feedback loop involving *wnt3a, sp8* and *fgf8* (Mercader et al., 2006). The timeline indicates that these are successive events. Grey indicates that induction cascade is not activated. FL, forelimb, HL, hindlimb.

to induce extra limb/fin fields. Whether Bmp regulates *wnt2ba* expression directly or indirectly, e.g. via the ventral *vox/vent* genes, awaits further investigation. The quick response observed (at 7 hpf) could argue for a direct involvement, whereas regulatory networks analyzed in cancer suggested an indirect involvement (Katoh and Katoh, 2009). Simultaneous with induction of a new limb/fin field, the DV axis of the embryo or tadpole, and consequently the expression boundary of *en1*, also shift ventrally. This shift could explain why the ectopic limb/fins are always positioned ventral to the endogenous ones.

Alterations in fin induction after overactivation or inhibition of the RA pathway in presence of Bmp inhibition indicated that Bmp acted upstream or in parallel to RA signalling during fin field induction. To differentiate between the two possibilities, we studied RA-modulating genes using real-time PCR. As none of the studied RA-modifying genes were differentially expressed after Bmp inhibitor treatment, we concluded that the two pathways act in parallel and cooperate for successful fin induction.

Putting these results together, we propose the incorporation of Bmp in the limb induction cascade. We suggest that Bmp and RA signalling pathways integrate at the level of *wnt2b/wnt8c* regulation, with RA conferring limb-forming ability by activating *wnt2b/wnt8c* indirectly in the intermediate mesoderm and Bmp restricting the expression of *wnt2b/wnt8c* to dorsal mesoderm, either by inhibiting expression at high levels (Fig. 6A) and/or by activating expression at lower levels (Fig. 6B). At the same time as inducing new limb/fin fields and starting the induction cascade for distal outgrowth, the DV boundary becomes shifted ventrally when Bmp signalling is lowered.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table S1. Forward and reverse primers used for real-time PCR

Gene	Forward	Reverse
Limb field inducers		
wnt2ba	GGTACGACACCACCCGTGTAA	ATCGAGCCAGTCGGGTTTCT
fgf8a	ACACGGCTCTGCAGAATGTG	CCCTTGGGCAACCTCTTCAT
fgf8 b	ATAGCCGAACCAGCGGTAAA	TTTTGCCCCTCTGATTCGAA
Fgf10	GCGAGGGATTTCGGCATT	GGCGCTCAGACCTACGAACA
fgf24	AAGGAGACACCCCGATTGAA	CATGAGGCTGTTTCGCGTTT
sp8	GCCACTCTGCCTTCAGTACCA	GACCCCCTGTCAACATGGAA
RA modifiers		
Aldh1a2	CCGGCACCGTCTGGATTA	TTGAATCCTCCGAAAGGACACT
Aldh1a3	GCCGTGCTCGCGACTT	GAACGCGTGCAGGAAAGGT
crabpI	GCCGACGACGTGGTTTG	CCGATTCGCCCTCATTCTC
crabpII	GACGTCCCTGCACGAGCTT	TCTGTTCGCAGCTAATCTTGCT
Cyp26	CTTGCCGTTCATTGGAGAAAC	ATGCGCAGAAACTTCCTTCTCT
Normaliser genes		
S18	TCGCTAGTTGGCATCGTTTATG	CGGAGGTTCGAAGACGATCA
SDHA	TGTCCTATGTGGATCCCGAAA	AGGGCGGAGTCAGCTTAGGT
HPRT1	GAAGGCTCTTCTGGATCACGTT	AAGGCATAGCCGACCACAAA