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Blimp-1 is an essential component of the genetic program controlling development of the pectoral limb bud

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Abstract

Formation of paired limbs in vertebrate embryos has long been a particularly useful paradigm for the study of pattern formation. Here, we show that Blimp-1, a SET domain and zinc finger-containing transcriptional factor, plays an important role in the development of the pectoral fins of the zebrafish structures that are homologous to forelimbs of amniotes. The *blimp-1* gene is expressed dynamically in the mesenchyme as well as the ectodermal cells of the early fin bud, and later, in the cells of the apical ectodermal ridge (AER) of the outgrowing fin. Consistent with this expression profile, loss of Blimp-1 activity severely impairs fin outgrowth and patterning. We present evidence that *blimp-1* functions downstream of *tbx5* and *fgf24* and therefore is not required for the initial specification of the fin bud primordia. Subsequently, however, its function is necessary for the induction of *fgf10* and *sonic hedgehog* in the mesenchyme. In addition, Blimp-1 activity is absolutely critical for the proper induction of gene expression in the ectoderm and establishment of the AER. Taken together, these results identify an additional layer of control in the genetic pathway that operates in the developing limb and provides novel insights into regulatory mechanisms that organize its pattern.

Keywords: Blimp-1; Limb bud; Zebrafish; Pectoral fin; *tbx5*; *fgf24*; *fgf10*; Apical ectodermal ridge; Zone of polarizing activity; *Sonic hedgehog*

Introduction

Vertebrate limbs begin their development as localized protrusions called limb buds in the lateral plate mesoderm (LPM) that lies along the flank of the embryo. Embryological manipulations in the chick, together with genetic analysis in the mouse, have now provided a basic framework of the kinds of tissue interactions and gene activity that underlies the allocation of cells to the limb primordia and their subsequent growth and differentiation (reviewed in Capdevila and Izpisua Belmonte, 2001). Tbx5, a T-box containing transcriptional regulator, is an evolutionarily conserved and a central determinant of the forelimb bud developmental pathway. Expression of the *Tbx5* gene is thought to be induced in mesenchymal cells of the LPM in response to signaling by the Fgf8 and Wnt2B proteins that emanate from the adjacent intermediate mesoderm (IM) (Cohn et al., 1995; Vogel et al., 1996; Crossley et al., 1996; Kawakami et al., 2001; Ng et al., 2002). It should be noted, however, that recent genetic studies with the mouse do not corroborate such an early role for Fgf8 signaling from the IM in limb bud initiation (Boulet et al., 2004; Perantoni et al., 2005). The Tbx5 expressing cells form the inner core of the limb bud and are enveloped by an epithelial layer of ectodermal cells. Tbx5 is required not only for the initiation of the forelimb bud primordia, but it also controls the outgrowth of the limb (for example, see Rallis et al., 2003; Agarwal et al., 2003) — a process that is dependant on the transfer of information from the mesenchymal cells to the overlying ectoderm and the establishment therein of a signaling center, the AER.

A number of studies have now demonstrated that the formation of the pectoral fins in the zebrafish is regulated by developmental processes that, in many ways, have been conserved during evolution. For instance, a homolog of *tbx5* is expressed in an equivalent domain and acts in a similar manner in the determination of the fin primordia (Tamura et al., 1999; Begemann and Ingham, 2000; Ruvinsky et al., 2000; Ahn et al., 2002; Garrity et al., 2002). Furthermore, the zone of

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polarizing activity (ZPA), another important signaling source which secretes Sonic hedgehog (Shh), that polarizes the limb bud along the antero-posterior axis, is also active in the zebrafish fin bud (Neumann et al., 1999). Despite these and several other similarities, there are also notable differences in the mechanism of morphogenesis of fins and tetrapod limbs. In the latter, AER formation is triggered by inductive signaling mediated by Fgf10 that is secreted from the limb bud mesenchyme. In response to Fgf10, the AER expresses two other Fgf signaling molecules, Fgf4 and Fgf8, which not only maintain the expression of *Fgf10* in the underlying mesenchyme, but also direct the establishment of the ZPA. In mouse *Fgf10* mutants, AER formation does not occur at all and *Shh* is never activated in the ZPA (Min et al., 1998; Sekine et al., 1999). Moreover, conditional inactivation of *Fgf8* function in the early limb ectoderm results in mice with substantial defects in limb formation (Lewandoski et al., 2000). By contrast, zebrafish *fgf10* has a comparatively subservient role in fin development, functioning largely as a maintenance factor for the AER (Norton et al., 2005). As for *fgf8*, it is a relatively late AER marker that does not appear to have a vital role in the formation of the fin (Reifers et al., 1998). These significant points of differences between zebrafish pectoral fin and amniote forelimb development have been postulated to center, largely, on the involvement of an additional Fgf family member, Fgf24, early in the genetic cascade controlling fin bud specification (Fischer et al., 2003). Fgf24 not only directs the expression of *fgf10* in the mesenchyme, but also is essential for establishing *shh* expression in the ZPA. Additionally, Fgf24 is an early marker of the AER, making it a candidate signal responsible for the maintenance of *fgf10* and *shh* expression in the mesenchyme, a role that is analogous to Fgf8 of amniotes.

Regardless of all of these advancements from investigations in different vertebrates, our understanding of many aspects of the limb development pathway is far from complete. In particular, we do not fully understand how distinct patterns of gene expression are established in response to the variety of signals that have been recognized to function in the developing limb bud. During the differentiation of B-cells of the immune system, the transcription factor Blimp-1 (for B-lymphocyte-inducing maturation protein) plays an important role in promoting their conversion into antibody secreting plasma cells (Shapiro-Shelef and Calame, 2004). Our previous work with the zebrafish homolog of Blimp-1, U-boot (Ubo), has shown that its activity is also required for the specification of the slow-twitch muscle fibers in the myotome and the neural crest progenitor cells at the boundary between the epidermis and the neural plate (Roy et al., 2001; Roy and Ng, 2004; Baxendale et al., 2004). *blimp-1* expression is extremely dynamic in embryos of all species examined, indicating that it has multiple functions in a variety of cell and tissue-types during development (de Souza et al., 1999; Chang et al., 2002; Ha and Riddle, 2003; Baxendale et al., 2004; Wilm and Solnica-Krezel, 2005; Vincent et al., 2005; Ng et al., 2006). In line with this, the gene has also been shown to regulate patterning of the gastrula in fish and frogs and differentiation of the tracheal system in the *Drosophila* embryo (de Souza et al., 1999; Wilm and Solnica-Krezel, 2005; Ng et al., 2006). Furthermore, targeted deletion of the *Blimp-1* locus in the mouse results in embryonic lethality, with severe defects in the development of the branchial arches and a complete absence of the primordial germ cells (Vincent et al., 2005; Ohinata et al., 2005). In this report, we demonstrate that *blimp-1* has an additional role in regulating the development of the pectoral fins in the zebrafish embryo. Here, its activity is critically required for the establishment of the ZPA and the AER through the coordination of gene expression programs in the mesenchymal cells as well as the ectoderm. Consequently, in the absence of Blimp-1 activity, specification of the fin primordia progresses normally, but subsequent events of fin outgrowth and patterning are completely arrested.

Materials and methods

Zebrafish strains

The *ubo*^{tp39} and the *shh* null mutant strain *sonic you* (*syu*^{t4}) were isolated in mutagenesis screens at the Max-Planck-Institut für Entwicklungsbiologie, Tübingen (van Eeden et al., 1996; Schauerte et al., 1998). The strain carrying a complete loss-of-function allele of the zebrafish *smoothened* (*smo*) gene, *slow-muscle-omitted* (*smu*^{b641}), was kindly provided by S. Devoto (Barresi et al., 2000).

Morpholino injections

The following antisense morpholinos (MOs) were used: *blimp-1* splice site targeting MO and *fgf24* and *tbx5* MOs targeting their respective translational start sites (Ahn et al., 2002; Fischer et al., 2003; Baxendale et al., 2004). The oligonucleotides were solubilized in sterile water and injected into newly fertilized zebrafish eggs at concentrations ranging from 5 to 10 ng/embryo. Efficacy of the *blimp-1* splice MO was determined by RT-PCR, using primers that are complementary to sequences in exon 1 and exon 5 of the zebrafish *blimp-1* gene. The sequences of the primer pair are as follows: forward primer 5′-TCACTTACCATCTGGACTAGCA-3′, reverse primer 5′-CTTCGGT-TGCTTGCTGCTTG-3′. Sequencing of the amplified band obtained from the morphant embryos revealed the retention of the whole of intron 2 in their mis-spliced mRNA.

In situ hybridization and Alcian blue staining

Whole-mount in situ hybridization was performed following routine protocols. For colorimetric analyses, Digoxigenin (DIG) labeled antisense RNA probes for the following genes were used: *blimp-1* (Baxendale et al., 2004), *tbx5* (Ruvinsky et al., 2000), *fgf24* (Fischer et al., 2003), *fgf10* (Ng et al., 2002), *shh* (Krauss et al., 1993), *dlx2a* (Akimenko et al., 1994) and *fgf8* (Reifers et al., 1998). DIG antisense RNAs, together with those labeled with Fluorescein, were used for the simultaneous detection of *blimp-1* and *tbx5* and *blimp-1* and *dlx2a* expression in the fin primordia. For these double fluorescent in situ hybridization reactions, signals were developed using the Tyramide Signal Amplification (TSA) kit (Molecular Probes), according to the manufacturer's instructions. Alcian blue staining of the fin endoskeleton was done as described previously (Grandel and Schulte-Merker, 1998).

Image analysis and figure preparation

Stained embryos were examined and photographed using a Zeiss compound microscope (Axioplan 2) equipped with a Nikon camera (DMX1200) for digital image capture. Optical sections of the fluorescent in situ hybridization stainings were obtained using a Zeiss LSM confocal microscope. Figures were assembled using Adobe Photoshop 6.01.

Results

blimp-1 is dynamically expressed in the developing zebrafish pectoral fin bud

During embryogenesis in the zebrafish, *blimp-1* is expressed in a variety of cells and tissues that include the developing pectoral fin anlagen, although the details of the pattern have not been reported previously (Baxendale et al., 2004; Wilm and Solnica-Krezel, 2005). We performed whole-mount *in situ* hybridization to fully document the temporal profile and the spatial domain of *blimp-1* expression in the pectoral fin bud. Expression in this region is first detected very weakly at approximately 20 h post fertilization (hpf); the levels gradually intensify, such that by 24 hpf, prominent *blimp-1* expression can be observed in a distinct cluster of cells at the site of the forming fin bud (Fig. 1A). Lateral views of stained embryos at this stage clearly reveal that the expression is localized to the inner mesenchymal layer (Fig. 1B). *blimp-1* expression also appears to be present in the overlying ectodermal cells (Fig. 1B). The ectodermal expression is even more apparent at 30 hpf, when it assumes a crescent-shaped pattern, whereas the expression in the mesenchyme dramatically decreases by this time (Fig. 1D). This is in contrast to the *tbx5* gene, whose expression is always restricted exclusively to the mesenchyme (Fig. 1C). By 36 hpf, *blimp-1* expression evolves into stronger levels and localizes to the apical fin fold — the zebrafish equivalent of the AER of amniote embryos (Fig. 1E). The expression in the AER refines into a narrow fringe by 48 hpf, at the extreme edge of the outgrowing fin (Fig. 1F). At 72 hpf, *blimp-1* expression is almost completely extinguished from the differentiating pectoral fin (Fig. 1G).

To more precisely visualize the spatial domains of this dynamic profile of *blimp-1* expression, we performed double label fluorescent in situ hybridization. At 24 hpf, we found *blimp-1* transcripts in the ectoderm and in the underlying mesenchyme, where it colocalized with *tbx5* (Figs. 2A–C). At 30 hpf, however, the crescent-shaped pattern of *blimp-1* superimposed almost entirely with *dlx2a* (Figs. 2D–F), a zebrafish homologue of the *distal-less* family of homeobox genes that is an early marker of the fin ectoderm and is expressed in the AER of all vertebrates (Akimenko et al., 1994). These observations unequivocally confirm the notion that *blimp-1* is expressed in the fin bud mesenchyme as well as the ectoderm in a developmental stage-dependent manner. In the early fin bud, *blimp-1* in transcribed in the mesenchyme and

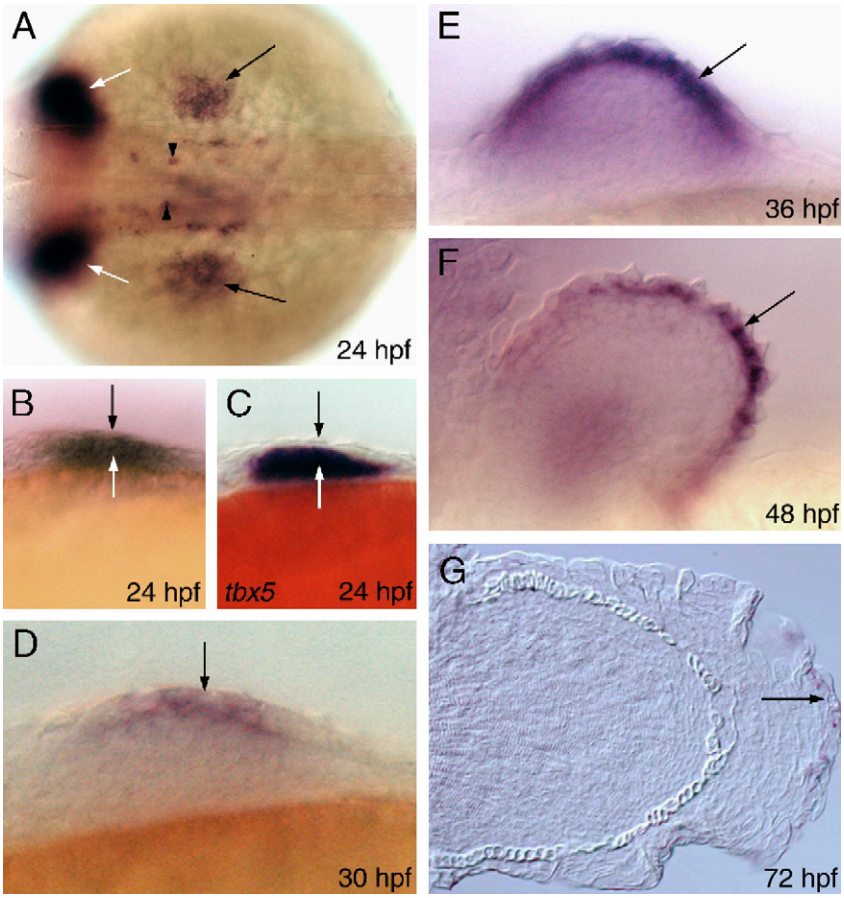

Fig. 1. Spatio-temporal profile of *blimp-1* expression in the developing pectoral fin. (A) A wild-type embryo, showing *blimp-1* expression in the fin bud (black arrows). Expression in the pharyngeal endoderm (white arrows) and spinal cord neurons (arrowheads) is also indicated. (B) Expression at this stage is evident in the ectoderm (black arrow) and the mesenchyme (white arrow). (C) *tbx5* expression shows localization only to the mesenchyme (white arrow) and is excluded from the ectoderm (black arrow). (D) *blimp-1* expression in the mesenchyme decreases and strengthens in the ectoderm (arrow). (E–G) *blimp-1* expression in succeeding stages of embryogenesis. Panel A depicts dorsal view, all others depict lateral views. All panels of this and subsequent figures are oriented anterior to the left.

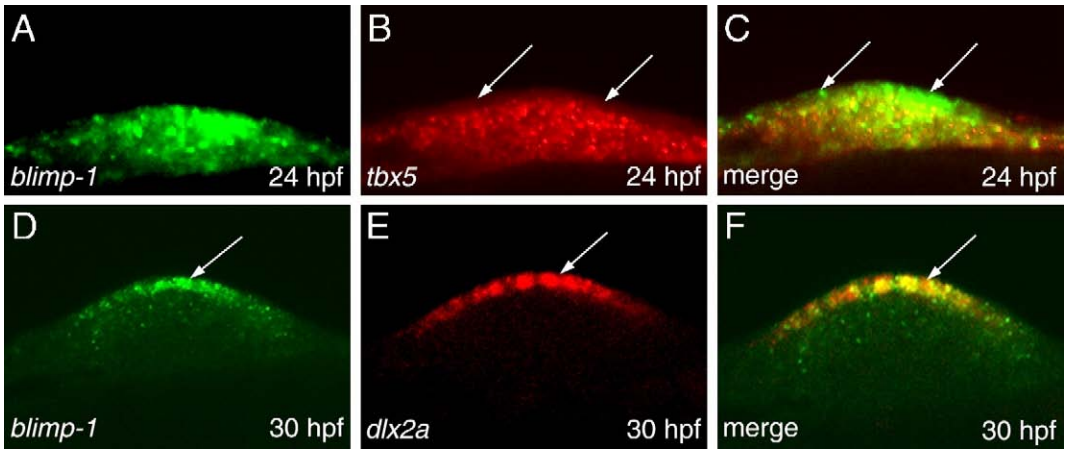

Fig. 2. Comparative analysis of the mesenchymal and ectodermal components of *blimp-1* expression with respect to *tbx5* and *dlx2a* using double label fluorescent in situ hybridization. (A) A wild-type embryo, showing *blimp-1* expression in the fin bud. (B) The same embryo showing *tbx5* expression in the fin bud. The ectoderm, which is devoid of *tbx5* transcripts, is indicated (arrows). (C) Superimposition of the images depicted in panels A and B, showing colocalization of *blimp-1* and *tbx5* signals in the mesenchyme, while ectodermal cells only contain *blimp-1* transcripts (arrows). (D–F) *blimp-1* and *dlx2a* are co-expressed in cells of the AER from 30 hpf (arrow). All panels depict lateral views.

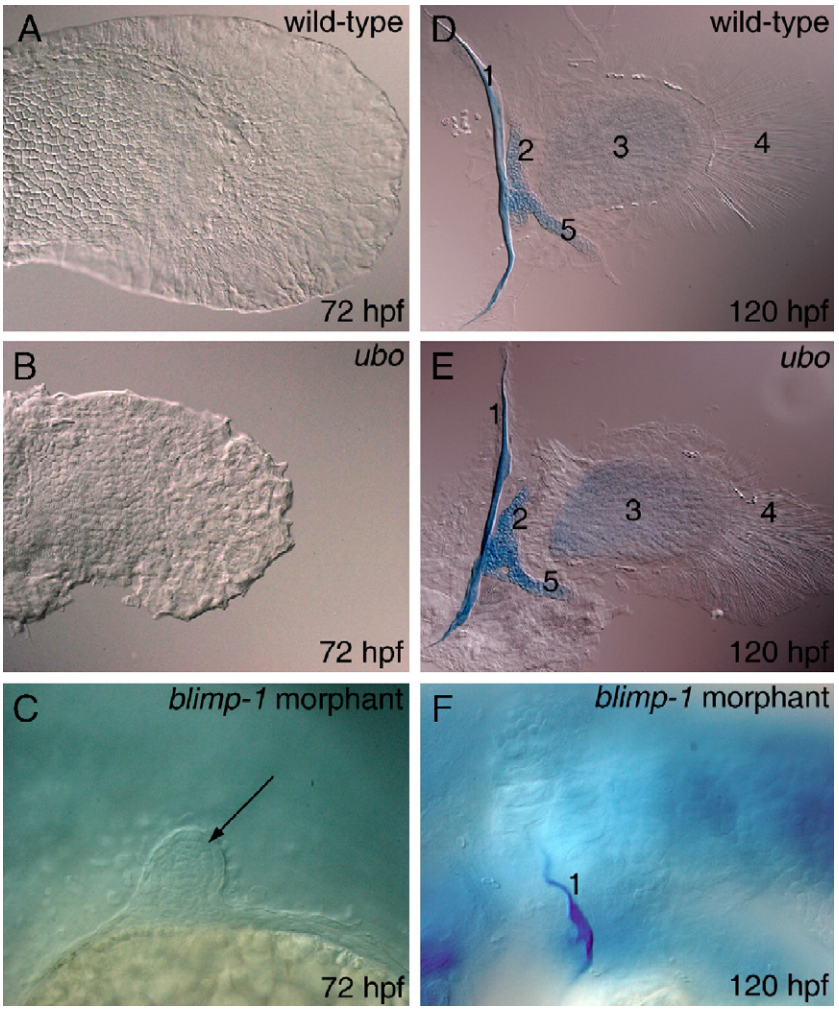

Fig. 3. Pectoral fin development is affected in embryos compromised in Blimp-1 activity. (A) Normarski image of a wild-type fin. (B) Fin of a homozygous *ubo* mutant embryo. (C) Rudimentary fin bud of a *blimp-1* morphant embryo. (D–F) Alcian blue stain of the pectoral fin skeletal elements of a wild-type, *ubo* mutant and *blimp-1* morphant embryo. 1 = cleithrum, 2 = scapulocoracoid, 3 = endoskeletal disk, 4 = actinotrichs, 5 = postcoracoid process. All panels show lateral views.

in the ectoderm, whereas during later period of fin outgrowth and differentiation, its expression is limited to the ectodermal cells of the AER.

blimp-1 is required for the development of the pectoral fin

The dynamic pattern of *blimp-1* expression in the fin mesenchymal cells as well as the ectoderm is suggestive of its requirement for the specification and/or the proper outgrowth of the fin bud. To analyze this, we examined the pectoral fins of embryos homozygous for a hypomorphic allele of the *ubo* locus that impairs wild-type activity of the Blimp-1 protein (Roy et al., 2001; Baxendale et al., 2004). At 72 hpf, these embryos exhibit variably shortened pectoral fins with very irregular edges compared to that of their normal siblings, indicating that Blimp-1 function is indeed necessary for proper development of the pectoral fin (Figs. 3A, B; see Table 1 for a quantitative analysis of this and all other fin phenotypes described in this paper).

We reasoned, however, that the hypomorphic nature of the *ubo* mutation is likely to mask a more critical function of *blimp-1* in the formation of the pectoral fin. We have previously demonstrated that the mis-specification phenotype of the slow-twitch muscle precursors as well as the progenitors of the neural crest is less severe in *ubo* embryos and contrasts with the more dramatic effects that are observed in these cells when expression of the Blimp-1 protein is "knocked down" using antisense MOs against the *blimp-1* gene (Roy and Ng, 2004; Baxendale et al., 2004). Indeed, RT-PCR analysis revealed the occurrence of aberrant splicing of *blimp-1* pre-mRNA in the morphants (i.e. embryos injected with splicing inhibitory anti-*blimp-1* MOs), leading to the retention of the whole of intron 2 (Figs. 4A, B). If translated, such a mis-spliced mRNA is predicted to produce a severely truncated and non-functional Blimp-1 protein (Fig. 4B). Consistent with this, the morphant embryos almost completely lack any visible signs of fin outgrowth; when examined at 72 hpf, these embryos exhibit a very small and undifferentiated tubercular structure in the region where the pectoral fin is normally located (Fig. 3C; see also Wilm and Solnica-Krezel, 2005). The internal skeleton of the wild-type fin consists of a series of cartilaginous elements — the cleithrum, scapulocoracoid and endoskeletal disk, arranged in that order along the proximo-distal axis, respectively (Fig. 3D) (Grandel and Schulte-Merker, 1998). Although fins of *ubo* mutants contain all of these elements (Fig. 3E), albeit sometimes reduced in size, the rudimentary fin buds of *blimp-1* morphant embryos

Table 1
Quantitative analysis of the effects of the different mutants and morphants on gene expression in the developing fin bud and morphology of the differentiated pectoral fin

Phenotype	Genotype and number examined (*n*)	Penetrance (and expressivity)
Fin morphology at 72 hpf	*ubo*=18	100% with fin truncation (extent of fin truncation was variable)
	blimp-1 morphants=35	100% with rudimentary fin bud
Fin skeleton at 120 hpf	*ubo*=8	37.5% showed shortened scapulocoracoid (shortening was variable) the rest appeared wild-type
	blimp-1 morphants=7	100% showed absence of elements distal to cleithrum
tbx5 expression at 30 hpf	*blimp-1* morphants=12	100% showed wild-type like expression
tbx5 expression at 36 hpf	*blimp-1* morphants=10	40% showed slight reduction in levels, the rest appeared wild-type
tbx5 expression at 48 hpf	*blimp-1* morphants=11	63.6% showed reduction in levels, the rest appeared wild-type
fgf24 expression at 24 hpf	*blimp-1* morphants=12	100% showed wild-type like expression
fgf24 expression at 48 hpf	*blimp-1* morphants=14	100% showed absence of expression in ectoderm and sustained expression in mesenchyme
blimp-1 expression at 24 hpf	*tbx5* morphants=21	100% showed complete absence from the fin bud region
	fgf24 morphants=12	100% showed complete absence from the fin bud region
	syu=15	100% showed wild-type like expression
	smu=15	100% showed wild-type like expression
blimp-1 expression at 36 hpf	*syu*=12	100% showed reduction in levels
	smu=10	100% showed reduction in levels
blimp-1 expression at 48 hpf	*syu*=8	100% showed reduction in levels
	smu=10	100% showed reduction in levels
fgf10 expression at 26 hpf	*ubo*=12	66.6% showed reduced expression, the rest appeared wild-type
	blimp-1 morphants=17	100% showed strong reduction
fgf10 expression at 30 hpf	*ubo*=19	20% showed reduced levels, the rest appeared wild-type
	blimp-1 morphants=16	100% showed strong reduction
fgf10 expression at 36 hpf	*ubo*=12	100% showed more or less wild-type levels of expression
	blimp-1 morphants=19	31.6% showed complete absence, the rest showed very strong reduction
shh expression at 30 hpf	*ubo*=11	100% showed reduced levels of expression
	blimp-1 morphants=18	100% showed complete absence of expression
shh expression at 48 hpf	*ubo*=9	100% showed reduced expression
	blimp-1 morphants=21	100% showed complete absence of expression
dlx2a expression at 32 hpf	*ubo*=18	100% showed reduced expression
	blimp-1 morphants=17	100% showed complete absence of expression
dlx2a expression at 36 hpf	*ubo*=13	100% showed reduced expression
	blimp-1 morphants=24	100% showed absence of expression
fgf8 expression at 38 hpf	*ubo*=18	66.7% embryos showed strong reduction in levels, the rest showed complete absence of expression
	blimp-1 morphants=15	100% showed absence of expression

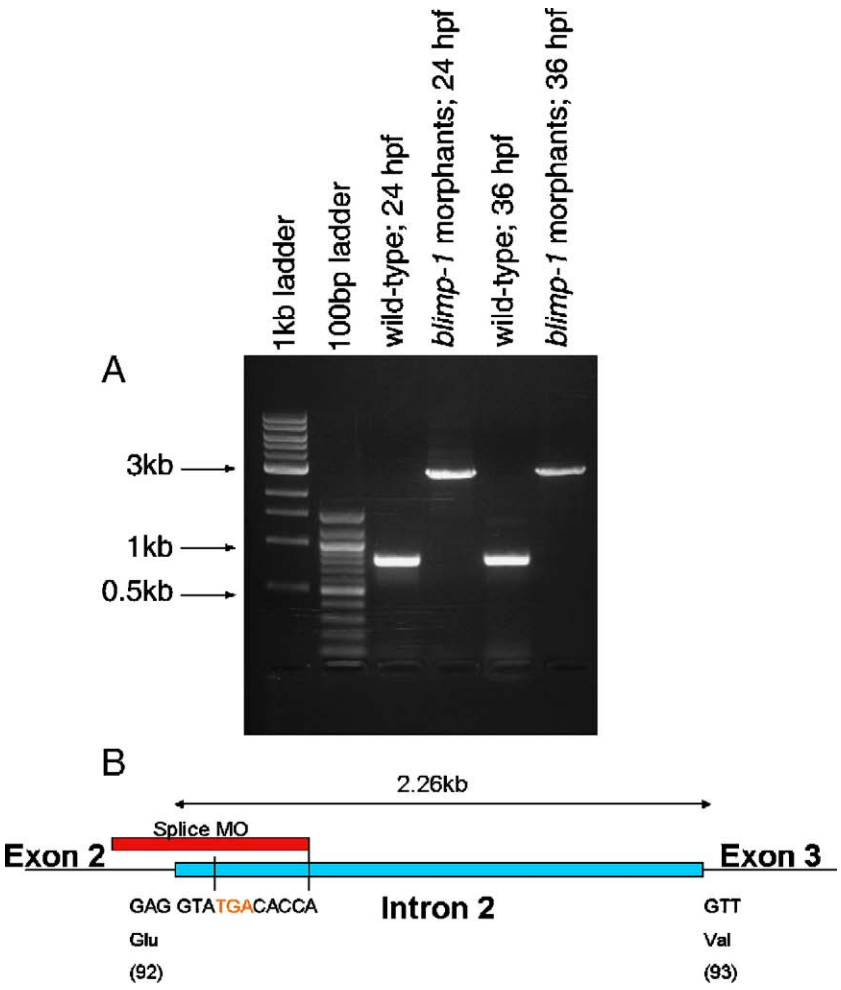

Fig. 4. Splice junction targeted anti-*blimp-1* MOs effectively block splicing of *blimp-1* pre-mRNA. (A) RT-PCR of mRNA extracted from wild-type embryos and *blimp-1* morphants, showing the expected 783 bp band in the wild-type lanes and an approximately 3 kb band in those of the morphants. The primer pair used for the PCR reaction amplifies across exons 1–5 (see Materials and methods). (B) Diagram illustrating the target site of the MO, at the junction between exon 2 and intron 2. The last codon of exon 2 and the first codon of exon 3, together with their corresponding amino acid, are indicated. The premature stop codon in the mis-spliced *blimp-1* mRNA is highlighted in red.

completely lack the skeletal structures distal to the cleithrum (Fig. 3F). Based on all of these observations, we conclude that Blimp-1 plays a crucial role in the development of the pectoral fin.

blimp-1 acts downstream of tbx5 and fgf24 in the development of the fin primordium

In order to position *blimp-1* in the genetic pathway that regulates the specification and patterning of the pectoral fin, we first analyzed the expression of two important genes, *tbx5* and *fgf24*, that act early in the induction of the fin primordium, in embryos compromised in Blimp-1 activity. A comparative study of the onset of expression of the three genes, i.e. *tbx5*, *fgf24* and *blimp-1*, suggests that *blimp-1* is likely to function downstream from *tbx5* as well as *fgf24*. While the earliest time point of *blimp-1* expression in the fin mesenchyme is 20 hpf, *fgf24* is observed in this region from 18 hpf (Fischer et al., 2003) and *tbx5* from 17 hpf (Begemann and Ingham, 2000; Ruvinsky et al., 2000). Consequently, we found that the expression of *tbx5* at early stages of fin primordia formation occurs normally in *ubo* mutants as well as *blimp-1* morphant embryos, confirming that the activation of this gene in the fin mesenchyme is independent of Blimp-1 activity (Figs. 5A, B; data not shown). Later in development, although the pattern of *tbx5* in *ubo* mutants and their wild-type siblings appears indistinguishable, the levels are discernibly reduced and the domain of expression smaller in the *blimp-1* morphant embryos (Figs. 5C–F; data not shown). In this respect, our results contradict an earlier preliminary observation that had implicated a role for *blimp-1* upstream of *tbx5* (Wilm and Solnica-Krezel, 2005).

The expression of *fgf24* in the fin mesenchymal cells of *ubo* embryos and *blimp-1* morphants appears identical to wild-type embryos at 24 hpf (Figs. 5G, H; data not shown). Unlike *tbx5*, whose expression remains confined to the mesenchyme throughout fin development, *fgf24* expression normally declines in these cells between 28 and 30 hpf and reappears

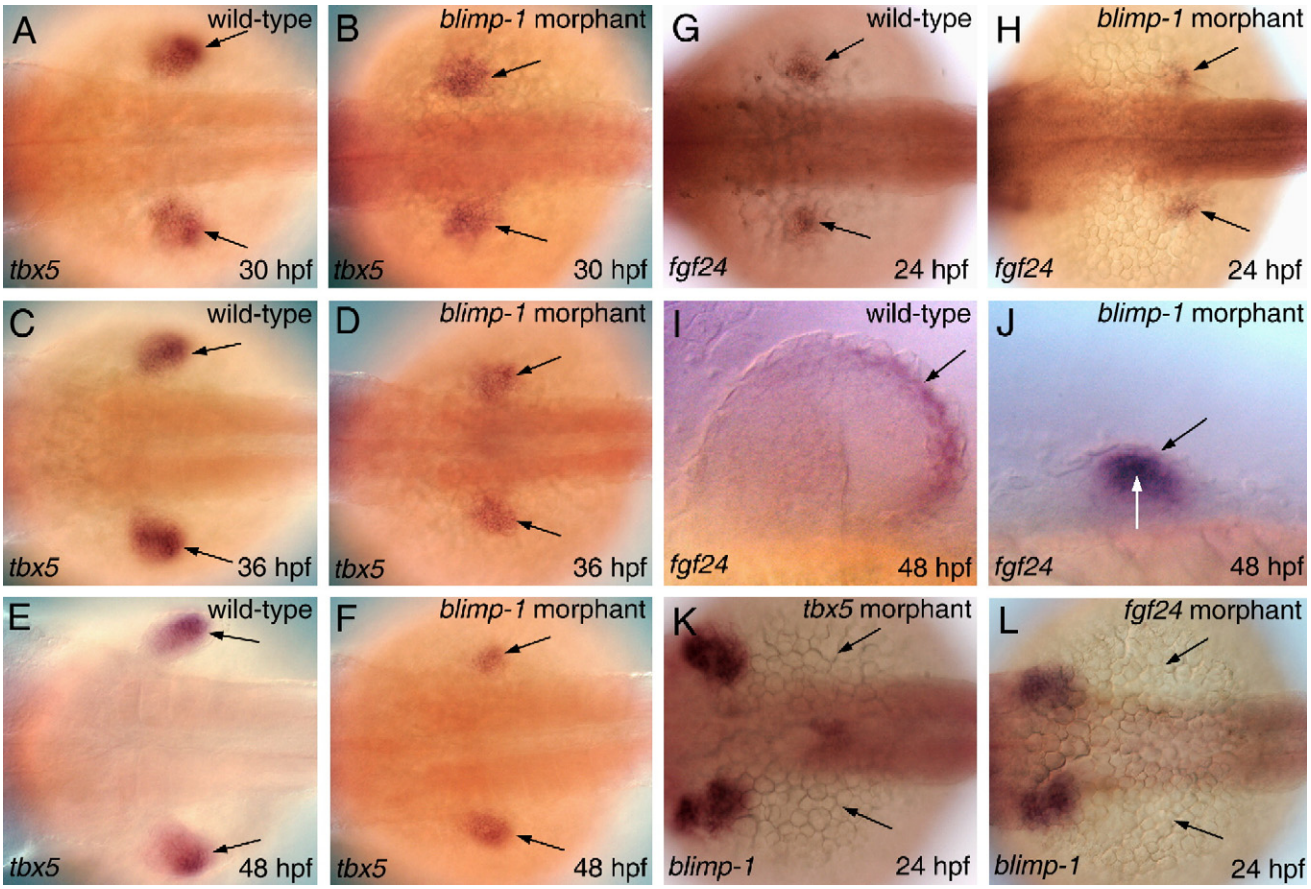

Fig. 5. *blimp-1* acts downstream of *tbx5* and *fgf24*. (A–F) *tbx5* expression in the fin buds (arrows) of wild-type and *blimp-1* morphants. (G and H) *fgf24* expression (arrows) in a wild-type embryo and a *blimp-1* morphant. (I) A wild-type fin, showing *fgf24* expression in the AER (arrow). (J) A *blimp-1* morphant at the same stage, showing absence of *fgf24* from the ectoderm (black arrow) and its continued expression in the mesenchyme (white arrow). (K and L) *blimp-1* expression is absent (arrows) from the prospective fin buds of *tbx5* and *fgf24* MO injected embryos. All panels depict dorsal views, except panels I and J, which depict lateral views.

in the AER (Fischer et al., 2003). In line with this, when wild-type embryos were examined at 48 hpf, we found prominent expression of *fgf24* in the AER and complete absence from the fin mesenchyme (Fig. 5I). A similar pattern of *fgf24* was observed in *ubo* mutant embryos, although some of them showed a lower level of expression than that apparent among their wild-type siblings (data not shown). Strikingly, in *blimp-1* morphants, *fgf24* sustains its expression in the mesenchymal cells and fails to get activated in the fin bud ectoderm (Fig. 5J).

We have also made the reciprocal analysis of the status of *blimp-1* transcription in the fin primordia of embryos that are depleted of the Tbx5 and Fgf24 proteins. We failed to observe any *blimp-1* expression in *tbx5* and *fgf24* morphants in the region of the prospective fin bud at all stages of embryogenesis (Figs. 5K, L; data not shown). These data confirm the view that *blimp-1* operates downstream of *tbx5* and *fgf24* in the early genetic cascade that specifies the pectoral fin bud.

Absence of Blimp-1 function prevents proper induction of fgf10 in the mesenchyme

We next investigated the effects of the loss of Blimp-1 on the expression of *fgf10* in the fin bud mesenchyme. During normal development, *fgf10* expression follows *fgf24* and can be first detected in the mesenchymal cells at 24 hpf (Ng et al., 2002; Fischer et al., 2003). Moreover, loss of *fgf24* completely inhibits *fgf10* expression (Fischer et al., 2003). Since *blimp-1* expression also requires Fgf24 activity, this would indicate that Blimp-1 could be needed for inducing the expression of *fgf10* in the mesenchyme, in response to Fgf24 signaling. In line with such a possibility, we found that, at 26 hpf, the levels of *fgf10* transcripts are reduced in the fin buds of *ubo* mutant embryos compared to their wild-type siblings (Figs. 6A, B), although the expression levels appeared more or less comparable later, at 30 and 36 hpf (Figs. 6D, E, G, H). In *blimp-1* morphants, which represent a much stronger loss-of-function condition, there was a considerable reduction of *fgf10* expression in the mesenchymal cells at all of these stages of development (Figs. 6C, F, I). Thus, a primary defect in the fin buds of embryos lacking Blimp-1 activity is their inability to properly institute the expression of *fgf10* in the mesenchyme.

Blimp-1 induces shh in the ZPA and requires Hh signaling for the maintenance of its own expression

In amniotes, induction of *Shh* expression and formation of the ZPA in the posterior mesenchymal cells are directed by the Fgf proteins secreted from the AER (Sun et al., 2002;

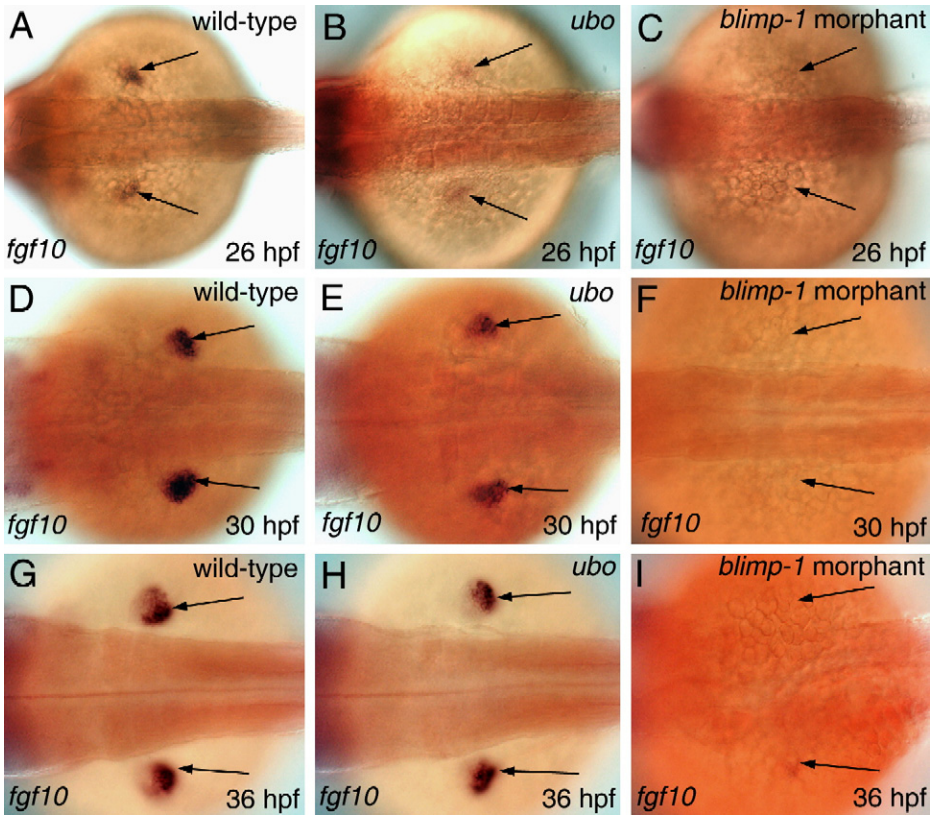

Fig. 6. Blimp-1 activity is required for *fgf10* expression in the fin bud mesenchyme. (A–I) *fgf10* expression in the fin mesenchyme (arrows) of wild-type, *ubo* and *blimp-1* morphant embryos at specific developmental stages. All panels depict dorsal views.

Boulet et al., 2004). Instead, in the zebrafish embryo, *shh* expression is initiated differently, by Fgf24 signaling, derived from the mesenchyme (Fischer et al., 2003). Here, the AER seems to be only involved in the maintenance of *shh* expression in the ZPA. Since Blimp-1 functions downstream of *fgf24* and is required for the full activation of *fgf10* expression, we explored whether embryos lacking *blimp-1* function also show a loss of *shh* expression from the ZPA. *shh* is expressed at lower levels in *ubo* embryos compared to their wild-type counterparts at all developmental stages analyzed, indicating that activation of the *shh* gene requires a threshold level of Blimp-1 activity that is reduced in the *ubo* embryos (Figs. 7A–E). Consistent with this, in the *blimp-1* morphants, *shh* expression is never observed in the pectoral fin primordia (Figs. 7C, F). Thus, Blimp-1 is also required for the activation of *shh* expression and the establishment of the ZPA in the posterior mesenchymal cells of the developing fin bud.

We also examined the reciprocal consequence: that of the loss of Hh signaling on *blimp-1* expression in the fin bud. We have previously shown that *blimp-1* is activated in the precursors of the slow-twitch muscles within the somites of the zebrafish embryo in response to Hh signaling that emanates from midline tissues (Baxendale et al., 2004). It is apparent from data presented here that, in the pectoral fin buds, *blimp-1* expression precedes the onset of *shh* in the ZPA. Whereas *blimp-1* initiates as early as 20 hpf, *shh* is first detected in the ZPA around 28 hpf (Krauss et al., 1993). This would implicate that, in the context of the fin, Hh signaling is not required for the induction of *blimp-1*. Indeed, embryos lacking activity of Shh or Smoothened (Smo), a transmembrane protein that is essential of the intracellular transduction of the Hh signal, showed normal levels and pattern of *blimp-1* transcription in the fin primordia at 24 hpf (Figs. 7G, J). However, Hh activity does play a role in the maintenance of *blimp-1* expression through the succeeding stages of fin development as evidenced by the progressive decline in *blimp-1* transcription in the absence of Hh pathway activity (Figs. 7H–L).

Blimp-1 activity is essential for the specification of the AER

Since Fgf10 signaling relays inductive information from the mesenchyme to the ectoderm and its activity is necessary for the proper development of the AER, we reasoned that loss of Blimp-1 function, which affects *fgf10* expression, should also affect the AER. More importantly, *blimp-1* is itself actively transcribed in the ectoderm and in the AER from early stages of fin development, signifying that it could directly influence the expression of marker genes in the ectoderm and, consequently, the formation of the AER. The fact that ectodermal gene expression indeed does get affected in the absence of Blimp-1 function is already borne out from our earlier observation that *fgf24* fails to get activated in the

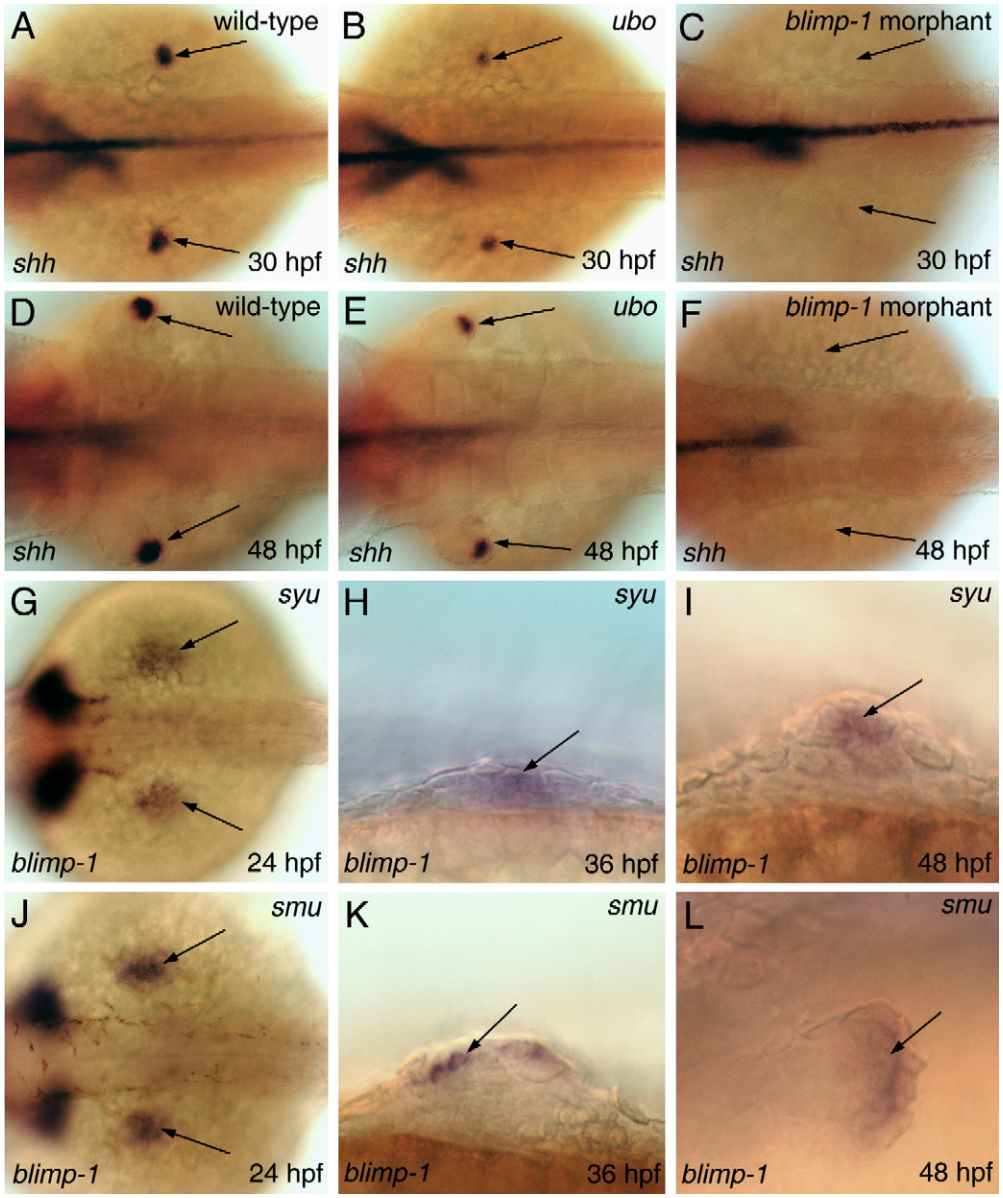

Fig. 7. Blimp-1 function is necessary for *shh* expression whereas Hh signaling is required for maintenance, but not the initiation, of *blimp-1* expression. (A–F) *shh* expression (arrows) in the ZPA of wild-type, *ubo* mutant and a *blimp-1* morphant embryos at specific developmental stages. (G–I) *blimp-1* expression (arrows) in embryos lacking Shh activity. (J–L) *blimp-1* expression (arrows) in embryos lacking Smo activity. Panels A–F, G and J depict dorsal views; panels H, I, K and L show lateral views.

ectoderm in *blimp-1* morphant embryos (Fig. 5J). Analysis of the expression of *dlx2a* showed that it is quite noticeably reduced in the fin buds of the *ubo* mutants and is totally undetectable in the *blimp-1* morphant embryos (Figs. 8A–F).

The *fgf8* gene, which is the definitive marker of the AER, is activated in the zebrafish fin bud much later compared to that in birds and mammals (Reifers et al., 1998). In wild-type embryos, *fgf8* is first detectable in cells of the AER around 38 hpf, at about the time the AER becomes morphologically distinguishable (Fig. 8G). *fgf8* expression is barely visible in the fin buds of *ubo* mutants and is completely absent from those of the *blimp-1* morphants (Figs. 8H, I). These dramatic effects on *dlx2a* and *fgf8* expression underscore a pivotal role for Blimp-1 in the specification of the AER.

Discussion

We have identified that the transcription factor Blimp-1 is a novel component of the regulatory pathway that directs the development of the pectoral fin in the zebrafish embryo. The spatio-temporal expression of *blimp-1* in the fin bud mesenchyme and epistasis analysis allowed us to position the gene downstream of *tbx5* and *fgf24*, but upstream of *fgf10* in the fin development pathway. Accordingly, we have shown that loss of Blimp-1 interrupts fin development at an early stage, immediately following the establishment of the fin primordia, precluding proper initiation of *fgf10* expression in the fin bud mesenchyme and the establishment of the ZPA. As a consequence, ectoderm and AER markers genes like *dlx2a*, *fgf24*

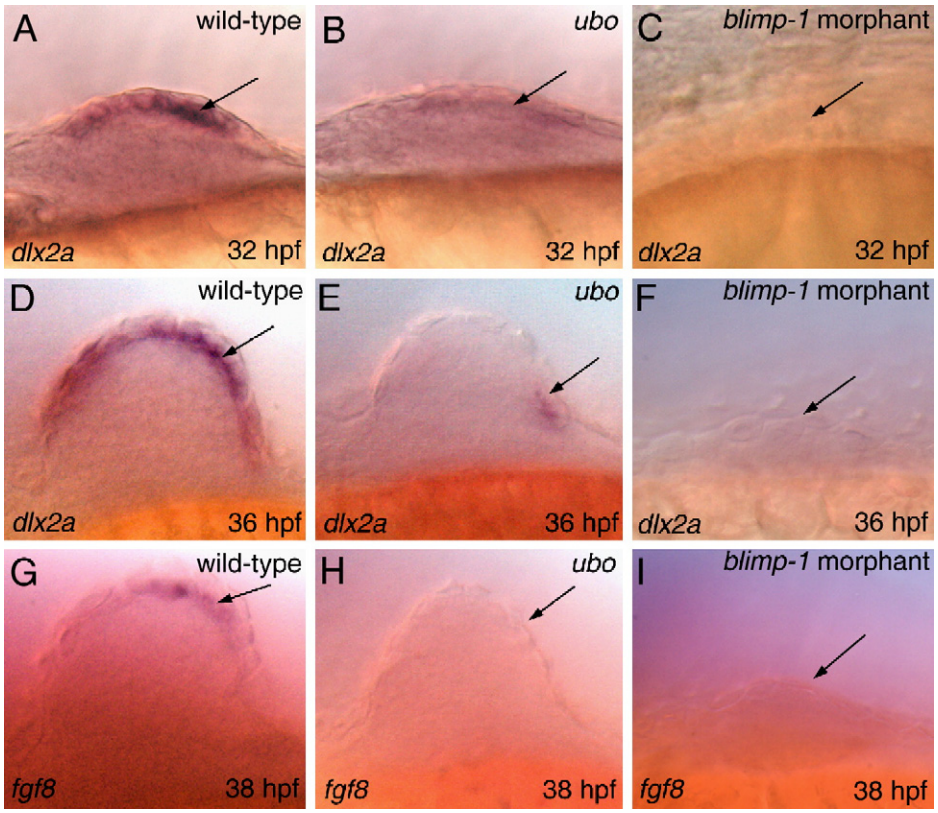

Fig. 8. Blimp-1 activity is crucial for the establishment the AER. (A–F) *dlx2a* expression in the AER (arrow) of a wild-type, *ubo* mutant and a *blimp-1* morphant embryo. (G–I) *fgf8* expression in the AER (arrow) of a wild-type, *ubo* mutant and a *blimp-1* morphant embryo. All panels depict lateral views of developing pectoral fin buds.

and *fgf8* that depend on Fgf10 signaling are completely down-regulated. However, our observation that *blimp-1* is also one of the earliest markers of the fin ectoderm and, subsequently, the AER itself, denotes that the Blimp-1 protein has an independent role in activating gene expression here that is distinct from its function in the mesenchyme. Evidence for such a scenario is derived from the examination of AER development in embryos homozygous for the hypomorphic *ubo* mutation that reduces, but does not completely eliminate, the activity of the Blimp-1 protein. In these animals, specification of the fin bud and induction of *fgf10* progresses almost normally, but the expression of AER-specific genes such as *dlx2a* and *fgf8* are appreciably reduced and distal elements of the mature fin are truncated.

To integrate all of our findings, we propose that Fgf24 signaling is responsible for the activation of the *blimp-1* gene in the mesenchyme as well as in the ectodermal cells of the fin bud. The Blimp-1 protein then participates, directly or indirectly, in the induction of the expression of *fgf10* and *shh* in the mesenchymal cells, as well as genes such as *dlx2a* and *fgf24* in the ectoderm of the early fin bud. Further progression of fin development then becomes dependant on signaling by Fgf10 from the mesenchyme. In this model, maintenance of *blimp-1* expression in the AER by Fgf10 will result in the maintenance of *fgf24* in this tissue, and subsequently, in the induction of *fgf8* and *fgf4*. Secretion of all of these Fgf proteins from the AER will, in turn, ensure the continued expression of *fgf10* in the mesenchyme and *shh* in the ZPA, eliciting the feedback loop of gene expression that is necessary for the outgrowth and differentiation of the fin. Veracity of this model comes from the observation of fin development in the recently described *fgf10* mutants that are devoid of the Fgf10 protein (Norton et al., 2005). Here, in contrast to Blimp-1 deficient embryos, the early phase of gene expression in the fin ectoderm and the initiation of the AER and the ZPA occur almost normally. However, all of these fail to be maintained in the succeeding stages of embryogenesis. Ultimately, they are totally lost, and fin outgrowth is completely inhibited. On similar lines, we have shown that, although the initiation of *blimp-1* expression in the fin primordia precedes the onset of *shh* in the ZPA and is regulated independently of Shh activity, Hh signaling is nevertheless required for the continued expression of *blimp-1* through the later stages of fin outgrowth.

We note that the homologs of *blimp-1* have previously been observed to be expressed in both fore- and the hindlimb buds of the developing chick and the mouse embryo (Chang et al., 2002; Ha and Riddle, 2003; Vincent et al., 2005). In the chick, expression is always restricted to the ectoderm — it originates in the dorsal ectoderm and then becomes prominent in the AER. In the mouse, *Blimp-1* transcripts are first present throughout the limb buds and then shift to the posterior region that includes the ZPA. Expression is also prevalent in the AER. These species-specific disparities in the expression pattern parallel the differences that are evident in the requirement of the gene in the limb development program of different animals. For example, *Blimp-1* activity is not only dispensable for the

initial events of limb bud specification in the mouse, but unlike in the zebrafish, it also appears not to be necessary for the formation of the ZPA and the AER (Vincent et al., 2005). However, the possibility does remain that *Blimp-1* has an important later role in patterning mammalian limbs that is obscured by the premature lethality of the mutant embryos. Ablation of its activity specifically in the limb precursor cells should help to fully clarify this issue. Nevertheless, irrespective of what the precise role of Blimp-1 might be in the limb buds of amniotes, the lack of an early phenotype in the mouse helps to reinforce the idea that alterations in the regulation and function of genes and their networks are likely to be the developmental basis for the morphological diversification of appendages apparent in the different groups of vertebrates.

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