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A study of Xlim1 function in the Spemann-Mangold organizer

LAURENT KODJABACHIAN[1,#], ALEXANDER A. KARAVANOV[1,#], HIROKI HIKASA[2,3], NEIL A. HUKRIEDE[1], TAZU AOKI[2], MASANORI TAIRA[2,3] and IGOR B. DAWID*[,1]

[1]National Institutes of Health. National Institute of Child Health and Human Development, Laboratory of Molecular Genetics, Bethesda, MD, USA, [2]Laboratory of Molecular Embryology. Department of Biological Sciences, Graduate School of Science, University of Tokyo, Japan and [3]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Japan

ABSTRACT The Spemann-Mangold organizer is required in amphibian embryos to coordinate cell fate specification, differentiation of dorsal cell types and morphogenetic movements at early stages of development. A great number of genes are specifically expressed within the organizer, most of them encoding secreted proteins and transcription factors. The challenge is now to uncover genetic cascades and networks of interactions between these genes, in order to understand how the organizer functions. The task is immense and requires loss-of-function approaches to test the requirement for a given factor in a specific process. For transcription factors, it is possible to generate inhibitory molecules by fusing the DNA binding region to a repressor or activator domain, which should in principle antagonize the activity of the endogenous protein at the level of the DNA targets. We used this strategy to design activated and inhibitory forms of the LIM homeodomain transcription factor Lim1, which is encoded by an organizer gene involved in head development, as revealed by analyses of knockout mice. We found that Lim1 is a transcriptional activator, and can trigger dorso-anterior development upon ventral expression of hyperactive forms, in which Ldb1 is fused to Lim1. Using inhibitory Lim1 fusion proteins, we found that *Lim1*, or genes closely related to it, is required for head formation as well as for notochord development. Co-expression experiments revealed that Lim1 is required downstream of the early organizer factor Siamois, first, to establish the genetic program of the organizer and second, to mediate the action of organizer agents that are responsible for blocking ventralizing activities in the gastrula.

KEY WORDS: *Spemann-Mangold organizer, Xenopus, Xlim1, anteroposterior axis, axial mesoderm.*

Introduction

During gastrulation in vertebrates, a complex series of events occurs, which leads to the correct spatial positioning of the three embryonic germ layers along the body axis, and to their patterning by localized and often reciprocal inductions. The Spemann-Mangold organizer (also called the dorsal organizer or gastrula organizer), a relatively small dorsal region of the gastrula in amphibians, coordinates these complex events. The graft of an early gastrula dorsal blastopore lip, where the Spemann-Mangold organizer forms, into the ventral region of a host embryo, leads to the development of a secondary body axis, which includes head features (reviewed in Lemaire and Kodjabachian, 1996; Harland and Gerhart, 1997). In contrast, grafting the same region explanted from a late gastrula only gives rise to tail duplication. These differences served as evidence for the existence of independent head and trunk organizers. Molecules involved in organizer ontogeny or activity have been isolated on the basis of their early dorsal expression, or their ability to induce aspects of dorsal axis development upon over-expression (reviewed in Lemaire and Kodjabachian, 1996; Niehrs, 1999). So far, the homeoprotein Siamois and its close relative Twin, are unique zygotic factors which can trigger the development of a complete secondary axis, implicating these factors in organizer establishment (Lemaire *et al.*, 1995; Laurent *et al.*, 1997). Various molecules are able to stimulate either head or trunk development, and are therefore believed to act downstream of Siamois in specific compartments of the organizer.

The LIM homeodomain (LHX) protein Xlim-1/Lim1 is expressed in the Spemann-Mangold organizer, and can induce partial axis duplication upon ectopic expression on the ventral side of the *Xenopus* embryo (Taira *et al.*, 1992; Taira *et al.*,

***Address correspondence to:** I.B. Dawid. National Institutes of Health, National Institute of Child Health and Human Development, Laboratory of Molecular Genetics, Bldg 6B, Rm 420, 9200 Rockville Pike, 20892 Bethesda, MD, USA. FAX: +1-301-496-0243. e-mail: idawid@nih.gov

[#] Both authors contributed equally to this work

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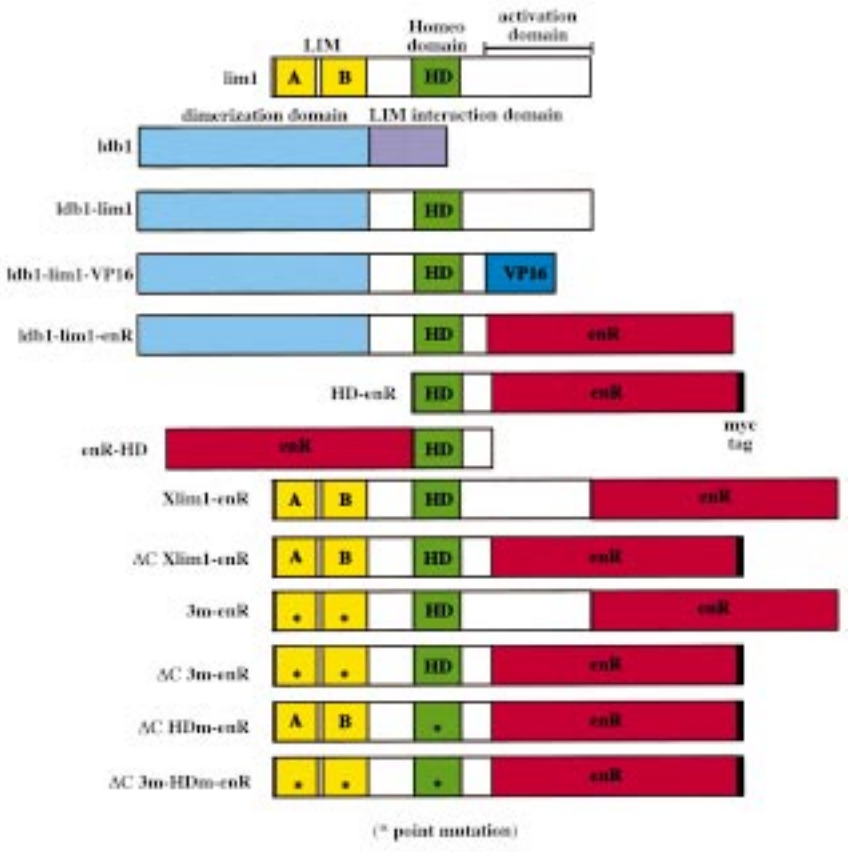

Fig. 1. Schematic representation of the constructs used in this study. *(See text for details).*

1994a). Analyses of mice mutant for the *Lim1* gene have revealed its essential function in the head organizer, as these animals lack most of the anterior brain (Shawlot *et al.*, 1995). This dramatic phenotype indicates that Lim1 is a central regulator during the early phases of axial development, and it is therefore important to understand how this gene functions. However, analyses of mutant mouse embryos have only revealed partial aspects of the mode of action of this factor, and we reasoned that important information could be gained by studying Lim1 in the more accessible *Xenopus* system.

Functional domains in the Lim1 protein have been quite extensively characterized, allowing three domains to be distinguished: an N-terminal pair of LIM domains which seem to play an adapter role, a central homeodomain with DNA-binding activity, and a C-terminal transactivation domain (Agulnick *et al.*, 1996; Breen *et al.*, 1998). The LIM domain is a double-zinc finger motif, which can interact with various protein domains (reviewed in Dawid *et al.*, 1998). LIM domains can interact with a LIM domain binding protein, Ldb1 or NLI/CLIM-2, which is required to allow Lim1 to exert its dorsalizing potential in frog embryos (Agulnick *et al.*, 1996). A similar situation exists in *Drosophila* where Chip, a homologue of Ldb1, is required for the normal activity of the LHX factor Apterous (Morcillo *et al.*, 1997; Fernandez-Funez *et al.*, 1998). Ldb1/Chip is known to contain a homodimerization domain (Jurata *et al.*, 1997; Breen *et al.*, 1998), and further studies in *Drosophila* have revealed that tetramers composed of two Ldb and two LHX molecules are functional *in vivo*. In particular, it was shown that a chimera in which the dimerization domain of Chip replaces the LIM domains of Apterous, fully rescues the *apterous* mutant, suggesting that the main role of Chip is to bridge two Apterous molecules via their LIM domains (Milan and Cohen, 1999; Van Meyel *et al.*, 1999).

In the present study we designed activator and inhibitory Lim1 fusion proteins and assayed their function upon ectopic expression in early frog embryos. Based on these studies, we present evidence that Lim1 is involved at multiple steps of organizer formation and activity in *Xenopus*.

Results

Xlim1 is a transcriptional activator

Ectopic expression of mutant forms of Xlim1, where LIM domains have been deleted or mutated, leads to dorsalization of mesoderm and the formation of partial secondary axes (Taira *et al.*, 1994a). Similarly, co-expression of Xlim1 and Ldb1 induces partial ectopic axes, neuralizes ectoderm and induces organizer gene expression in animal cells (Agulnick *et al.*, 1996; Breen *et al.*, 1998). In order to test whether vertebrate LHX activity is regulated similarly to *Drosophila* Apterous, we made a fusion between the dimerization domain of Ldb1, as defined by Breen *et al.* (1998), and the Lim1 protein from which LIM domains were deleted (Ldb1-lim1; Fig. 1). Here, we present data obtained with the zebrafish versions of Ldb1 and Lim1, but similar results were obtained with the *Xenopus* proteins (Hiratani and Taira, submitted). A range of phenotypes was observed upon ventral injection of *ldb1-lim1* in frog embryos. Low doses (200 pg to 1 ng) induced partial secondary axes similar to co-injection of *ldb1* and *lim1* (Fig. 2 A,B), while high doses (4 to 6 ng) led to the formation of ectopic cement gland or eye without clear axis formation (Fig. 2C). In fact, high doses of this construct also impaired axis elongation upon dorsal injection (Fig. 2D), suggesting that Lim1 could repress trunk fates when it is hyperactive. Interestingly, anterior fates were never observed upon injection of *Xlim1-3m* (Taira *et al.*, 1994a) or co-injection of *ldb1* and *lim1* (Agulnick *et al.*, 1996), indicating that the Ldb1-lim1 fusion protein is more active in this assay. It is important to note the activity of Lim1 in inducing anterior fates, as it is in agreement with the requirement for this gene in head development in the mouse (Shawlot and Behringer, 1995).

The experiments described above did not reveal whether Lim1 acts as a transcriptional activator or repressor. To address this question, additional constructs were made where various domains of Lim1 are fused to the transactivation domain of the viral protein VP16 (VP16; see Kessler, 1997) or the repressor domain of *Drosophila* Engrailed (enR; Han and Manley, 1993) (Fig. 1). In a previous study a putative transcriptional activation domain was found in Lim1 between amino acids 266 and 403 (Breen *et al.*, 1998), and this region was therefore deleted in most of our fusion proteins.

First, we found that the fusion protein Ldb1-lim1-VP16 acted similarly to Ldb1-lim1, as it induced partial axes at low doses (100 pg) and cement gland at higher doses (500 pg) (Fig. 2 E,F). This

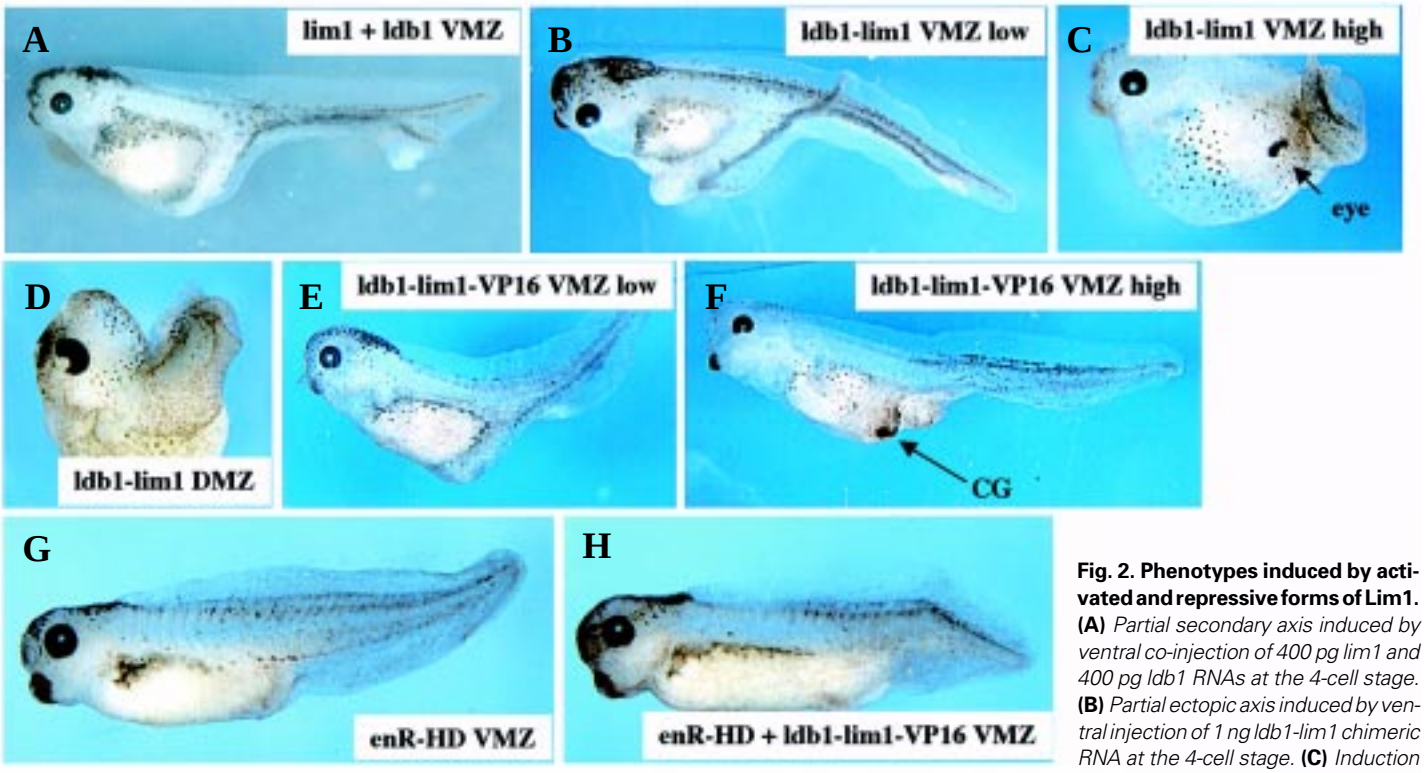

Fig. 2. Phenotypes induced by activated and repressive forms of Lim1. **(A)** *Partial secondary axis induced by ventral co-injection of 400 pg lim1 and 400 pg ldb1 RNAs at the 4-cell stage.* **(B)** *Partial ectopic axis induced by ventral injection of 1 ng ldb1-lim1 chimeric RNA at the 4-cell stage.* **(C)** *Induction of a single eye by ventral injection of 6 ng ldb1-lim1 chimera RNA at the 4-cell stage. Note that this embryo shows deficient secondary axis formation.* **(D)** *Dorsal injection at the 4-cell stage of 1 ng ldb1-lim1 chimera RNA leads to suppression of trunk/tail structures.* **(E)** *Partial ectopic axis induced by ventral injection of 100 pg ldb1-lim1-VP16 chimera RNA at the 4-cell stage.* **(F)** *Injection of 500 pg ldb1-lim1-VP16 chimera RNA at the 4-cell stage stimulates cement gland formation (CG) but not axis development.* **(G)** *Ventral injection at the 4-cell stage of 40 pg enR-HD chimera RNA does not provoke any visible phenotype.* **(H)** *Secondary axis formation mediated by ldb1-lim1-VP16 chimera RNA (200 pg) is suppressed by the repressive enR-HD chimera RNA (40 pg) upon ventral co-injection at the 4-cell stage.*

result indicates that Lim1 acts as a transcriptional activator, at least in ventral territories of the embryo. Moreover, axis induction by *ldb1-lim1-VP16* was found to be antagonized by co-injection of *enR-HD* encoding one of the repressor versions of Lim1 used in this study (Fig. 2H). Consistently, axis induction by Xlim1-3m or Xlim1 and Ldb1 was also suppressed by the co-expression of this repressor form of Lim1 (not shown). As enR-HD is not believed to contain any domain able to interact with Ldb1-lim1-VP16, the observed antagonism between these molecules is likely to take place at the level of the target DNA. This is illustrated by an experiment where gene expression was measured by northern analysis in animal caps loaded with different combinations of RNAs (Fig. 3). Co-expression of Ldb1 and Xlim1 in animal cells activated the transcription of the organizer genes *chordin* (*chd*; Sasai *et al.*, 1994), *otx2* (Pannese *et al.*, 1995; Blitz and Cho, 1995) and *goosecoid* (*gsc*; Cho *et al.*, 1991), and the presence of the repressive form of XLim1, ΔC Xlim1-enR, blocked this effect (Fig. 3). As Xlim1 in the presence of Ldb1 has been shown to activate directly the transcription of *gsc* (Mochizuki *et al.*, 2000), we reasoned that the repressive forms of Xlim1 might constitute good reagents to prevent the normal function of this gene in frog embryos.

Xlim1 is required for dorso-anterior development

Similar to findings in *Lim1* knockout mice, we observed that dorsal expression of the repressive forms of Xlim1 antagonized anterior development, as shown in Fig. 4B. Using markers expressed at different positions in the central nervous system, we could determine that the embryonic axis was truncated anterior to rhombomere 5 (Fig. 4 C,D). This position is roughly consistent with the level of truncation observed in mutant mice, as brain was missing rostral to rhombomere 3 (Shawlot *et al.*, 1995). However, we found an additional phenotype, which was not seen in *Lim1*-/- mouse mutants, as notochord development was strongly impaired in embryos injected with the HD-enR repressive form of Xlim1 (Fig. 4 E,F). Importantly, injected embryos underwent normal blastopore closure, which ruled out a defect in convergence-extension as being the primary cause for defective notochord development. In contrast to defective notochord development, somite formation could take place in presence of repressive Xlim1, as somitic tissue actually developed at the midline of injected embryos. However, we do not know whether this mislocalized somitic tissue formed de novo at the expense of axial mesoderm, or whether somites simply fused at the midline due to the lack of a physical barrier. As Xlim1 is clearly expressed in the developing notochord in *Xenopus* (Taira *et al.*, 1994b; Karavanov *et al.*, 1996), it is not surprising to find that this gene is required for normal notochord formation, although this is not apparently the case in mouse.

We next compared the activity of different repressive versions of Xlim1 in antagonizing anterior development, in order to determine which regions are functionally important in the Lim1 molecule.

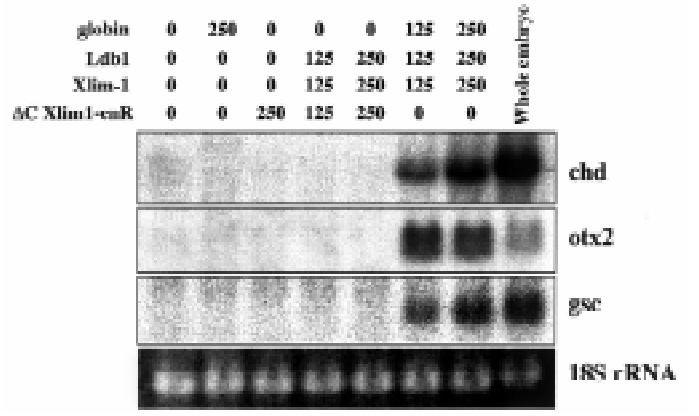

Fig. 3. ΔC Xlim1-enR suppresses the expression of dorsal marker genes activated by Xlim-1 and Ldb1 in animal caps. *Animal caps were dissected before gastrulation from embryos injected at the 2-cell stage with mRNAs as indicated. Explants were cultured until control sibling embryos reached stage 11. Total RNA was extracted from 15 animal caps and subjected to Northern blot analysis using chordin (chd), otx2 and goosecoid (gsc) probes. Globin RNA was injected as a control. 18S rRNA stained with ethidium bromide serves as a loading control.*

Thus, we injected similar amounts of each of these RNAs (100 and 200 pg/blastomere) repeatedly in three independent experiments, and scored the resulting phenotypes as headless (most severely truncated, with no eyes and no cement gland), microcephalic (no cement gland, small or single eye), or normal (Fig. 5). All repressive forms of Lim1 could trigger a similar range of phenotypes, but with variable penetrance. A number of conclusions can be drawn from this test, as follows. (1) The respective orientation between the DNA binding domain and the repressor domain has virtually no influence on the severity of the phenotypes, as HD-enR and enR-HD are about equally active. (2) We confirmed that the carboxy-terminal region of Lim1 contains an activator domain functional *in vivo*, as its presence reduces the severity of phenotypes in Xlim1-enR injected embryos compared to ΔC Xlim1-enR injected embryos. (3) A mutation in the homeodomain dramatically suppresses the inhibitory activity of ΔC HDm-enR, indicating that the repression requires the DNA binding activity of our chimeras, and does not merely depend on titration of Lim1 cofactors. However, it should be noted that this mutant is not totally inert suggesting that the presence of the LIM domains and possibly other parts of the molecule could sequester cofactors required for normal activity. This is also consistent with the fact that 3m-enR and ΔC 3m-enR are less active than their counterparts without mutation in the LIM domains. Further support for this idea comes from observations made in zebrafish, where over-expression of Islet-3 LIM domains triggered inhibitory effects (Kikuchi *et al.*, 1997), probably by trapping essential LIM binding factors, such as Ldb1. (4) The presence of the amino-terminal region of Lim1 decreases the severity of phenotypes in ΔC Xlim1-enR injected embryos compared to HD-enR injected embryos, suggesting that this region could contain an activator domain. This is supported by the fact that in a yeast one-hybrid system, a fusion of the entire Xlim-1 protein to the GAL4 DNA binding domain is more active than a fusion of the C-terminal activation domain of Xlim-1 to the GAL4 DNA binding domain (Breen *et al.*, 1998). (5) Dimerization is probably not required for repression as HD-enR and enR-HD constructs, which are not believed to dimerize, are as active as the Ldb1-lim1-enR construct. (6) The phenotypes observed cannot be attributed to the presence of a myc-tag in some of our constructs, as severe phenotypes are also generated by constructs lacking this epitope.

We next wanted to address the question of the specificity of action of the repressive Lim1 molecules in the embryo. The most

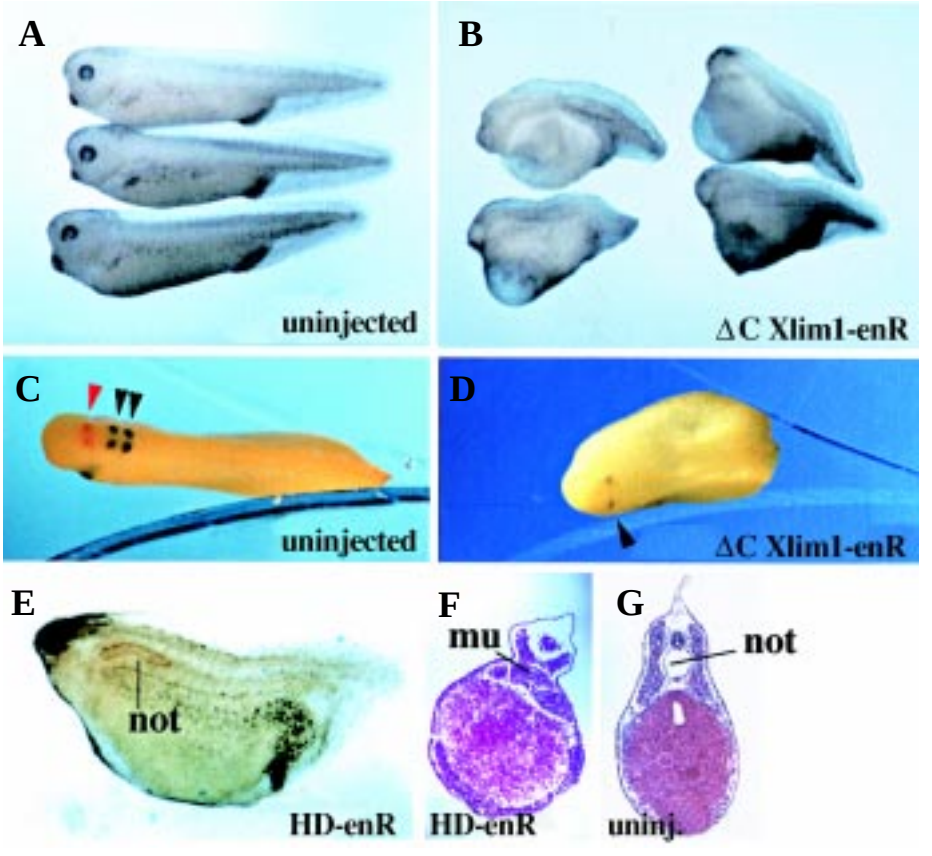

Fig. 4. Phenotypes elicited by expression of repressive forms of Lim1. *Dorsal injection at the 4-cell stage of 250 pg ΔC Xlim1-enR mRNA provokes anterior truncation* **(B,D)** *compared to uninjected siblings* **(A,C)**. *Embryos were subjected to two-color whole-mount in situ hybridization analysis with two head markers, krox20 (rhombomeres 3 and 5; dark blue) and en2 (isthmus; red) at the tailbud stage. The head in ΔC Xlim1-enR injected embryos is truncated anterior to rhombomere 5* **(D)**. *Dorsal injection at the 4-cell stage of 100 pg HD-enR RNA also leads to head truncation and deficiency in notochord, as revealed by staining with the MZ15 antibody* **(E)** *or histological section* **(F)**. **(G)** *Control section of an uninjected embryo. Note that somitic tissue is present in headless embryos and actually expands in the region normally occupied by the notochord (F). mu, muscle; not, notochord.*

appropriate test for establishing specificity is the rescue of mutant phenotypes by co-expression of the normal protein. Despite substantial efforts we could not obtain a significant level of rescue of anterior structures in whole embryos, or of marker gene expression in explants, by using such a strategy. In short, any of the repressive Lim1 molecules acted dominantly in our assays over any of the activated versions of Lim1 (not shown). Although we cannot provide a full explanation for this phenomenon, we do not think that these constructs act in a nonspecific manner, and we carried out a number of tests to support this view. As Xlim1 is a homeodomain factor, we tested whether the repressive forms of Lim1 antagonized the activity of unrelated homeodomain proteins. The homeodomain protein Otx2, when expressed in ventral ectoderm, induces ectopic formation of cement gland (Bradley *et al.*, 1996); this effect was not antagonized by co-expression of repressive HD-enR (Fig. 6 A,B). This experiment demonstrates that this inhibitory form of Lim1 does not interact with the targets of another homeodomain factor, indicating that repression shows a certain degree of specificity. If repressive versions of Lim1 behaved specifically they should not trigger any phenotypes in tissues which do not express Xlim1. This is the case when repressive Lim1 is expressed ventrally (Fig. 2G), where very little *Xlim1* mRNA is present (Taira *et al.*, 1992). Another such situation can be artificially created when the homeodomain factor Siamois is ectopically expressed in naïve ectoderm, as this protein stimulates the expression of many organizer genes, but not of *Xlim1*, in animal cap assays (Carnac *et al.,* 1996). Dorsalization of the ectoderm by Siamois results in the formation of large cement glands, and this effect was not suppressed upon co-expression of repressive HD-enR (Fig. 6 C,D). Again, this experiment suggests that repressive Lim1 constructs act specifically, since cement gland development is impeded when the repressor construct is expressed in tissues normally expressing Xlim1 (Fig. 4B).

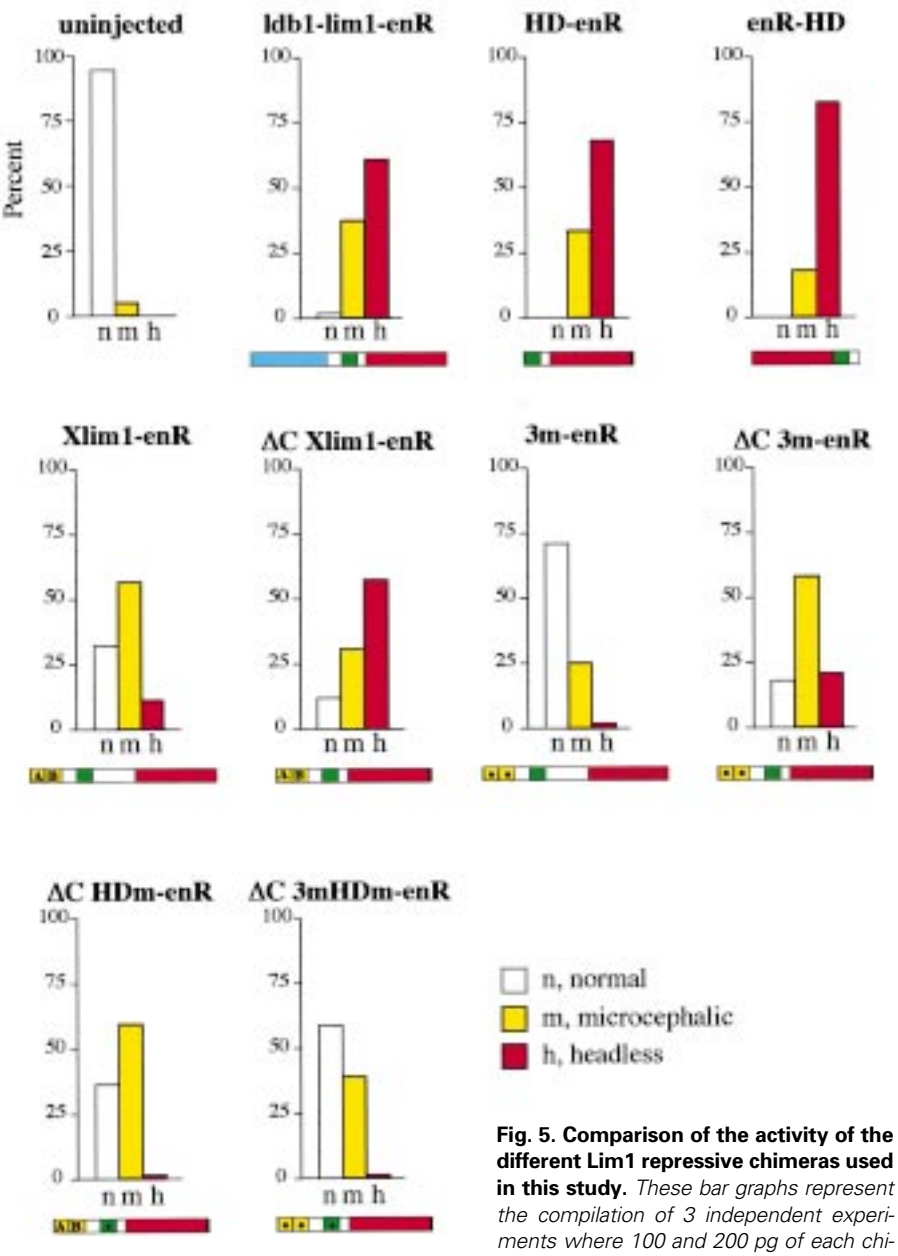

Fig. 5. Comparison of the activity of the different Lim1 repressive chimeras used in this study. *These bar graphs represent the compilation of 3 independent experiments where 100 and 200 pg of each chimera RNA were injected dorsally at the 4-cell stage. The phenotypes induced were scored as headless (no cement gland, no eyes) or microcephalic (no cement gland, small or single eye). Refer to the text for an interpretation of these data.*

Xlim1 is required for organizer gene expression

To analyze further the function of the *Xlim1* gene at early stages of development, we looked at organizer gene expression in embryos injected with repressive enR-HD (Fig. 7). Recent studies have shown that secretion of inhibitors of BMP, Wnt and Nodal factors by the Spemann-Mangold organizer is required for the correct patterning of the embryonic axis (Glinka *et al.*, 1997; Piccolo *et al.*, 1999). We found that inhibitory forms of Lim1 blocked the expression of the Wnt inhibitors Dkk1 (Glinka *et al.*, 1998) and Frzb (Leyns *et al.*, 1997; Wang *et al.*, 1997), the BMP inhibitors Chordin (Piccolo *et al.*, 1996) and Noggin (Zimmerman *et al.*, 1996), as well as the expression of Cerberus which has a triple inhibitory function on BMP, Wnt and Nodal signaling (Piccolo *et al.*, 1999). In addition the organizer genes *goosecoid*, *otx2* and *Xnr3* (Smith *et al.*, 1995) were also repressed, and ADMP, a specific inhibitor of Follistatin recently found to be involved in trunk development (Moos *et al.*, 1995; Dosch and Niehrs, 2000), was repressed as well. In order to study the fate of the cells injected with repressive Lim1, we looked at expression of the ventroposterior marker *PV.1* (Ault *et al.*, 1996) and found that the presumptive dorsal organizer was not ventralized, at least during gastrulation (Fig. 7A). This is consistent with the observation that

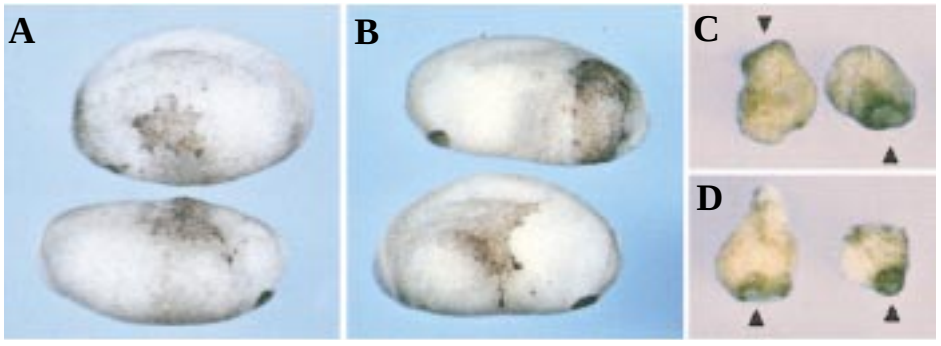

Fig. 6. Repressive forms of Lim1 do not antagonize activity of Otx2 and Siamois in animal tissues. *(A,B) Embryos were injected in ventral-animal position at the 4-cell stage with* **(A)** *800 pg otx2 RNA or* **(B)** *a mixture of 800 pg otx2 and 100 pg HD-enR RNAs. Over-expression of otx2 leads to the formation of cement gland tissue in the epidermis, and this effect is not antagonized by co-expression of HD-enR. (C,D) Embryos were injected animally at the 2-cell stage with* **(C)** *20 pg Siamois RNA, or* **(D)** *a mixture of 20 pg Siamois and 100 pg HD-enR RNAs. Animal caps were dissected at stage 9 and cultured until siblings reached tadpole stages. Siamois expression in animal cells stimulates cement gland formation, and this effect is not suppressed by co-expression of HD-enR. Arrowheads point at cement glands.*

the somites and neural tube still form in HD-enR injected embryos (Fig. 4F). In conclusion, although the exact fate of dorsal cells lacking Xlim1 function is not clear, it appears that organizer formation does not occur in the absence of this gene.

The general requirement for Xlim1 in dorsal gene expression could indicate that this gene is acting very early in the genetic cascade leading to the formation of the Spemann-Mangold organizer. The earliest known zygotic actor in this cascade is the gene *Siamois*, which is directly activated by maternal cues, can trigger complete axis formation, and is required for the establishment of all dorsal fates (Lemaire *et al.*, 1995; Fan and Sokol, 1997; Kessler, 1997; Darras *et al.*, 1997). Although *Xlim1* expression is first detected several hours after the onset of *Siamois* expression (Lemaire *et al.*, 1995), it was possible that repressive forms of Lim1 artificially blocked the activation of *Siamois* in our injections. However, RT-PCR analysis in embryos injected with high doses of enR-HD demonstrated that *Siamois* expression was not modified compared to uninjected embryos, over a period of 3 hours. This result indicates that the *Lim1* family of genes is required for establishing the organizer program downstream or in parallel to the zygotic factor Siamois.

Xlim1 position in the Spemann-Mangold organizer cascade

In an effort to determine which steps of organizer function depend on Xlim1 activity, we carried out co-injection experiments with repressive Lim1 and factors known to stimulate sequential steps in axis formation. Ventral expression of Siamois led to the development of a complete secondary axis, and the co-expression of repressive HD-enR completely suppressed this effect (Fig. 8 A,B). This experiment confirms that Lim1 is required downstream of Siamois during organizer establishment, as suggested by their respective period of expression. It has been shown that activated forms of Lim1 stimulate expression of the BMP inhibitor Chordin in animal cells (Taira *et al.*, 1994a; Fig. 3), and we show here that Lim1 function is required for normal dorsal expression of *chordin* (Fig. 7A). These data suggest that Lim1 could act downstream of Siamois to establish the program of expression of organizer specific secreted inhibitors. However, it was not known whether these factors can act in absence of Lim1 function to drive dorso-anterior development. Thus, we tested whether axis induction by ventral co-expression of the truncated BMP receptor and the Wnt inhibitor Frzb required Xlim1 function. Unexpectedly, we found that HD-enR could suppress secondary axis development in such conditions, indicating that Lim1 is necessary to relay the action of organizer agents. Consistent with this result, we could show that expression of the resident *Xlim1* gene is activated by this combination of factors (Fig. 8 E,F). In a similar experiment an inhibitory form of Siamois, enR-Sia (Darras *et al.*, 1997), did not antagonize axis formation by these factors (not shown), supporting the notion that the requirement for Xlim1 in this assay is specific. Hence, it appears that Xlim1 is required sequentially, first to induce organizer gene expression downstream of *Siamois*, and second to relay the activity of inhibitors of ventralization.

Discussion

Prior to this study it was not known whether Lim1 was a transcriptional activator or repressor. Here, we show that the Ldb1-lim1-VP16 fusion protein could generate phenotypes similar to those induced by the combination of Xlim1 and Ldb1, indicating that Xlim1 acts as a transcriptional activator in this context. However, we cannot rule out that Lim1 could also act as a transcriptional repressor on particular promoters. Further progress into this issue requires the identification of direct transcriptional targets of Lim1. Likely candidates for such targets include the organizer genes *chordin* and *otx2*, whose expression can be ectopically induced by Xlim1 in animal cells, *goosecoid*, whose regulatory elements contain Xlim1 binding motifs (Mochizuki *et al.*, 2000), and *cerberus*, as expression of *cerr-l*, a murine *cerberus* homologue, depends on Lim1 activity (Shawlot *et al.*, 1998). Here, we show that putative additional targets may exist as the inhibitory versions of Xlim1 can antagonize expression of most organizer genes examined, with the notable exception of *Siamois*. Thus, it will be essential to determine which genes Lim1 directly regulates *in vivo* in order to understand the networks of interactions required for axis formation. This issue can be best addressed in *Xenopus* with the help of inducible versions of Lim1 fusion proteins (Gammil and Sive, 1997; Tada *et al.*, 1998).

We found that the activated version of Lim1 containing the Ldb1 dimerization domain could induce anterior features upon ventral ectopic expression, which has not been observed in previous studies with wild type or mutant Xlim1 (Taira *et al.*, 1994a; Agulnick *et al.*, 1996). This result suggests that this fusion protein acts more potently in antagonizing ventralizing factors, arguing for the critical importance of dimerization of vertebrate LIM homeodomain proteins as described for their *Drosophila* counterparts (Milan and Cohen, 1999; Van Meyel *et al.*, 1999). It is interesting to note that activated Lim1 constructs can induce

trunk development at low doses, and anterior features with no signs of normal trunk formation at higher doses (Fig. 2). These data suggest that Lim1 could be involved in initiating both head and trunk development, and that these two developmental programs are mutually exclusive, as revealed by studies with normal and inhibitory Otx2 proteins (Andreazzoli *et al.*, 1996; Isaacs *et al.*, 1999). This idea is further supported by our finding that Lim1 is required both for head development and for trunk axial fate determination in *Xenopus*. Phenotypic analysis in *Lim1* mutant mice, however, did not reveal a requirement for this gene in notochord formation. A similar situation has been described for another organizer gene, *goosecoid*, which is required for notochord development in *Xenopus*, based on the phenotypes induced by inhibitory versions of this protein (Ferreiro *et al.*, 1998), while no such phenotypes are visible in mutant mice defective for *goosecoid* (Rivera-Perez *et al.*, 1995). This apparent difference between our study and previous work might come from the respective strategies used to abolish the function of Lim1. In our study, it is likely that the activity of Lim1 related proteins are antagonized by the presence of antimorphic forms of Lim1. Thus, the observed phenotypes could be the result of the blockage of multiple Lim1 related proteins in *Xenopus* embryos, while inactivation of a single *Lim1* gene might not be sufficient to generate the same phenotypes in mouse embryos. Moreover, functional redundancy could also occur between Lim1 and transcription factors belonging to different families if they are collectively required to activate target genes (see Perea-Gomez *et al.*, 1999).

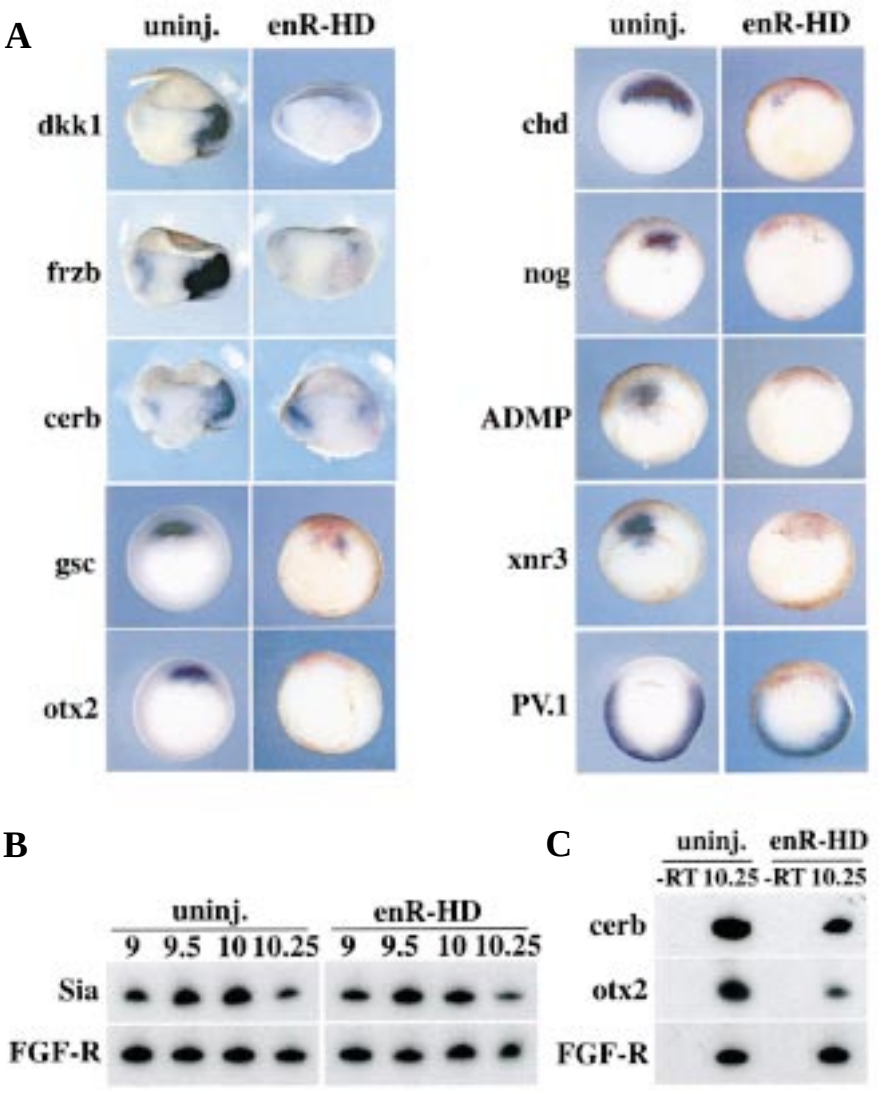

Fig. 7. Repressive form of Lim1 inhibits expression of organizer genes except *Siamois*. *Embryos were injected at the 4-cell stage with a mixture of 100 pg enR-HD and 400 pg lacZ RNAs.* **(A)** *After revealing β-galactosidase activity with a red substrate at stage 10.25/10.5, embryos were processed for in situ hybridization with the indicated probes. In the cases of dkk1, frzb and cerberus, embryos were bisected before hybridization to enhance staining in the deep layers of the embryo. All dorsal genes examined are repressed by enR-HD. However, the ventral-posterior gene PV.1 is not ectopically expressed dorsally. Dorsal is right for dkk1, frzb and cerb panels, and dorsal is up in all other panels.* **(B,C)** *RT-PCR experiments on embryos injected at the 4-cell stage in a dorsal vegetal position with 100 pg enR-HD RNA. This site of injection targets cells that express the organizer gene Siamois. Panel B shows that expression of Siamois is unchanged in enR-HD injected embryos between stages 9 and 10.25. Panel C shows that the same embryos exhibit reduced levels of expression of cerberus and otx2, confirming that enR-HD was active in this experiment. FGF-R is used as a loading control.*

An important flaw to our demonstration of the developmental requirements for Xlim1 is the lack of phenotypic rescue by co-expression of inhibitory and activator forms of the protein. We could show that the two types of proteins acted antagonistically both in animal cells and in ventral marginal cells. However, this mutual antagonism is not apparent upon dorsal co-expression. A possible complication to this assay is the fact that activated Lim1 molecules generate gastrulation defects on their own, thus combinatorial deleterious effects could prevent phenotypic rescue. However, this is not the only problem, as co-expression of activated and repressive Lim1 constructs in animal explants treated with Activin did not result in the recovery of dorsal marker gene expression (data not shown). Thus, the inhibitory form of Lim1 is dominant over the activator form in every case tested, suggesting that control elements of target genes are irreversibly resistant to activation once they contact a Lim1-enR chimeric protein. Possibly, in the case when several Lim1 binding sites exist in a target gene (see Mochizuki *et al.*, 2000), the presence of a single repressor form of Lim1 could shut off expression of this gene, making rescue difficult to achieve. Although we cannot provide a totally satisfying explanation for the apparent lack of rescue, we do not think that our chimeras act non-specifically, as they do not antagonize other homeoproteins such as Otx2 and Siamois in contexts where Xlim1 is not normally expressed (Fig. 6). Moreover, the phenotypes generated by expression of inhibitory forms of Xlim1 are consistent with the expression pattern of this gene, in particular from a temporal point

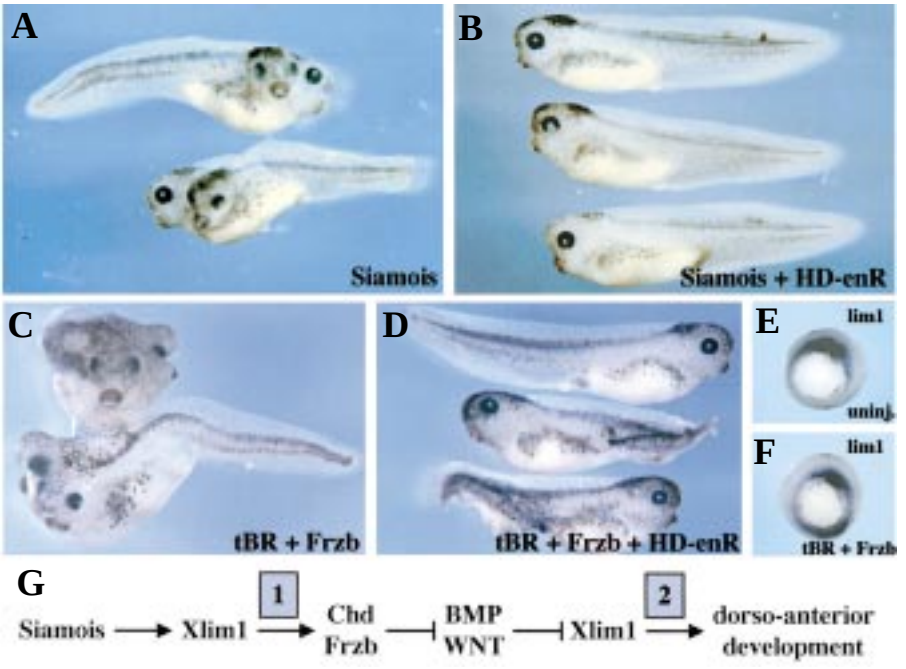

Fig. 8. Position of Lim-1 in the genetic cascade of the Spemann-Mangold organizer. *(A,B) Embryos were injected ventrally at the 4-cell stage with* **(A)** *20 pg Siamois RNA or* **(B)** *with a mixture of 20 pg Siamois and 100 pg HD-enR RNAs. Expression of Siamois leads to formation of a complete secondary axis, and co-expression of the repressive form of Lim1 suppresses this effect. (C,D) Embryos were injected ventrally at the 4-cell stage with* **(C)** *500 pg truncated BMP receptor (tBR) and 400 pg frzb RNAs or* **(D)** *with a mixture of 500 pg tBR, 400 pg frzb and 100 pg HD-enR RNAs. Co-expression of the BMP and Wnt antagonist's tBR and frzb promotes formation of a complete secondary axis, and this effect is inhibited by co-expression of the repressive form of Lim1. (E,F) in situ hybridization with XLim1 probe on* **(E)** *stage 10.5 uninjected embryos or* **(F)** *embryos injected as in C, reveals activation of XLim1 by co-expression of BMP and Wnt antagonists.* **(G)** *Model for XLim1 position within the organizer genetic cascade based on its requirement downstream of known regulators of organizer function. Lim1 activity is critical downstream of Siamois in establishing the organizer (phase 1), and is needed to relay the activity of organizer's inhibitors to allow dorso-anterior development (phase 2).*

of view. We showed that inhibitory forms of Lim1 do not suppress the expression of Siamois, which appears to be the earliest zygotic gene involved in axis formation, and which is normally expressed prior to Xlim1 (Lemaire *et al.*, 1995). In contrast, inhibitory forms of Lim1, in agreement with the fact that Lim1 is normally required for organizer gene expression, suppress axis induction by Siamois. Less anticipated was the observation that Lim1 is also required downstream of organizer factors whose expression is regulated by this gene. These factors, such as Chordin and Frzb, serve to limit or prevent ventralizing activities of BMP and Wnt acting during gastrulation (Jones *et al.*, 1996). We show here that these dorsal factors can induce the ectopic expression of Xlim1 during gastrulation, thereby suggesting the existence of a positive feedback between these genes, in order to enhance the dorsal expression program. Consistent with this idea, axis development is prevented when inhibitory forms of Lim1 are expressed during gastrulation under the control of the cytomegalovirus promoter (data not shown). Thus, our data indicate that Lim1 is a central regulator, required at two critical steps during early development: First, during the short period of organizer establishment where it participates in the activation of early genes. Second, during the phase where the organizer functions to antagonize ventralizing activities and to allocate dorsal fates. It will be interesting to determine whether Lim1 targets are the same during both phases, which would indicate that this factor is required for initiation and maintenance of organizer gene expression. Alternatively, Lim1 could activate a different set of targets at different times, arguing for a sequential progression towards the acquisition of anterior dorsal fates. Interestingly, the second view is supported by recent observations made in *Lim1* mutant mice. Using chimeric mice and explant recombination, Shawlot and colleagues (1999) put forward a double assurance model whereby Lim1 is required in different tissues at different developmental stages in order to impart anterior identity to the embryo. However, targets of Lim1 activity during this process are not known, and the functional analysis of chimeric Lim1 proteins in *Xenopus* should help elucidate this question.

Materials and Methods

Methods used in this paper are standard and were previously described in the following articles. Preparation and microinjection of *Xenopus* embryos: Taira *et al.*, 1994a. Northern blot analysis of animal cap assays: Taira *et al.*, 1992; Taira *et al.*, 1994a. Whole-mount *in situ* hybridization: Gawantka *et al.*, 1995. Whole-mount immunostaining: Darras *et al.*, 1997. RT-PCR: Darras *et al.*, 1997. The primers used were:

otx2 forward	5' GCA CCC AGT CGG TGG GAT ATC 3'
otx2 reverse	5 'CCA CTC TCC GAG CTC ACT TC 3'
siamois forward	5' AAA CCA CTG ATT CAG GCA GAG G 3'
siamois reverse	5' GTA GGG CTG TGT ATT TGA AGG G 3'
cerberus forward	5' GCT TGC AAA ACC TTG CCC TT 3'
cerberus reverse	5' CTG ATG GAA CAG AGA TCT TG 3'
FGF-R forward	5' TTG AAG TCT GAT GCG AGT GA 3'
FGF-R reverse	5' GGG TTG TAG CAG TAC TCC AT 3'

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