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Crossveinless 2 is an essential positive feedback regulator of Bmp signaling during zebrafish gastrulation

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Signaling by bone morphogenetic proteins (Bmps) plays a pivotal role in developmental and pathological processes, and is regulated by a complex interplay with secreted Bmp binding factors, including Crossveinless 2 (Cvl2). Although structurally related to the Bmp antagonist Chordin, Crossveinless 2 has been described to be both a Bmp agonist and antagonist. Here, we present the first loss-of-function study of a vertebrate *cvl2* homologue, showing that zebrafish *cvl2* is required in a positive feedback loop to promote Bmp signaling during embryonic dorsoventral patterning. In vivo, Cvl2 protein undergoes proteolytic cleavage and this cleavage converts Cvl2 from an anti- to a pro-Bmp factor. Embryonic epistasis analyses and protein interaction assays indicate that the pro-Bmp function of Cvl2 is partly accomplished by competing with Chordin for binding to Bmps. Studies in cell culture and embryos further suggest that the anti-Bmp effect of uncleaved Cvl2 is due to its association with the extracellular matrix, which is not found for cleaved Cvl2. Our data identify Cvl2 as an essential pro-Bmp factor during zebrafish embryogenesis, emphasizing the functional diversity of Bmp binding CR-domain proteins. Differential proteolytic processing as a mode of regulation might account for anti-Bmp effects in other contexts.

Key words: Zebrafish, Crossveinless 2, Bmp

INTRODUCTION

Signaling by secreted Bone morphogenetic proteins (Bmps) regulates numerous processes of embryogenesis and organogenesis during vertebrate and invertebrate development (Hogan, 1996). Bmps are members of the transforming growth factor β (Tgfβ) superfamily of proteins, which signal through transmembrane receptor complexes consisting of type I and type II serine/threonine kinases. Activation of the receptors leads to phosphorylation of type I by type II receptors, followed by phosphorylation of intracellular R-Smad proteins by type I receptor kinases. Upon their phosphorylation, R-Smads dissociate from the receptors, form complexes with Co-Smads, like Smad4, and translocate to the nucleus to participate in the transcriptional regulation of target genes (Baker and Harland, 1997; Heldin et al., 1997).

Given the high sensitivity of developing cells to Bmp signals, the activity of Bmps is under tight spatial and temporal regulation. One important level of regulation occurs in the extracellular space, mediated by various secreted Bmp-binding proteins (Balemans and Van Hul, 2002). Some of these have rather complex functions. Chordin for example binds to Bmps and prevents the interaction of Bmps with their receptors, accounting for Bmp inhibition (Piccolo et al., 1996). In addition, Chordin can have subtle long-range pro-Bmp effects, as demonstrated for the *Drosophila* Chordin and Bmp homologues Sog and Dpp: Sog/Dpp complexes seem to diffuse within the extracellular space, with Sog carrying Dpp away from the Sog source (Ashe and Levine, 1999; Decotto and Ferguson, 2001). By cleaving Bmp/Dpp-bound Chordin/Sog, Tolloid metalloproteases can then release biologically active Bmps from the complex (Piccolo et al., 1997), resulting in increased Bmp/Dpp levels at the sites of Tolloid activity (Ashe and Levine, 1999; Blader et al., 1997; Decotto and Ferguson, 2001). Another factor with a similar dual effect on Bmp signaling is Twisted gastrulation, which promotes binding of Chordin to Bmps (anti-Bmp effect), but also the cleavage of Chordin by Tolloid (pro-Bmp effect) (Chang et al., 2001; Larrain et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001; Shimmi and O'Connor, 2003). These examples show that Bmp-binding proteins cannot be generally categorized as anti- or pro-Bmp factors, but rather can have differential functions depending on the molecular context.

In the early zebrafish and *Xenopus* embryo, ventrally expressed Bmps and dorsally expressed Bmp inhibitors establish a ventral-to-dorsal gradient of Bmp activity, which is required for patterning of the dorsoventral axis (De Robertis et al., 2000; Hammerschmidt and Mullins, 2002; De Robertis and Kuroda, 2004; Schier and Talbot, 2005). Accordingly, the zebrafish mutants *bmp2b/swirl*, *bmp7/snailhouse*, *alk8/lost-a-fin* (a type I Bmp receptor), *smad5/somitabun* and *tolloid/minifin* display dorsalized phenotypes (Bauer et al., 2001; Connors et al., 1999; Dick et al., 2000; Hild et al., 1999; Kishimoto et al., 1997; Mintzer et al., 2001; Nguyen et al., 1998; Schmid et al., 2000). By contrast, the *chordin/dino* mutant is ventralized (Fisher et al., 1997; Schulte-Merker et al., 1997), with additional subtle dorsalized traits in ventral-most derivatives, reflecting the dual anti- and pro-Bmp function of Chordin described above (Hammerschmidt and Mullins, 2002; Wagner and Mullins, 2002). The effects of loss of Tsg function on dorsoventral patterning of the zebrafish embryo are less clear. Although according to one report, Tsg is a Bmp antagonist (Ross et al., 2001), other studies come to the opposite conclusion, indicating a pro-Bmp effect of Tsg (Little and Mullins, 2004; Xie and Fisher, 2005).

The Bmp binding of Chordin is mediated by CR repeats that are characterized by ten cysteine residues with a conserved spacing pattern (Larrain et al., 2000). CR domains are also present in Crossveinless 2 (Cvl2 or Cv-2). Although in vertebrates, the role of Cvl2 had not been analyzed via loss-of-function studies as yet, analyses of *Drosophila* mutants indicate that it is required to enhance

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Bmp signaling during wing vein development (Conley et al., 2000). *cvl2* gain-of-function experiments in vertebrate cell culture systems and *Xenopus* embryos led to conflicting results, showing either pro- or anti-Bmp effects (Binnerts et al., 2004; Kamimura et al., 2004; Moser et al., 2003; Coles et al., 2004).

Here, we describe the first loss-of-function study for a vertebrate *crossveinless 2* homologue. We show that zebrafish *cvl2* acts as a Bmp signaling-promoting factor during early embryonic development and that this activity has Chordin-dependent and -independent aspects. We also show that proteolytic cleavage can convert Cvl2 from an anti- to a pro-Bmp factor and modifies its interaction with the extracellular matrix. These findings might help to explain the controversies about the role of Cvl2 reported in previous gain-of-function studies.

MATERIALS AND METHODS

Fish strains

Mutant strains used were: *swirl*^{ta72}; *minifin*^{tc263}; *lost-a-fin*^{tm110} (Mullins et al., 1996) *dino*^{tt250}; *ogon*^{tm305} (Hammerschmidt et al., 1996a) and *ogon*^{m60} (Solnica-Krezel et al., 1996). The generation of *hsp70::bmp2b* and *hsp70::noggin3* transgenic fish lines will be described elsewhere (F.R. and M.H., unpublished).

Isolation of zebrafish *crossveinless2*, RT-PCR and generation of expression constructs

Conditions and PCR primers used for cloning of zebrafish *cvl2*, for the generation of expression constructs, for temporal RT-PCR analyses and for genomic mapping of *cvl2* are available from the authors upon request. *cvl2-N* contains amino acids 1-355, in *cvl2-CM* residues 350-355 (VFGDPH) are replaced by LVPRGS.

Morpholino oligonucleotides

The sequences of the used *cvl2* antisense morpholino oligonucleotides are: TTA CTG GAG GAG ACA GAC ACA GCA T (ATG-MO=MO1) corresponding to nucleotides +1 to +25 of the cDNA; CTA AAT TCG CTC CAG ACG CAC GGG (UTR-MO=MO2) corresponding to nucleotides –25 to –2 in the 5′UTR. Sequences of the respective mismatch control MOs were: ACa GGA cGA GAC AGA CAg AGC tTC C (–2 to +23); CTA AAT TCc CTg CAc ACG gAC cGG. Unless stated otherwise, 1 nl containing 2.4 ng MO was injected per embryo.

In situ hybridization and immunohistochemistry

In situ hybridization and whole-mount immunostaining was performed as described previously (Hammerschmidt et al., 1996a). Staining for phosphorylated Smads was done using an anti-pSmad1/5/8 antibody (Cell Signaling Technology) at a concentration of 1:200 and a Cy3-coupled secondary antibody (Jackson ImmunoResearch Labs). Graphical illustrations of staining intensities were generated from Photoshop images of vegetal views, using ImageJ software (http://rsb.info.nih.gov/ij/).

For Western blot analyses, dechorionated midgastrula embryos were dissociated in Ca-free Ringer's solution; cells were separated from yolk via centrifugation, and protein extracts were prepared by standard procedures (Westerfield, 1994). Extracts were separated on 8% polyacrylamide gels. Myc-tagged fusion proteins were detected with anti c-myc antibody 9E10 (Santa Cruz Biotechnology), anti-pSmad1/5/8 antibody (Cell Signaling Technology) was used at a concentration of 1:1000.

Biacore analyses

A detailed description of the expression and purification of recombinant proteins, microsequencing of the Cvl2 fragments and interaction assays is available from the authors upon request. Recombinant Chd protein was obtained from R&D systems (Minneapolis, MN).

Cell culture and heparin binding experiments

Plasmids pCS2-*cvl2-CM*, pCS2-*cvl2-WT* and pCS2-*cvl2-N* were transfected into HEK293 cells with the calciumphosphat method, efficiency was controlled by co-transfection of pCS2-*gfp*. Transfected cells at ~60% confluence were incubated with serum-free medium for 48 hours. Separation of supernatant, cells and ECM was carried out as described previously (Novikova et al., 2000). Briefly, the supernatant was collected and the cells were washed off with cold PBS. The ECM attached to the culture dish was washed off with 95°C sample buffer (50 mM Tris, pH 6.8, 1% SDS, 4% glycerol).

Heparin binding assays were carried out as described by Jasuja et al. (Jasuja et al., 2004), using proteins from cell extracts obtained in the transfection experiments.

RESULTS

Zebrafish *cvl2* is co-expressed with Bmps

We isolated the complete coding region of a zebrafish homologue of *crossveinless2*, consisting of a signal peptide, five chordin-like cysteine rich domains (CR-domains, also called von Willebrand Factor type C domains) and a partial von Willebrand Factor type D (vWFd) domain (Fig. 1A; GenBank Accession Number AY847871; see Fig. S1 in the supplementary material for alignments). RT-PCR analysis at different stages of embryonic development failed to detect maternally supplied *cvl2* transcripts. *cvl2* expression commenced at late blastula stages, ~1 hour before the onset of gastrulation, and continued during all later developmental stages investigated (Fig. 1B).

Whole-mount in situ hybridization revealed that during blastula stages, *cvl2* was broadly expressed at rather low levels (not shown). During gastrula stages (shield, 60% epiboly, 80% epiboly), *cvl2* transcripts were predominantly found on the ventral side of the embryo, with from ventral to dorsal decreasing mRNA levels (Fig. 1C,D,I). Additionally, transcripts became detectable around nuclei of the yolk syncytial layer (YSL, Fig. 1E,K) and in the prechordal plate (arrow in Fig. 1D). At the tailbud stage (end of gastrulation), *cvl2* was most prominently expressed in the lateral mesoderm along the entire anteroposterior length of the embryo (Fig. 1E,F). During segmentation stages, this domain became restricted to the anterior half of the embryo (Fig. 1G,H) and to the tailbud mesoderm (see Fig. S2A in the supplementary material). Interestingly, all these expression domains correspond to areas of *bmp* expression: *bmp2b*, *bmp4* and *bmp7* are expressed ventrally, *bmp2b* in the YSL, and *bmp4* in the prechordal plate (Dick et al., 2000; Nikaido et al., 1997; Schmid et al., 2000).

cvl2 expression depends on Bmp signaling

Analysis of *cvl2* expression in mutants with altered Bmp activity revealed a positive regulation of *cvl2* by Bmp signaling. In *chordin/dino* mutants, ventral expression of *cvl2* was expanded to the dorsal side (Fig. 1I,J). By contrast, in *bmp2b/swirl* mutant embryos, ventral expression was absent (Fig. 1K). These data suggest that *cvl2* expression is under the positive control of Bmp signaling. The same appears to be true for later stages of zebrafish development, when *cvl2* was expressed in a variety of different tissues, including the otic vesicles and the pharyngeal pouches (Fig. 1L and Fig. S2 in the supplementary material). Using transgenic zebrafish lines expressing *bmp2b* or the Bmp inhibitor *noggin3* under the control of a heatshock-inducible promoter (Halloran et al., 2000) (F.R. and M.H., unpublished), we obtained complete loss of this late *cvl2* expression upon blockage of Bmp signaling (Fig. 1N). However, *bmp2b* overexpression strongly increased *cvl2* transcript levels without inducing *cvl2* expression at ectopic sites (Fig. 1M).

Cvl2 is required to promote Bmp signaling during dorsoventral patterning of the zebrafish embryo

To study whether Cvl2 is required for early zebrafish development, we carried out loss-of-function experiments, using antisense morpholino oligonucleotides (MO) (Nasevicius and Ekker, 2000).

Used MOs efficiently inhibited translation of *cvl2* transcripts, as revealed via western blotting of protein extracts from embryos co-injected with cvl2 MOs and mRNA encoding a Cvl2-Myc fusion protein (Fig. 2A). Phenotypically, such MO-mediated loss of Cvl2 function led to reduced Bmp signaling and moderate dorsalization of the embryo.

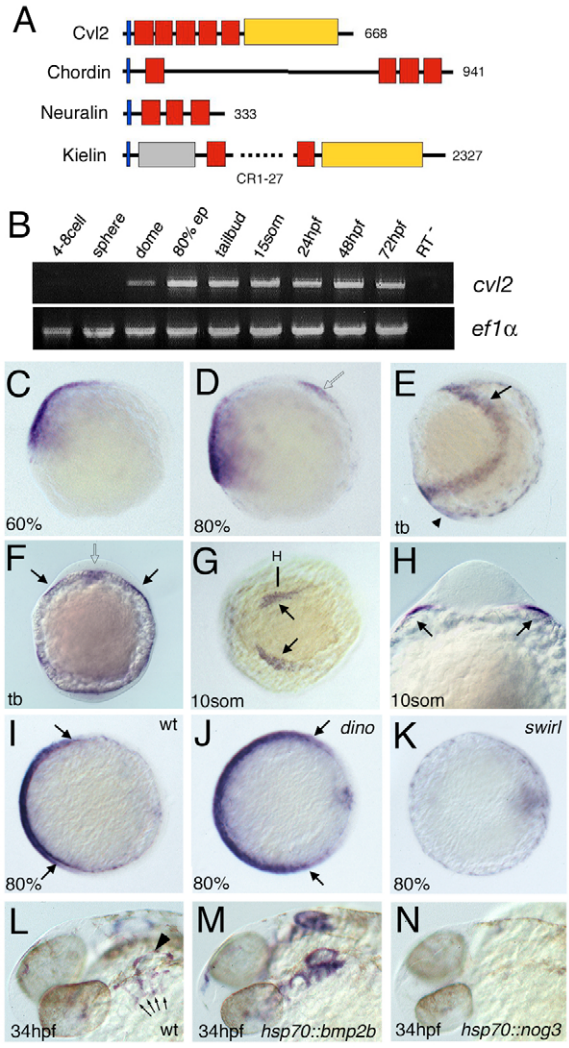

Fig. 1. *cvl2* is co-expressed with *bmps* and positively regulated by Bmp signaling. (**A**) Domain composition of Cvl2 compared with the CR domain proteins Chordin (Sasai et al., 1994), Neuralin (Coffinier et al., 2001) and Kielin (Matsui et al., 2000). Blue boxes indicate signal peptide; red boxes indicate CR domains; yellow boxes indicate vWFd domain; gray box indicates the Thrombospondin domain; numbers one the right indicate total number of amino acid residues. (**B**) Temporal expression profile of *cvl2* determined by RT-PCR. *cvl2* is only zygotically expressed. (**C-K**) Spatial expression pattern determined by in situ hybridization; developmental stage is indicated in lower left-hand corner; genotype of embryos is indicated on the right-hand side. (C-E) Lateral views, ventral leftwards. (F,H) Frontal views, focal plane of H is indicated in G. (G) Animal pole view on head, ventral leftwards. Filled arrows in E-H indicate anterior lateral mesoderm; open arrows in D,F indicate prechordal plate; arrowhead in E indicates ventral tailbud mesoderm. (I-K) Vegetal views, ventral leftwards, with dorsal borders of the expression domain indicated by arrows. (I,J) *cvl2* expression is expanded dorsally in *chordin* mutants. (I,K) Ventral expression is absent in *bmp2b* mutants. (**L-N**) Late *cvl2* expression at 34 hpf in otic vesicle (arrowhead in L) and pharyngeal pouches (arrows in L) is elevated in *hsp70::bmp2b* transgenic (M), but downregulated in *hsp70::noggin3* transgenic (N) embryos after a 30 minutes heatshock (28°C to 39°C) at 26 hpf.

To detect and measure Bmp signaling activity, we examined the phosphorylation state of Smad1/5 proteins, as a direct readout of Bmp type I receptor kinase activity. Western blotting (Fig. 2B) and whole-mount immunostaining (Fig. 2C,D) at midgastrula stages revealed a significant reduction in the levels of phosphorylated Smad1/5 of *cvl2* morphant embryos compared with embryos injected with mismatch control MO. Graphical illustrations of pSmad1/5 signal intensities along the dorsoventral axis of the midgastrula embryo showed that in *cvl2* morphants Bmp signaling was reduced over the entire ventrolateral domain (Fig. 2E,F). Consistently, *cvl2* morphants displayed reduced expression of ventral marker genes such as *eve1* (ventral mesoderm) (Joly et al., 1993) (Fig. 2G,H), *cvl2* itself (Fig. 2I,J), *gata2* (ventral ectoderm) (Detrich et al., 1995) (Fig. 2K,L) and *tfap2a* (ventral ectoderm) (Knight et al., 2003) (see Fig. S3E,F in the supplementary material) at midgastrula stages, and a reduced number of ventral cell types such as blood precursors (marked by expression of *gata1*) (Detrich et al., 1995) (Fig. 2O,P) at early segmentation stages. By contrast, expression of *tbx6*, a marker for the entire non-axial mesoderm largely under the control of Wnt8 (Szeto and Kimelman, 2004; Ramel et al., 2005), was only moderately affected in midgastrula *cvl2* morphants (Fig. S3C,D), while the expression domains of dorsal markers like *otx2* (Li et al., 1994) (Fig. 2M,N), which mark anterior neuroectoderm, were ventrally expanded. Accordingly, at segmentation stages, *cvl2* morphants displayed an expansion of dorsal and paraxial cell types, as apparent by a broadening of hindbrain rhombomeres 3 and 5, revealed by *krox20* expression (Fig. 2Q,R), and by a broadening of the anterior somites, revealed by *myoD* expression (see Fig. S3G,H in the supplementary material). These data indicate that Cvl2 is required for ventral specification both in the mesoderm and the ectoderm. Similar results were obtained with two different *cvl2* MOs, but not with the corresponding mismatch control MOs. The effect of used MOs was specific, as shown by the rescue of the morphant phenotype to near wild-type condition upon co-injection of low doses of mRNA encoding zebrafish Cvl2 (for the UTR-MO) or *Drosophila* Cvl2 (for both the UTR-MO and the ATG-MO; Fig. 2S,T; and data not shown). The rescue with *Drosophila cvl2* mRNA further indicates that the function of Cvl2 proteins has been conserved across invertebrate and vertebrate species.

Partial loss of Cvl2 synergistically enhances dorsalization of Bmp pathway mutants

To further analyze the functional interaction of *cvl2* with the Bmp pathway, we injected suboptimal amounts of *cvl2* MO into mutants with weakly reduced Bmp activity, such as *tolloid/minifin*. *minifin* embryos, as well as embryos injected with low doses of *cvl2* MO (0.8 ng per embryo), display a very subtle expansion of the neuroectoderm, as shown by *krox20* staining (Fig. 2U-W). Injection of the same low doses of *cvl2* MO into *minifin* embryos, however, resulted in strongly enhanced dorsalization (Fig. 2X). The same enhancement was obtained upon co-injection of low doses of *cvl2* MO with low doses of *chordin*, *noggin* or *twisted gastrulation* RNA, or upon injection into weakly dorsalized *alk8/lost-a-fin*^{tm110} mutants (confirmed by genotyping; data not shown). Together, these results strengthen the notion that during early dorsoventral patterning, Cvl2 has an essential ventralizing function and functionally interacts with Bmp signaling.

Loss of Cvl2 function has differential and moderate effects in chordin mutants

We next analyzed the functional interaction of *cvl2* with Chordin, performing epistasis analyses by injecting *cvl2* MOs into homozygous *chordin* mutants to generate double-deficient embryos. *chordin/dino* mutants display elevated Bmp signaling and are ventralized, as morphologically indicated by a reduction of the brain, a derivative of the dorsal ectoderm, and an enlargement of the blood islands, a derivative of the ventral mesoderm (Hammerschmidt et al., 1996a; Hammerschmidt et al., 1996b). The phenotype in the ventral tail fin, a derivative of the ventral ectoderm, is more complex. Although other ventralized mutants such as *sizzled/ogon* (Martyn and Schulte-Merker, 2003; Wagner and Mullins, 2002; Yabe et al., 2003) display a duplication of the ventral tail fin over its entire length (see Fig. S3J in the supplementary material), a variable percentage of *chordin* mutants show only duplications in posterior-most regions of the ventral tail fin, whereas more anteriorly, ventral tail fin tissue is missing (Fig. 3A-D). This feature reflects the additional subtle pro-Bmp activity of Chordin in regions of the ventral ectoderm that require peak Bmp levels (see Introduction).

To quantify the effects of *cvl2* MOs in the ventral tail fin, we classified *chordin* mutants according to the length of the fin gap (class I: no gap, Fig. 3B; class II: partial gap, Fig. 3C; class III: complete gap, Fig. 3D), reflecting the degree of reduction of Bmp activity in this tissue. Injection of low doses of *cvl2* MO (0.8 ng/embryo), which had no effect in wild-type siblings, significantly enhanced the loss of ventral tail fin tissue of *chordin* mutants (see numbers in Fig. 3B-D). However, ventralized traits at other sites of *chordin* mutants appeared less sensitive to Cvl2 levels. Thus, the blood islands of mutants even injected with highest doses of *cvl2* MOs (3 ng/embryo) remained their increased size, as also confirmed by *gata1* staining at the 10-somite and 24 hpf stage (data not shown). Similarly, injection of highest doses of *cvl2* MOs into *dino* mutants or *chordin* morphants only led to a very subtle alleviation of the reduced *krox20* expression (Fig. 3E). The unresponsiveness of these phenotypic traits of *chordin* mutants to loss of Cvl2 indicates that here, Chordin is epistatic to Cvl2, suggesting that Cvl2 fulfills part of its pro-Bmp function via an inhibitory effect on Chordin. However, the enlarged gap in the ventral tail fin of *chordin* mutants injected with *cvl2* MO suggests that Cvl2 in addition promotes Bmp signaling independently of a putative Chordin-antagonizing function.

In contrast to *chordin* mutants, *sizzled/ogon* mutants displayed a more widespread response to *cvl2* MOs. Thus, injected *ogon* mutants not only showed a rescue of the ventral tail fin duplication (Fig. S3I-K), but also a significant lateral expansion of the *krox20* expression domains (Fig. 3F), suggesting that Cvl2 acts largely independent of Sizzled.

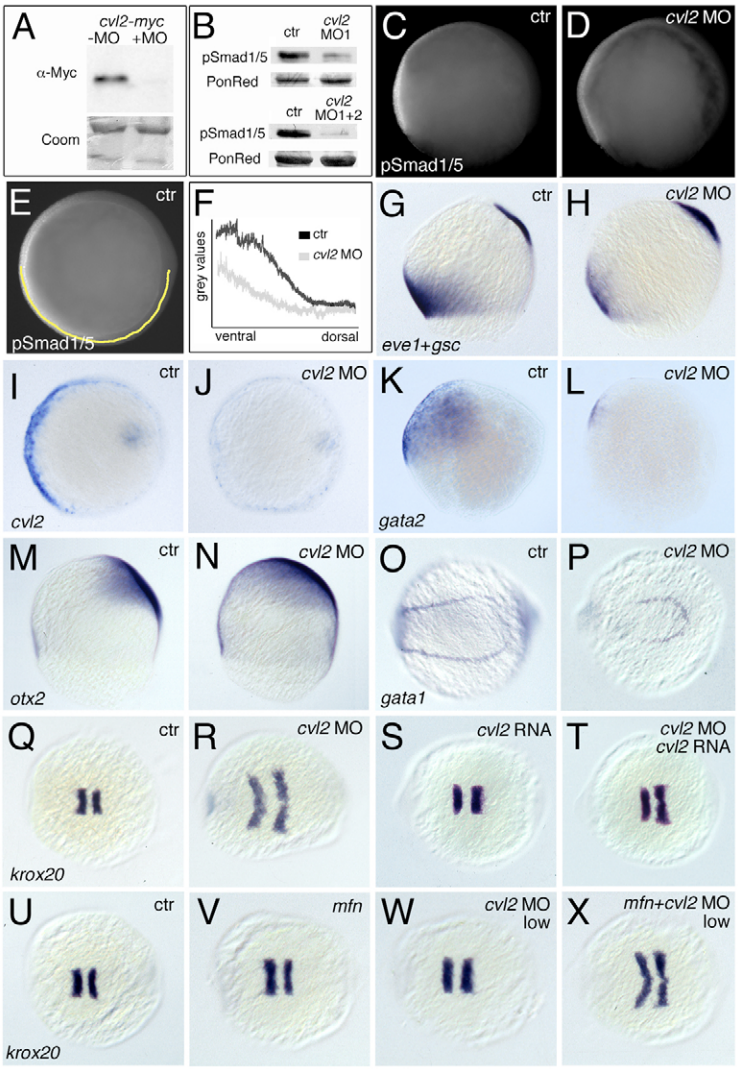

Fig. 2. *cvl2* is required for ventral specification and Bmp signaling. (**A**) Western blot; translation of myc-tagged *cvl2* mRNA is suppressed by *cvl2* ATG-MO. (**B**) Western blots, revealing strongly reduced pSmad1/5 levels in embryos of 80% epiboly stage after injection of *cvl2* MO1 (upper panel) or co-injection of *cvl2* MO1+2 (lower panel). (**C**,**D**) Whole-mount immunostaining, lateral view at 80% epiboly; *cvl2* MO1 leads to a reduction of ventral α-pSmad1/5 immunoreactivity. (**E**,**F**) p-Smad1/5 intensity along dorsoventral axis of *cvl2* morphant (gray in F) and control sibling (black in F) at 80% epiboly stage. Signal intensities were measured from vegetal views, along a yellow line indicated in E for one of the investigated controls (n=7). (G-X) Whole-mount in situ hybridization with probes indicated in lower left corners. (**G-N**) Eighty percent epiboly stage. (G,H,K-N) Lateral views; (I,J) vegetal views, dorsal rightwards. Ratios of affected embryos were: *eve1*, 33/39; *cvl2*, 66/71; *gata2*, 31/32; *otx2*, 45/53. (J) Only the ventral expression of *cvl2* is reduced in the morphant, while expression in the prechordal plate is normal. (**O**,**P**) Ten-somite stage, view of tailbud, anterior leftwards; expression domain of the blood precursor marker *gata1* is reduced in *cvl2* morphant (29/37 embryos). (**Q-X**) Five-somite stage, anterior leftwards; the *krox20* expression domains are ventrally expanded in *cvl2* morphant (R, 53/68 embryos), but rescued to near wild-type condition in embryo co-injected with *cvl2* MO and *Drosophila cvl2* mRNA (T, 46/47 embryos). (U-X) Low doses of *cvl2* MO1 enhance the mild dorsalization of *tolloid/minifin* mutants (40/43 in cross of two *mfn* homozygotes, 22/97 in cross of two *mfn* heterozygotes), while having no or a much weaker effect in wild-type embryos injected in parallel (0/33). Control embryos are injected with a mismatch *cvl2* morpholino. Abbreviations: Coom, Coomassie Blue staining of gel loaded with same amounts of protein; PonRed, Ponceau Red staining of blots after immunoreaction.

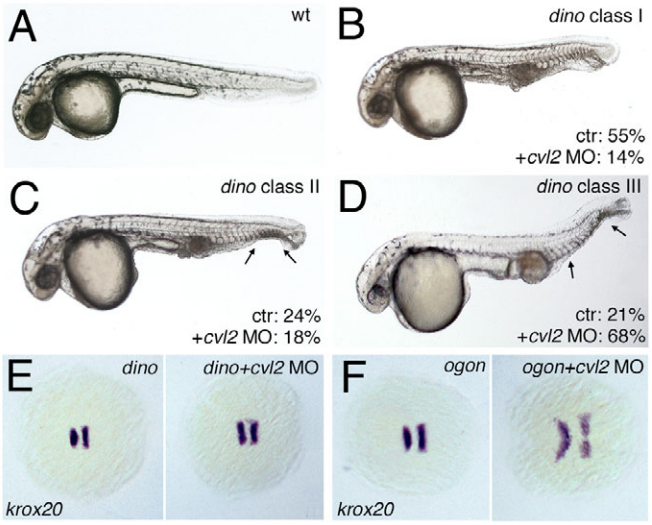

Fig. 3. Loss of *cvl2* only weakly affects *chordin/dino* mutants. (**A-D**) Lateral views of 32 hpf embryos; different classes of *dino* mutants displaying an increasing loss of ventral tail fin tissue (arrows in C,D). Percentages indicate the fraction of embryos displaying the shown tail fin phenotype among uninjected mutants (n=132) and mutants from the same crosses injected with *cvl2* MO (n=215). Injection of *cvl2* MO2 increases the percentage of *dino* mutants with class III phenotype, reflecting a stronger dorsalization within the ventral tissue fin tissue. (**E**,**F**) *krox20* staining, five-somite stage, animal view, anterior leftwards. *cvl2* MO1 clearly expands the neuroectoderm in *ogon*^{tm305} mutants (F, 41/42 embryos), but only has a weak effect in *chordin* morphants (E, 55/55 embryos), indicating that ventralized traits of chordin can neither be rescued nor enhanced upon loss of Cvl2 function.

Overexpression of wild-type cvl2 can moderately dorsalize or ventralize

Based on the reduction of Bmp activity in the loss-of-function assays, gain of function would be expected to yield increased Bmp activity and ventralization. However, upon overexpression of *cvl2* mRNA we observed a small fraction of embryos with signs of mild ventralization, while a larger fraction displayed weak to moderate dorsalization, both by morphological (Fig. 4D; Table 1) and marker expression criteria (Fig. 4H,L). The fraction of dorsalized embryos was even higher, when more RNA was injected (Table 1). To better understand this unexpected and seemingly inconsistent result, we investigated the protein products deriving from the injected *cvl2* mRNA.

Cvl2 is proteolytically cleaved between the N-terminal CR repeats and the C-terminal vWFd domain

Injection of mRNA encoding C-terminally myc-tagged Cvl2 into zebrafish embryos, and anti-Myc western blotting of protein extracts from injected embryos at late gastrula stages, revealed a C-terminal fragment (~45 kDa including the Myc-tag) in addition to full-length Cvl2 protein (~90 kDa including the Myc-tag) (Fig. 4A, left lane). The smaller fragment was obtained only under reducing electrophoresis conditions, whereas under non-reducing conditions, only one band corresponding to full-length protein was visible (Fig. 4A, right lane). The size of full length Cvl2 is slightly larger than the calculated mass (86 kDa including the Myc-tag), presumably owing to glycosylation of the N-terminal part (see Kamimura et al., 2004). Together, these data indicate that a large region of zebrafish Cvl2 is cleaved into two fragments that remain associated via disulfide bonds, similar to the situation recently reported for human Cvl2 (Binnerts et al., 2004).

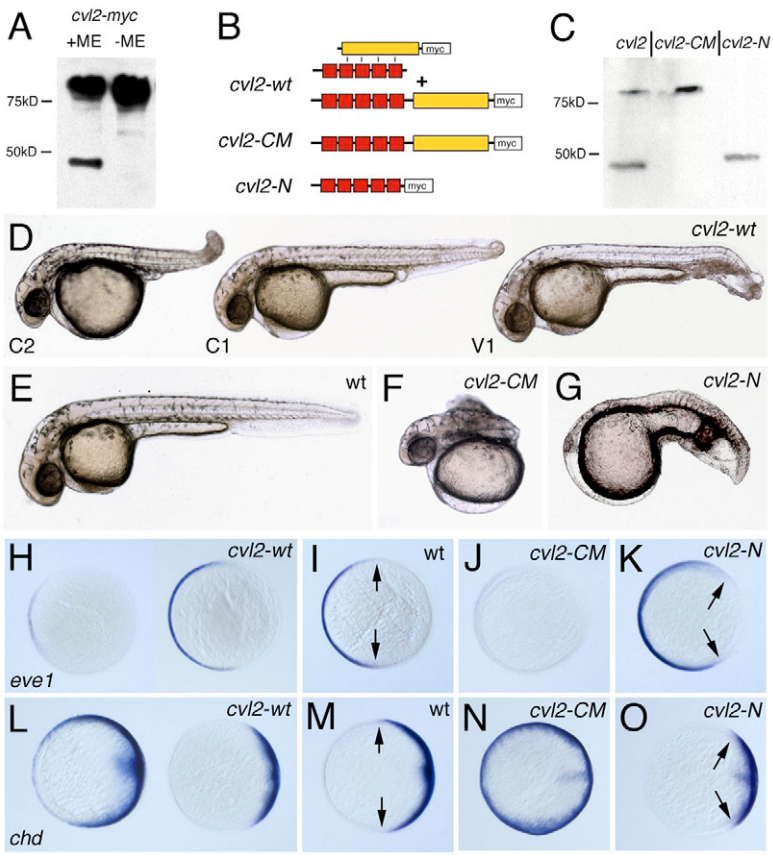

Fig. 4. Proteolytic cleavage converts Cvl2 from an anti- to a pro-Bmp factor. (**A**) Western blot with anti-Myc antibody after SDS-PAGE with embryonic protein extracts of *cvl2*-myc mRNA-injected embryos. Under reducing conditions [+mercaptoethanol (ME)], full-length Cvl2 and a C-terminal cleavage fragment are detectable. Under non-reducing conditions (without ME), the cleavage products remain associated, and only the high molecular weight band is detectable. (**B**) Illustration of Cvl2 proteins encoded by the different mRNAs. The protein encoded by *cvl2-WT* is either cleaved, with the two fragments remaining associated via disulfide bounds of thus far unidentified residues (top), or uncleaved, resembling Cvl2-CM (lower panel). (**C**) Western Blot showing that Cvl2-CM is not cleaved in embryos, and that Cvl2-N is readily synthesized. (**D-G**) Lateral views of 32 hpf embryos; (**H-O**) animal views, dorsal rightwards, after in situ hybridization at 80% epiboly for the ventral marker *eve1* (H-K) or the dorsal marker *chordin* (*chd*; L-O) (Schulte-Merker et al., 1997). Injection of *cvl2-wt* mRNA leads to both weakly ventralized (V1 in D and right embryos in H,L) and weakly (C1 in D) or moderately dorsalized embryos (C2 in D and left embryos in H,L). Injection of *cvl2-CM* mRNA causes strong dorsalization (F,J,N), while *cvl2-N* mRNA causes ventralization (G,K,O). Arrows in I,K,M,O indicate borders of the *eve1* or *chd* expression domains. Ratios of obtained phenotypes were: (D-G) see Table 1; (H) dorsalization, 17/44; wild type, 22/44; mild ventralization, 5/44; (J) 52/55; (K) 21/43; (L) dorsalization, 21/56; wild type, 29/56; mild ventralization 6/56; (N) 45/48; (O) 21/36.

Table 1. Effects of indicated mRNAs or MOs after (co)injection of indicated amounts into fertilized wild-type zebrafish eggs

Injected RNA or MO	pg per embryo	n	V4 (%)	V3 (%)	V1-2 (%)	WT (%)	C1-2 (%)	C3-4 (%)	C5 (%)
cvl2-WT RNA	30	132	0	0	6.8	76.5	16.7	0	0
cvl2-WT RNA	100	81	0	0	2.5	45.7	43.2	8.6	0
cvl2-CM RNA	30	293	0	0	0	25.3	16.4	51.5	6.8
cvl2-N RNA	150	165	1.8	6.6	38.9	52.7	0	0	0
cvl2-CM RNA + *cvl2-N* RNA	30 + 150	180	0	0	5.4	74.3	20.3	0	0
cvl2-CM Δ393-396 RNA	30	213	0	0	0	63.4	17.8	17.4	1.4
bmp2b MO	800	166	0	0	0	0	1.2	12	86.8
bmp2b MO + *cvl2-N* RNA	800 + 150	170	0	0	0	0	2.4	11.8	85.8
chd RNA	20	97	0	0	0	6.2	22.7	43.3	27.8
chd RNA + *cvl2-N* RNA	20 + 150	158	0	0	3.8	62.0	20.3	8.8	5.1
chd MO	2000	85	0	0	100	0	0	0	0
chd MO + *cvl2-N* RNA	2000 + 150	115	0	98.3	1.7	0	0	0	0

Phenotypic classification of dorsalization (C) and ventralization (V) strength was determined using morphological criteria at 32 hpf, according to Mullins et al. (1996) and Kishimoto et al. (1997).

The same Cvl2 protein pattern was obtained when zebrafish *cvl2* was expressed in SF9 cells, using the baculovirus transfection system (Fig. 6A). After purification of Cvl2 protein from the supernatants of transfected SF9 cells (Fig. 6A), microsequencing the ends of the Cvl2 fragments identified the Cvl2 cleavage site as $VFGD_{353\text{-}354}PHYN$, located between the fifth CR repeat and the vWFd domain of the full-length protein (see Fig. S1 in the supplementary material).

Proteolytic cleavage converts Cvl2 from a dorsalizing to a ventralizing factor

In order to analyze the impact of this proteolytic cleavage on the biological activity and the molecular properties of Cvl2, we generated two mutant constructs of Cvl2: a full-length construct in which the cleavage site is destroyed (Cvl2-CM) and a construct representing only the N-terminal half of Cvl2 containing the five CR domains (Cvl2-N, amino acids 1-355) (Fig. 4B). These constructs, when injected into zebrafish embryos or transfected into SF9 cells, led to protein bands of the expected sizes (Fig. 4C, Fig. 6A). In contrast to wild-type Cvl2-WT, which can both ventralize and dorsalize, these two opposite effects were completely separated in the two mutant versions of Cvl2. Although the mRNA encoding cleavage-resistant Cvl2-CM caused strong dorsalization (Fig. 4F,J,N and Table 1), injection of *cvl2-N* mRNA led to ventralized phenotypes only (Fig. 4G,K,O; Table 1). Co-injection of *cvl2-CM* and *cvl2-N* mRNA caused intermediate phenotypes, similar to the effect of *cvl2-wt* (Table 1). Together, these results indicate that uncleaved Cvl2 acts as an inhibitor of Bmp signaling, which upon proteolytic processing can be converted to a Bmp signaling-promoting factor, the apparent in vivo role of Cvl2 according to the loss-of-function studies described above.

The ventralizing effect of Cvl2-N strictly depends on Bmps and occurs in competition with the dorsalizing effect of Chordin

Our observation that *cvl2* morphants display reduced levels of phosphorylated Smad1/5 proteins suggests that Cvl2 acts by modulating Bmp signaling. To study whether this is also true under gain-of-function conditions, we injected *cvl2-N* mRNA into *bmp2b* morphant embryos. Indeed, while sibling embryos injected with *cvl2-N* mRNA alone were ventralized, embryos co-injected with *cvl2-N* mRNA and *bmp2b* MO were as strongly dorsalized as control embryos injected with *bmp2b* MO only (Fig. 5A-D; Table 1). This indicates that Cvl2 requires Bmps to fulfill its ventralizing activity, suggesting that it is a Bmp signaling-promoting factor that acts at the level or upstream of Bmp proteins. By contrast, embryos co-injected with *chordin* and *cvl2-N* mRNAs displayed a strong reduction of dorsalization compared with sibling embryos injected with *chordin* mRNA alone (Fig. 5E,F; Table 1). This shows that Cvl2 can rescue the blockage of Bmp signaling by Chordin, consistent with the results of the Cvl2-Chordin epistasis analyses described above (Fig. 3).

The ventralizing effect of Cvl2-N is partly independent of Chordin

Comparing the ventralized traits of *chordin* mutants in the absence or presence of Cvl2, we had concluded that Cvl2 has both Chordin-dependent and Chordin-independent pro-Bmp effects (Fig. 3). To examine the Chordin-independent effect under gain-of-function conditions, *cvl2-N* mRNA was co-injected with maximal amounts of *chordin* MOs. Nevertheless, *chordin* morphants co-injected with *cvl2-N* mRNA displayed an enhancement of all ventralized traits, including a further expansion of the blood island and a further

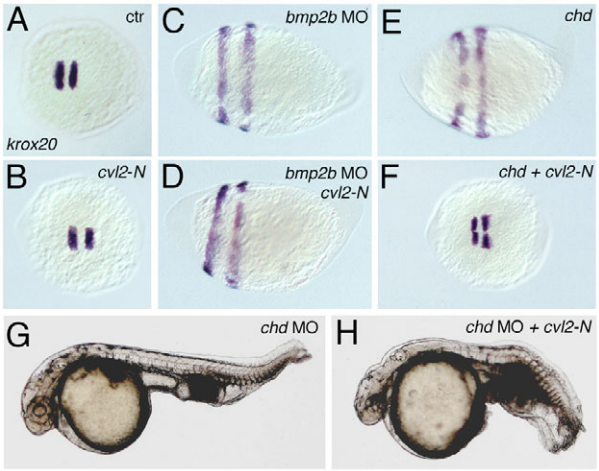

Fig. 5. The ventralizing effect of Cvl2-N depends on Bmp2b, but is partly independent of Chordin. (A-F) *krox20* staining. (**A**) Wild type, (**B**) wild type injected with *cvl2-N* mRNA, (**C**) *bmp2b* morphant, (**D**) *bmp2b* morphant injected with *cvl2-N* mRNA, (**E**) *chd* mRNA injected embryo and (**F**) *chd* plus *cvl-N* mRNA injected embryo. *cvl2-N* mRNA can rescue dorsalization caused by *chd* mRNA but not by *bmp2b* MO. (**G**,**H**) Lateral views of 32 hpf embryos. The ventralization of (G) *chd* MO injected embryos is enhanced by co-injection of (H) *cvl2*-N mRNA.

reduction of the size of the head, as well as a loss of the gap in the ventral tail fin (Fig. 5G,H; Table 1). Although we cannot rule out that some residual Chordin protein is present in morphants, this result suggests that Cvl2 has an additional, Chordin-independent pro-Bmp function.

Uncleaved and cleaved Cvl2 strongly bind Bmps

To gain insights into the molecular mechanisms underlying the effects of Cvl2 on Bmp signaling, we first studied the Bmp binding properties of the Cvl2 versions via Biacore analyses. Cvl2 proteins were isolated from baculovirus-transfected SF9 cells, and purified to ~90% purity (Fig. 6A). Comparing cleavage-resistant Cvl2-CM with the CR repeats-containing Cvl2-N fragment, and with wild-type Cvl2 (Cvl2-WT, ~80% cleaved/associated; Fig. 6A, lane 4), all three immobilized versions were found to bind Bmp2 with nearly identical high affinity (apparent K_D ~ 1.2 nM) (Table 2 and Fig. 6B). Similar affinities were determined for Bmp4 and Bmp7, while binding to the more distantly related growth and differentiation factor 5 (Gdf5) was about 25-fold weaker (Table 2). The affinities between Cvl2 and Bmp members are similar to the affinity between Bmp2 and the type I receptor BRIA (Keller et al., 2004). Together, these data indicate that Cvl2 strongly binds to Bmps, that this binding occurs via its N-terminal CR repeats, and that binding is not affected by the proteolytic cleavage of Cvl2.

Binding of Cvl2 and Chordin to Bmp2 is mutually exclusive

In the loss-of-function experiments, we had observed that the pro-Bmp activity of Cvl2 is strongly reduced in *chordin* mutants. As both Cvl2 and Chordin directly bind to Bmps, we sought to determine whether Cvl2 might function to prevent the inhibitory interaction between Chordin and Bmps. We therefore carried out competitive Biacore analyses, measuring the binding of Chordin to preformed Bmp2-Cvl2 complexes (K_D ~ 25 nM; Fig. 6B; Table 3). We found that saturation of Bmp2 with Cvl2-WT or Cvl2-N blocked binding of Chordin to Bmp2 (Fig. 6D), although Chordin readily bound to immobilized Bmp2 alone (K_D ~ 12 nM; Fig. 6C, Table 3), consistent with previous reports (Larrain et al., 2000). This shows that Cvl2 and Chordin bind to overlapping epitopes of Bmp2, and that direct binding of Cvl2 and Chordin to Bmps is mutually exclusive.

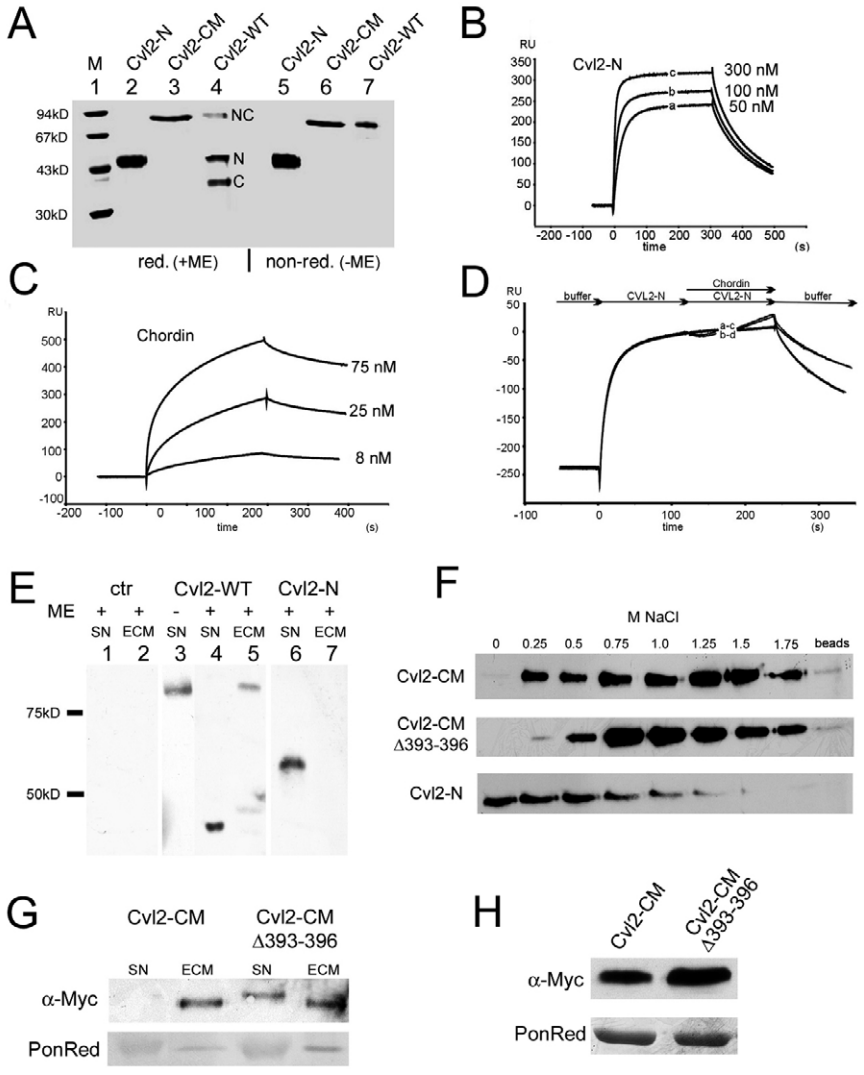

Fig. 6. Biochemical characterization of Cvl2 proteins. (**A**) SDS-PAGE gel showing purified Cvl2-N (lanes 2, 5), Cvl2-CM (lanes 3, 6) and Cvl2-WT (lanes 4, 7) proteins expressed in SF9 cells under reducing (lanes 2-4) and non-reducing (lanes 5-7) conditions; bands in lane 4 correspond to the uncleaved protein (NC), and the N- and C-terminal cleavage products. (**B**) Biacore sensograms showing the binding of 50 (a), 100 (b), 300 (c) nM Cvl2-N to immobilized Bmp2. (**C**) Sensograms showing binding of 8 (a), 25 (b) and 75 (c) nM Chordin to immobilized Bmp2. (**D**) Binding of Chordin to Cvl2-N saturated immobilized Bmp2. At time zero, perfusion with 500 nM Cvl2-N was initiated. The saturation binding of Cvl2-N was set as zero. After 120 seconds, perfusion was continued with 500 nM Cvl2-N plus Chordin. In different cycles, 25 (b) and 75 (d) nM Chordin were applied. In between, sensograms were recorded in the absence of Chordin (a,c). Perfusion with buffer started at 240 seconds. The subtle shift upon perfusion with highly concentrated Chordin (b,d) might result from a slow, equilibrium-driven replacement of Cvl2-N by Chordin. (**E-H**) Western blots with anti-Myc antibody. (E) Differential distribution of Cvl2 proteins in HEK 293 cell cultures. Cells were transfected with empty vector (lanes 1,2), or plasmids encoding Cvl2-WT (lanes 3-5) or Cvl2-N (lanes 6,7). Cleaved/associated Cvl2-WT is characterized by a high molecular weight band in the absence of ME (lane 3), and a low molecular weight band in the presence of ME (lane 4). It is found only in the supernatant, whereas uncleaved Cvl2-wt (high molecular weight band in presence of ME) is found only in the ECM fraction (lanes 4,5). Cvl2-N is present only in the supernatant (lanes 6,7). (F) Elution profiles of Cvl2 proteins from heparin-coated sepharose beads. Cvl2-CM, Cvl2-CMΔ393-396 and Cvl2-N display decreasing affinity to heparin. (G) Removal of a putative heparin binding site (amino acids 393-396) from Cvl2-CM leads to accumulation of the protein in the supernatant of HEK 293 cell cultures. (H) In injected zebrafish embryos, Cvl2-CM and Cvl2-CMΔ393-396 are present in similar amounts, although the dorsalizing effect of Cvl2-CMΔ393-396 is much weaker (compare with Table 1).

Table 2. Binding affinities of different Bmps to immobilized Cvl2-WT, Cvl2-N or Cvl2-CM

	Immobilized protein								
	Cvl2-WT			*Cvl2-N*			*Cvl2-CM*		
Ligand	$k_{on} \times 10^6$ ($M^{-1}s^{-1}$)	$k_{off} \times 10^{-3}$ (s^{-1})	K_D (nM)	$k_{on} \times 10^6$ ($M^{-1}s^{-1}$)	$k_{off} \times 10^{-3}$ (s^{-1})	K_D (nM)	$k_{on} \times 10^6$ ($M^{-1}s^{-1}$)	$k_{off} \times 10^{-3}$ (s^{-1})	K_D (nM)
Bmp2	2.3±0.40	3.2±0.52	1.4±0.32	6.0±0.71	6.1±0.64	1.0±0.18	5.5±0.67	6.2±0.35	1.2±0.17
Bmp4	2.3±0.42	4.5±0.70	2.0±0.28	1.0±0.26	2.0±0.27	2.1±0.52	2.5±0.22	4.8±0.40	1.9±0.38
Bmp7	2.3±0.44	7.9±0.61	3.5±0.50	1.2±0.17	2.7±0.56	2.3±0.68	2.6±0.32	7.5±0.47	2.9±0.41
Gdf5	1.5±0.10	52±2.5	34±2.0	0.7±0.11	21±1.6	30±3.3	1.4±0.20	51±3.0	32±2.2

Apparent K_D (in nM) of immobilized proteins is the quotient k_{off} / k_{on}. k_{off} and k_{on} were measured by Biacore analyses.

Uncleaved Cvl2 binds to the extracellular matrix, whereas cleaved Cvl2 does not

Despite the opposing activities of cleaved and uncleaved Cvl2 in overexpression assays, we were unable to detect significant differences in their binding properties. As many secreted signaling proteins, including Bmps themselves and their inhibitors Noggin and Chordin, can be tightly associated with components of the extracellular matrix (ECM) (Ruppert et al., 1996; Paine-Saunders et al., 2002; Jasuja et al., 2004), we wanted to examine whether differential affinity to the ECM might account for the opposite effects of uncleaved and cleaved Cvl2.

For this purpose, we transfected plasmids encoding Myc-tagged Cvl2-WT or Cvl2-N into HEK293 cells, cultured cells for 48 hours and isolated proteins from cells, supernatants and ECM. Upon transfection with Cvl2-WT, which gives rise to both cleaved/associated and uncleaved Cvl2 in cell extracts (data not shown), the two forms distributed differently in the extracellular domain. Whereas uncleaved Cvl2 was found in the ECM fraction only, cleaved Cvl2 was in the supernatant (Fig. 6E, lanes 3-5). Similarly, Cvl2-N, which lacks the C-terminal half, was present only in the supernatant, but not in the ECM fraction (Fig. 6E, lanes 6,7). This shows that the C-terminus containing the vWFd domain is required for binding of uncleaved Cvl2 to the ECM, while cleavage of Cvl2 strongly reduces ECM binding, although the N- and C-terminal fragments remain associated.

As association of Bmps, Chordin and Noggin with the ECM mainly occurs via heparan sulfate proteoglycans (HSPGs) (Ruppert et al., 1996; Paine-Saunders et al., 2002; Jasuja et al., 2004), we next tested whether Cvl2 can bind to heparin-coated sepharose beads. After incubation of Myc-tagged Cvl2-N and Cvl2-CM with the beads, proteins were eluted with increasing concentrations of NaCl. The elution profiles showed that Cvl2-CM binds to heparin with much higher affinity than Cvl2-N (elution maximum for Cvl2-CM at 1.25-1.5M NaCl, for Cvl2-N at 0-0.5M NaCl, Fig. 6G), suggesting that differential binding to HSPGs might account for the difference in binding to the ECM between cleaved and uncleaved Cvl2.

Table 3. Binding affinities of Cvl2-N, Cvl2-CM, Cvl2-WT and full-length Chordin to immobilized Bmp2

	Immobilized Bmp2		
Ligand	$k_{on} \times 10^5$ ($M^{-1}s^{-1}$)	$k_{off} \times 10^{-3}$ (s^{-1})	K_D (nM)
Cvl2-N	5.9±0.83	15±0.30	25±5.8
Cvl2-CM	1.8±0.29	4.4±0.61	26±3.7
Cvl2-WT	0.81±0.13	1.8±0.32	22±4.6
Chordin	2.8±0.24	3.4±0.27	12±2.4

K_D values for Bmp binding proteins determined via Biacore analyses can differ by one order of magnitude, depending on which of the tested partners is immobilized (compare with Table 2 and see Sebald et al., 2004, for other examples).

To study the functional relevance of heparin binding, we sought to interfere with the heparin and ECM binding of Cvl2-CM. Cvl2 contains 67 basic amino acids (Arg and Lys) as potential binding sites for HSPGs, making it difficult to completely abolish heparin binding. We focused on one site that conforms to a consensus heparin binding site (see Hileman et al., 1998), which is located at amino acids 393-396 (RRTR), 40 residues C-terminal of the cleavage site. Removal of these four residues from Cvl2-CM reduced the affinity for heparin beads (elution maximum at 0.75-1M NaCl; Fig. 6F) and led to the release of a significant fraction of the protein into the supernatant after transfection into HEK293 cells (Fig. 6G). Importantly, injection of mRNA for this construct (*cvl2-CMΔ393-396*) into zebrafish embryos had a strongly reduced dorsalizing activity compared with *cvl2-CM*, although both proteins were synthesized at equivalent levels (Table 1; Fig. 6H). These data suggest that association of uncleaved Cvl2 with HSPGs of the ECM significantly contributes to its anti-Bmp activity, possibly by tethering Bmps at the ECM, making them inaccessible for their transmembrane receptors.

DISCUSSION

Cvl2 is an essential part of a positive feedback loop enhancing Bmp signaling during zebrafish embryogenesis

Providing the first loss-of-function study of a vertebrate *crossveinless2* homologue, we show that Cvl2 is required to promote Bmp signaling during dorsoventral patterning of the zebrafish embryo. We further show that *cvl2* is co-expressed with *bmp*s on the ventral side of the gastrulating embryo, and that its transcription is positively regulated by Bmp signaling, constituting a positive feedback loop of Bmp signaling.

During gastrula stages, Bmps are expressed in a graded fashion with highest levels at the ventral pole. According to the morphogen concept, this Bmp gradient defines positional values and differential cell fates along the dorsoventral axis. Cvl2 promotes Bmp signaling at all positions of the gradient: in the presence of the Cvl2-dependent positive feedback loop, this gradient reaches higher maximum levels at the ventral pole and is steeper, as revealed by pSmad1/5 immunostaining (Fig. 2B). Clearly, an increased slope should yield a higher spatial resolution of positional values along the dorsoventral axis, thereby facilitating the translation of the gradient into differential cell fate. In addition, the Cvl2-dependent positive feedback should ensure higher gradient stability over time. A similar role of Cvl2 to stabilize territories of Bmp signaling might also occur during later processes of vertebrate development, when *cvl2* displays a highly dynamic, but restricted expression at various sites that are supposed to be under the control of Bmps (Fig. 1; see Fig. S2 in the supplementary material) (Coffinier et al., 2002; Coles et al., 2004; Kamimura et al., 2004). Such a role of zebrafish Cvl2 to locally elevate Bmp signaling levels is consistent with the function of its

counterpart *crossveinless2* during wing development in *Drosophila* (Conley et al., 2000). Here, the developing crossveins of the pupal wing show increased Bmp signaling activity, revealed by increased Mad (the *Drosophila* Smad1/5 homologue) phosphorylation, although neither the *Drosophila bmp* homologs *decapentaplegic* and *glass bottom boat*, nor their type I receptor *thick veins* show elevated expression in this region. However, *cvl2* expression is upregulated in this territory, and in *cvl2* mutants, the elevated Mad phosphorylation is lost (Conley et al., 2000).

Chordin-dependent and -independent pro-Bmp effect of Cvl2

Our embryological epistasis analyses revealed a significant dependence of Cvl2 activity on Chordin function. Thus, we found that although loss of Cvl2 in wild-type embryos leads to reduced Bmp signaling and a dorsalization of the embryo, loss of Cvl2 in *chordin* mutants had only very moderate dorsalizing effects and failed to rescue particular ventralized traits of the mutants, as revealed by the persistent enlargement of the blood islands and the persistent reduction of the neuroectoderm (Fig. 3). This indicates that in the absence of Chordin, Cvl2 cannot display its full pro-Bmp effect, suggesting that it normally promotes Bmp signaling by antagonizing Chordin. This notion of Cvl2-Chordin competition is further supported by our overexpression studies (Fig. 5), showing that application of Cvl2-N can rescue the dorsalization caused by Chordin, whereas it cannot compensate for the loss of Bmp signaling caused by knockdown of Bmps.

In addition, we could detect Chordin-independent pro-Bmp effects of Cvl2 both in gain- and loss-of-function experiments. In the loss-of-function scenario, this effect was most apparent in the ventral tail fins, the tissue supposed to derive from the ventral-most ectoderm. In *chordin* mutants, it is reduced, pointing to a pro-Bmp effect of Chordin in ventral tail fin tissue. Recent experiments with temporally controlled Bmp inactivation indicate that Bmp function driving ventral tail fin formation is required during post-gastrulation stages (Pyati et al., 2005), and the same might be true for Chordin. In addition, Chordin might act during gastrula stages, transporting Bmps against their gradient into ventral-most regions to reach peak Bmp levels in concert with Tolloid function (Hammerschmidt and Mullins, 2002; Wagner and Mullins, 2002). In any case, our findings that both in *chordin* and in *tolloid* mutants, dorsalized traits of the ventral tail fin tissue are further enhanced by loss of Cvl2 suggests that here, Cvl2 has a pro-Bmp effect independently of the Chordin-Tolloid system (Figs 2, 3 and data not shown). Consistent results pointing to a Chordin-independent pro-Bmp effect of Cvl2 were obtained in our gain-of-function experiments, showing that Cvl2-N can further ventralize *chordin* morphant embryos (Fig. 5).

Molecular mechanisms of Cvl2 function: Bmp receptors and Tsg

Cvl2 contains five CR domains, which were initially characterized in Chordin, but are also present in other Bmp-regulating secreted proteins (Garcia Abreu et al., 2002). Accordingly, we found that both cleaved and uncleaved full length Cvl2, as well as its N-terminal fragment containing the CR domains, readily and specifically bind Bmps (Fig. 6). Binding affinities to immobilized Bmp2 were similar to that determined for Chordin under identical conditions (K_D ~25 nM for Cvl2, ~12 nM for Chordin; Table 3). We further found that direct binding of Chordin or Cvl2 to Bmp2 is mutually exclusive (Fig. 6), consistent with the opposing roles of Cvl2 and Chordin during zebrafish development. In this respect, it appears that the Chordin-dependent pro-Bmp effect found in the zebrafish embryo is due to an 'anti-Chordin' function, with Cvl2 competing with Chordin for free and possibly also for Chordin-prebound Bmps. Along the same lines, the Chordin-independent Bmp-promoting effect of Cvl2 could be due to competition with other Bmp inhibitors or Bmp-binding proteins.

We also searched for differences in the molecular properties of Cvl2-Bmp versus Chd-Bmp complexes that could account for the opposite effects of Cvl2 and Chd on Bmp signaling. One possibility could be that, in contrast to Chd (Larrain et al., 2000), binding of Cvl2 to Bmp does not block the interaction between Bmps and their transmembrane receptors, similar to how it has been previously reported for the anti-Bmp factor Follistatin (Iemura et al., 1998) and the CR domain-containing pro-Bmp protein Kcp (Lin et al., 2005). To test this notion, we performed competitive Biacore analyses between Chd or Cvl2, Bmp2 and type I (BmpRIA) or type II (BmpRII) receptors. However, obtained results were inconclusive. Thus, depending on which of the components were immobilized, each of the tested proteins (Cvl-N, Cvl-CM or Chd) either blocked Bmp-BmpR interaction, or was permissive, whereas we failed to detect crucial differences between Cvl-N on one side, and Cvl-CM and Chd on the other (J.Z. and W.S., unpublished).

Alternatively, Cvl2 could act at the level of other components of Bmp-containing complexes, such as Twisted gastrulation (Tsg). In gain-of-function experiments, Tsg can display both pro- and anti-Bmp effects, as has been described for Cvl2. As Cvl2, Tsg binds Bmps, mediated by a partial CR-like domain (Oelgeschlager et al., 2003). Tsg also binds Chordin, thereby facilitating binding of Chordin to Bmps, accounting for its anti-Bmp effect (Chang et al., 2001; Oelgeschlager et al., 2000). In addition, Tsg promotes cleavage of Chordin by the metalloprotease Tolloid, resulting in a pro-Bmp effect (Oelgeschlager et al., 2000; Scott et al., 2001; Larrain et al., 2001; Shimmi and O'Conner, 2003; Little and Mullins, 2004; Xie and Fisher, 2005). In view of this, we tested whether Cvl2 might act via Tsg, but Biacore analyses indicated that Cvl2 and Tsg do not physically interact (J.Z. and W.S., unpublished). However, we cannot rule out that Cvl2 binds to higher order complexes containing Chd and/or Tsg.

Molecular mechanisms of Cvl2 function: HSPGs and the ECM

Recently, it has been reported that the Bmp-antagonizing function of Chordin depends on the binding of Chordin to heparan sulfate proteoglycans (HSPGs) (Jasuja et al., 2004). HSPGs are either components of the extracellular matrix (ECM) or remain attached to the cell membrane, usually via GPI anchors. In *Drosophila*, the membrane-bound Glypican protein Dally has been shown to promote signaling by the Bmp homologue Dpp, acting as some kind of co-receptor (Fujise et al., 2003). In this light, both the Chordin-dependent and the Chordin-independent pro-Bmp effects could be explained by a role of Cvl2 to regulate the distribution of Bmps within the extracellular space, or the recruitment of Bmps to their cell-surface receptors. Here, we found that uncleaved Cvl2 binds to heparin with an affinity very similar to that reported for Chordin, and also to the ECM of transfected cells. Removal of a putative heparin binding site from uncleaved Cvl2 results in reduced affinity for heparin, reduced binding to the ECM and decreased anti-Bmp activity in embryos. Moreover, co-injection of *exostosin1* MOs, targeting a key enzyme of HSPG synthesis, strongly reduced the dorsalizing activity of *cvl2-CM* mRNA (F.R. and M.H., unpublished). This suggests that similar to Chordin, uncleaved Cvl2 requires binding to HSPGs to exert its anti-Bmp activity.

By contrast, cleavage of Cvl2 into two fragments that remain associated via disulfide bonds apparently leads to a conformational change that abolishes Cvl2 binding to the ECM (Fig. 6). As Bmps usually display strong affinity to the ECM (Reddi, 2002) and as *cvl2* is co-expressed with *bmp*s, binding of Bmps to cleaved Cvl2 could prevent sequestration of Bmps at the ECM, thereby helping to increase the availability of Bmps for receptor activation on target cells. Alternatively, or in addition, cleaved Cvl2 might facilitate the interaction between Bmps and their HSPG co-receptors.

In summary, our biochemical analyses indicate that Cvl2 binds to Bmps, that Cvl2 and Chordin compete for Bmp binding, and that cleaved Cvl2, in contrast to uncleaved Cvl2 and Chordin, lacks binding to the ECM. However, how this leads to the pro-Bmp effect of Cvl2 observed in vivo, remains largely unclear. In any case, our results highlight that binding of CR proteins to Bmps does not necessarily lead to an inhibition of Bmps, as in the case of Chordin (Picollo et al., 1996), but can, by contrast, be required to promote Bmp signaling, consistent with recent data obtained for the Kielin protein Kcp during renal regeneration in mouse (Lin et al., 2005).

Proteolytic processing of Cvl2 as a possible mechanism underlying its contrary effects in different contexts

Cvl2 gain-of-function studies in cell culture systems and in *Xenopus* embryos have yielded controversial results, revealing either pro- or anti-Bmp effects. Our findings that Cvl2 can be proteolytically cleaved, and that cleaved and uncleaved Cvl2 display similar affinities to Bmps, but different affinities to the ECM, might provide a biochemical explanation for this phenomenon. Our overexpression studies further indicate that Cvl2 must be present in the cleaved state to elicit its pro-Bmp effect, while the contrary phenotype obtained in our loss-of-function studies suggests that it is this cleaved version which is predominantly present during early zebrafish development to mediate the essential pro-Bmp effect of Cvl2. However, this might be different during other processes and/or in other organisms, where the uncleaved version might be present in excess, leading to a net anti-Bmp function of Cvl2. Such differences in the ratio of uncleaved versus cleaved protein could depend on the synthesis and turnover rate of Cvl2, or the abundance of Cvl2 proteases. According to our data, proteolytic activity is present in human (Fig. 6E) and insect (Fig. 6A) cell cultures, as well as in zebrafish embryos (Fig. 4A). The nature of the protease, if existent, remains unclear. We could not detect altered ratios of Cvl2 cleavage in *tolloid* gain-or loss-of-function experiments (data not shown), ruling out this apparent candidate. In any case, a spatial and temporal control of Cvl2 cleavage would offer an efficient and flexible system for the fine-tuning of Bmp activity, allowing context-dependent switching of a potent Bmp inhibitor to a Bmp agonist.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/5/801/DC1

References

Ashe, H. L. and Levine, M. (1999). Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature* **398**, 427-431.

Baker, J. C. and Harland, R. M. (1997). From receptor to nucleus: the Smad pathway. *Curr. Opin. Genet. Dev.* **7**, 467-473.

Balemans, W. and Van Hul, W. (2002). Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Dev. Biol.* **250**, 231-250.

Bauer, H., Lele, Z., Rauch, G. J., Geisler, R. and Hammerschmidt, M. (2001). The type I serine/threonine kinase receptor Alk8/Lost-a-fin is required for Bmp2b/7 signal transduction during dorsoventral patterning of the zebrafish embryo. *Development* **128**, 849-858.

Binnerts, M. E., Wen, X., Cante-Barrett, K., Bright, J., Chen, H. T., Asundi, V., Sattari, P., Tang, T., Boyle, B., Funk, W. et al. (2004). Human Crossveinless-2 is a novel inhibitor of bone morphogenetic proteins. *Biochem. Biophys. Res. Commun.* **315**, 272-280.

Blader, P., Rastegar, S., Fischer, N. and Strahle, U. (1997). Cleavage of the BMP-4 antagonist Chordin by zebrafish Tolloid. *Science* **278**, 1937-1940.

Chang, C., Holtzman, D. A., Chau, S., Chickering, T., Woolf, E. A., Holmgren, L. M., Bodorova, J., Gearing, D. P., Holmes, W. E. and Brivanlou, A. H. (2001). Twisted gastrulation can function as a BMP antagonist. *Nature* **410**, 483-487.

Coffinier, C., Tran, U., Larrain, J. and De Robertis, E. M. (2001). Neuralin-1 is a novel Chordin-related molecule expressed in the mouse neural plate. *Mech. Dev.* **100**, 119-122.

Coffinier, C., Ketpura, N., Tran, U., Geissert, D. and De Robertis, E. M. (2002). Mouse Crossveinless-2 is the vertebrate homolog of a Drosophila extracellular regulator of BMP signaling. *Mech. Dev.* **119**, S179-S184.

Coles, E., Christiansen, J., Economou, A., Bronner-Fraser, M. and Wilkinson, D. G. (2004). A vertebrate crossveinless 2 homologue modulates BMP activity and neural crest cell migration. *Development* **131**, 5309-5317.

Conley, C. A., Silburn, R., Singer, M. A., Ralston, A., Rohwer-Nutter, D., Olson, D. J., Gelbart, W. and Blair, S. S. (2000). Crossveinless 2 contains cysteine-rich domains and is required for high levels of BMP-like activity during the formation of the cross veins in Drosophila. *Development* **127**, 3947-3959.

Connors, S. A., Trout, J., Ekker, M. and Mullins, M. C. (1999). The role of tolloid/mini fin in dorsoventral pattern formation of the zebrafish embryo. *Development* **126**, 3119-3130.

De Robertis, E. M. and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu. Rev. Cell. Dev. Biol.* **20**, 285-308.

De Robertis, E. M., Larrain, J., Oelgeschlager, M. and Wessely, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat. Rev. Genet.* **1**, 171-181.

Decotto, E. and Ferguson, E. L. (2001). A positive role for Short gastrulation in modulating BMP signaling during dorsoventral patterning in the Drosophila embryo. *Development* **128**, 3831-3841.

Detrich, H. W., Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A. and Zon, L. I. (1995). Intra-embryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. USA* **92**, 10713-10717.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A. F., Talbot, W. S., Bouwmeester, T. and Hammerschmidt, M. (2000). Essential role of Bmp7 (snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. *Development* **127**, 343-354.

Fisher, S., Amacher, S. L. and Halpern, M. E. (1997). Loss of *cerebrum* function ventralizes the zebrafish embryo. *Development* **124**, 1301-1311.

Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S. and Nakato, H. (2003). Dally regulates Dpp morphogen gradient formation in the Drosophila wing. *Development* **130**, 1515-1522.

Garcia Abreu, J., Coffinier, C., Larrain, J., Oelgeschlager, M. and De Robertis, E. M. (2002). Chordin-like CR domains and the regulation of evolutionarily conserved extracellular signaling systems. *Gene* **287**, 39-47.

Halloran, M. C., Sato-Maeda, M., Warren, J. T., Su, F., Lele, Z., Krone, P. H., Kuwada, J. Y. and Shoji, W. (2000). Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **127**, 1953-1960.

Hammerschmidt, M. and Mullins, M. C. (2002). Dorsoventral patterning in the zebrafish: bone morphogenetic proteins and beyond. In *Pattern Formation in Zebrafish: Results and Problems in Cell Differentiation, Vol 40* (ed. L. Solnica-Krezel), pp. 72-95. Berlin: Springer-Verlag.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., van Eeden, F. J., Granato, M., Brand, M., Furutani-Seiki, M., Haffter, P., Heisenberg, C. P. et al. (1996a). dino and mercedes, two genes regulating dorsal development in the zebrafish embryo. *Development* **123**, 95-102.

Hammerschmidt, M., Serbedzija, G. and McMahon, A. P. (1996b). Genetic analysis of dorsoventral pattern formation in the zebrafish: requirement of a BMP-like ventralizing activity and its dorsal repressor. *Genes Dev.* **10**, 2452-2461.

Heldin, C. H., Miyazono, K. and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465-471.

Hild, M., Dick, A., Rauch, G. J., Meier, A., Bouwmeester, T., Haffter, P. and Hammerschmidt, M. (1999). The smad5 mutation somitabun blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo. *Development* **126**, 2149-2159.

Hileman, R. E., Fromm, J. R., Weiler, J. M. and Linhardt, R. J. (1998). Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins. *BioEssays* **20**, 156-167.

Hogan, B. L. (1996). Bone morphogenetic proteins in development. *Curr. Opin. Genet. Dev.* **6**, 432-438.
Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of Follistatin to a complex of Bone morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9337-9342.
Jasuja, R., Allen, B. L., Pappano, W. N., Rapraeger, A. C. and Greenspan, D. S. (2004). Cell-surface heparan sulfate proteoglycans potentiate chordin antagonism of bone morphogenetic protein signaling and are necessary for cellular uptake of chordin. *J. Biol. Chem.* **279**, 51289-51297.
Joly, J.-S., Joly, C., Schulte-Merker, S., Boulkebache, H. and Condamine, H. (1993). The ventral and posterior expression of the homeobox gene *eve1* is perturbed in dorsalized and mutant embryos. *Development* **119**, 1261-1275.
Kamimura, M., Matsumoto, K., Koshiba-Takeuchi, K. and Ogura, T. (2004). Vertebrate crossveinless 2 is secreted and acts as an extracellular modulator of the BMP signaling cascade. *Dev. Dyn.* **230**, 434-445.
Keller, S., Nickel, J., Zhang, J. L., Sebald, W. and Mueller, T. D. (2004). Molecular recognition of BMP-2 and BMP receptor IA. *Nat. Struct. Mol. Biol.* **11**, 481-488.
Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M. and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-4466.
Knight, R. D., Nair, S., Nelson, S. S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G.-J. and Schilling, T. F. (2003). lockjaw encodes a zebrafish tfap2a required for early neural crest development. *Development* **130**, 5755-5768.
Larrain, J., Bachiller, D., Lu, B., Agius, E., Piccolo, S. and De Robertis, E. M. (2000). BMP-binding modules in chordin: a model for signalling regulation in the extracellular space. *Development* **127**, 821-830.
Larrain, J., Oelgeschlager, M., Ketpura, N. I., Reversade, B., Zakin, L. and De Robertis, E. M. (2001). Proteolytic cleavage of Chordin as a switch for the dual activities of Twisted gastrulation in BMP signaling. *Development* **128**, 4439-4447.
Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). Expression of two zebrafish *orthodentical*-related genes in the embryonic brain. *Mech. Dev.* **48**, 229-244.
Lin, J., Patel, S. R., Cheng, X., Cho, E. A., Levitan, I., Ullenbruch, M., Phan, S. H., Park, J. M. and Dressler, G. R. (2005). Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. *Nat. Med.* **11**, 387-393.
Little, S. C. and Mullins, M. C. (2004). Twisted gastrulation promotes BMP signaling in zebrafish dorsal-ventral axial patterning. *Development* **131**, 5825-5835.
Martyn, U. and Schulte-Merker, S. (2003). The ventralized ogon mutant phenotype is caused by a mutation in the zebrafish homologue of Sizzled, a secreted Frizzled-related protein. *Dev. Biol.* **260**, 58-67.
Matsui, M., Mizuseki, K., Nakatani, J., Nakanishi, S. and Sasai, Y. (2000). Xenopus kielin: A dorsalizing factor containing multiple chordin-type repeats secreted from the embryonic midline. *Proc. Natl. Acad. Sci. USA* **97**, 5291-5296.
Mintzer, K. A., Lee, M. A., Runke, G., Trout, J., Whitman, M. and Mullins, M. C. (2001). Lost-a-fin encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation. *Development* **128**, 859-869.
Moser, M., Binder, O., Wu, Y., Aitsebaomo, J., Ren, R., Bode, C., Bautch, V. L., Conlon, F. L. and Patterson, C. (2003). BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. *Mol. Cell. Biol.* **23**, 5664-5679.
Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., Eeden van, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C.-P. et al. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81-93.
Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.
Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M. and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. *Dev. Biol.* **199**, 93-110.
Nikaido, M., Tada, M., Saji, T. and Ueno, N. (1997). Conservation of BMP signaling in zebrafish mesoderm patterning. *Mech. Dev.* **61**, 75-88.
Novikova, E. G., Reznik, S. E., Varlamov, O. and Fricker, L. D. (2000). Carboxypeptidase Z is present in the regulated secretory pathway and extracellular matrix in cultured cells and in human tissues. *J. Biol. Chem.* **275**, 4865-4870.
Oelgeschlager, M., Larrain, J., Geissert, D. and De Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature* **405**, 757-763.
Oelgeschlager, M., Reversade, B., Larrain, J., Little, S., Mullins, M. C. and De Robertis, E. M. (2003). The pro-BMP activity of Twisted gastrulation is independent of BMP binding. *Development* **130**, 4047-4056.
Paine-Saunders, S., Viviano, B. L., Economides, A. N. and Saunders, S. (2002). Heparan sulfate proteoglycans retain Noggin at the cell surface: a potential mechanism for shaping bone morphogenetic protein gradients. *J. Biol. Chem.* **277**, 2089-2096.
Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.
Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L. and De Robertis, E. M. (1997). Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* **91**, 407-416.
Pyati, U. J., Webb, A. E. and Kimelman, D. (2005). Transgenic zebrafish reveal stage-specific roles for Bmp signaling in ventral and posterior mesoderm development. *Development* **132**, 2333-2343.
Ramel, M.-C., Buckles, G. R., Baker, K. D. and Lekven, A. C. (2005). WNT8 and BMP2B co-regulate non-axial mesoderm patterning during zebrafish gastrulation. *Dev. Biol*. **287**, 237-248.
Reddi, A. H. (2000). Morphogenetic messages are in the extracellular matrix: biotechnology from bench to bedside. *Biochem. Soc. Trans*. **28**, 345-349.
Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* **410**, 479-483.
Ruppert, R., Hoffmann, E. and Sebald, W. (1996). Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur. J. Biochem.* **237**, 295-302.
Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
Schier, A. F. and Talbot, W. S. (2005). Molecular genetics of axis formation in zebrafish. *Annu. Rev. Genet.* **39**, 561-613.
Schmid, B., Furthauer, M., Connors, S. A., Trout, J., Thisse, B., Thisse, C. and Mullins, M. C. (2000). Equivalent genetic roles for *bmp7/snailhouse* and *bmp2b/swirl* in dorsoventral pattern formation. *Development* **127**, 957-967.
Schulte-Merker, S., Lee, K. J., McMahon, A. P. and Hammerschmidt, M. (1997). The zebrafish organizer requires chordino. *Nature* **387**, 862-863.
Scott, I. C., Blitz, I. L., Pappano, W. N., Maas, S. A., Cho, K. W. and Greenspan, D. S. (2001). Homologues of Twisted gastrulation are extracellular cofactors in antagonism of BMP signalling. *Nature* **410**, 475-478.
Sebald, W., Nickel, J., Zhang, J. L. and Mueller, T. D. (2004). Molecular recognition in bone morphogenetic protein (BMP)/receptor interaction. *Biol. Chem.* **385**, 697-710.
Shimmi, O. and O'Connor, M. B. (2003). Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. *Development* **130**, 4673-4682.
Solnica-Krezel, L., Stemple, D. L., Mountcastle-Shah, E., Rangini, Z., Neuhauss, S. C. F., Malicki, J., Schier, A., Stainier, D. Y. R., Zwartkruis, F., Abdelialah, S. et al. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development* **123**, 67-80.
Szeto, D. P. and Kimelman, D. (2004). Combinatorial gene regulation by Bmp and Wnt in zebrafish posterior mesoderm formation. *Development* **131**, 3751-3760.
Wagner, D. S. and Mullins, M. C. (2002). Modulation of BMP activity in dorsal-ventral pattern formation by the chordin and ogon antagonists. *Dev. Biol.* **245**, 109-123.
Westerfield, M. (1994). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish*. Eugene (OR): University of Oregon Press.
Xie, J. and Fisher, S. (2005). Twisted gastrulation enhances BMP signaling through Chordin-dependent and -independent mechanisms. *Development* **132**, 383-391.
Yabe, T., Shimizu, T., Muraoka, O., Bae, Y. K., Hirata, T., Nojima, H., Kawakami, A., Hirano, T. and Hibi, M. (2003). Ogon/Secreted Frizzled functions as a negative feedback regulator of Bmp signaling. *Development* **130**, 2705-2716.