Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction

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There were mistakes in the Materials and methods ‘Treatment with DEAB, SU5402’ section on p. 2806 of this paper. The whole section should therefore be replaced with the following.

4-Diethylaminobenzaldehyde (DEAB) (Fluka) and the FGF receptor inhibitor SU5402 (Calbiochem) were dissolved in dimethylsulfoxide (DMSO) and used at a concentration of 10 \( \mu \)M and 16 \( \mu \)M, respectively. DEAB treatment was performed from 30% epiboly onwards. Incubations were carried out in the dark at 28°C.

The authors apologise to readers for the mistakes.
Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction

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Vertebrate limb induction is triggered in the lateral plate mesoderm (LPM) by a cascade of signaling events originating in the axial mesoderm. While it is known that Fgf, Wnt and retinoic acid (RA) signals are involved in this cascade, their precise regulatory hierarchy has not been determined in any species. tbx5 is the earliest gene expressed in the limb bud mesenchyme. Recently, another transcription factor, Prdm1, has been shown to be crucial for zebrafish forelimb development. Here, we show that Prdm1 is downstream of RA, Wnt2b and Tbx5 activity. We find that RA activity, but not Fgf signaling, is necessary for wnt2b expression. Fgf signaling is required for prdm1 expression in the fin bud, but is not necessary for the initiation of tbx5 expression. We propose a model in which RA signaling from the somitic mesoderm leads to activation of wnt2b expression in the intermediate mesoderm, which then signals to the LPM to trigger tbx5 expression. Tbx5 is required for Fgf signaling in the limb bud leading to activation of prdm1 expression, which in turn is required for downstream activation of fgf10 expression.

KEY WORDS: Limb development, Prdm1, Tbx5, Retinoic acid, Fgf, Wnt2b

INTRODUCTION

The initial step in organogenesis is the specification of a small group of cells at a defined location within the embryo, which then develop into a mature organ. The vertebrate limb is an excellent model with which to study the genetic control of organ induction, as limb development is highly amenable to experimental and genetic manipulation in a range of model organisms (Capdevila and Izpisua Belmonte, 2001; Niswander, 2002; Tickle, 2002). Limbs arise from regions of the lateral plate mesoderm (LPM) at specific positions along the main anteroposterior body axis.

A number of studies have shown that the limb-inducing signal originates in the axial mesoderm, and is relayed from there to the LPM (reviewed by Capdevila and Izpisua Belmonte, 2001). An important signal shown to play a role in limb induction in mouse, chick and zebrafish is the Vitamin A derivative retinoic acid (RA) (Begemann et al., 2001; Berggren et al., 1999; Grandel et al., 2002; Mic et al., 2004; Niederreither et al., 1997; Niederreither et al., 1999; Stratford et al., 1997). RA is synthesized mainly by the enzyme Retinaldehyde dehydrogenase 2 (Raldh2), which is expressed in the LPM and early somites (Begemann et al., 1999; Niederreither et al., 1997). Inhibition of RA signaling during different time windows in zebrafish has revealed that it is required for limb initiation during a relatively short time span at the end of gastrulation, long before limb development commences in the LPM (Grandel et al., 2002). Also, mosaic experiments performed in zebrafish, where wild-type cells were transplanted into raldh2 mutant embryos, have shown that RA synthesis in somitic mesoderm is sufficient to trigger limb induction in the adjacent LPM (Linville et al., 2004). These experiments indicate that RA signaling acts very early in the cascade of genes controlling limb induction. They also show that the limb induction cascade is initiated in the somitic mesoderm, and suggest that the effect of RA on limb development is probably indirect, and likely to be mediated by secondary signals.

The T-box transcription factor Tbx5 is the earliest gene known to be expressed in the presumptive forelimb field (Gibson-Brown et al., 1996; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Simon et al., 1997; Tamura et al., 1999). Tbx5 is crucial for forelimb induction, as loss of Tbx5 activity causes failure of limb initiation in mouse, chicken and zebrafish (Agarwal et al., 2003; Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002; Rallis et al., 2003; Takeuchi et al., 2003). Conversely, ectopic overexpression of Tbx5 can trigger ectopic limb outgrowth in the interlimb LPM, indicating that Tbx5 is not only necessary, but also sufficient to initiate limb development (Takeuchi et al., 2003). A number of studies have shown that Tbx5 interacts both with Wnt and Fgf signals to direct limb induction. Thus, Tbx5 is required for activation of Fgf10 expression within the limb mesenchyme. Fgf10, in turn, signals to the overlying ectoderm to activate Fgf8 expression in the apical ectodermal ridge (AER) (Min et al., 1998; Norton et al., 2005; Ohuchi et al., 1997; Sekine et al., 1999). This event then leads to the establishment of a signaling feedback loop between ectodermal Fgf8 and mesenchymal Fgf10, which is crucial for subsequent limb outgrowth (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999).

In addition to its role in mediating AER signaling, Fgf8 has also been proposed to act at an earlier step in limb induction, as application of Fgf8 protein into the chicken flank is able to direct formation of an ectopic limb, and because Fgf8 is expressed in the intermediate mesoderm (IM) adjacent to the forelimb-forming region at the time of limb initiation (Crossley et al., 1996; Vogel et al., 1996). Arguing against this hypothesis, however, is the observation that conditional removal of Fgf8 activity from the IM has no effect on limb development in mice (Boulet et al., 2004; Perantoni et al., 2005). An alternative possibility may be that Fgf8 is functionally redundant with other members of the Fgf family expressed in the axial mesoderm. For example, fgf17b is co-expressed with fgf8 in the somites (Reifers et al., 2000). Because the mosaic analysis of raldh2 mutants indicates that the somitic mesoderm is crucial for limb induction (Linville et al., 2004), the somites could be a source of Fgf signaling required for limb induction. It is therefore presently not clear whether Fgf signaling participates in relaying the limb-inducing signal from the axial and paraxial mesoderm to the LPM.
The Wnt family of signaling molecules also plays an important role during limb initiation. In the chick, Wnt2b is expressed in the IM and LPM, and similar to Fgf protein application, ectopic expression of Wnt2b or β-catenin triggers the formation of an extra limb (Kawakami et al., 2001; Takeuchi et al., 2003). In zebrafish, wnt2b is only expressed in the IM, and knock down of Wnt2b with antisense morpholino oligonucleotides leads to failure of tbx5 expression activation in the LPM (Ng et al., 2002). Furthermore, injection of tbx5 messenger RNA (mRNA) can partially rescue Wnt2b knock-down embryos, whereas wnt2b mRNA injection fails to rescue Tbx5 knock-down embryos, suggesting that Wnt2b signaling is upstream of tbx5 during limb induction (Ng et al., 2002). In contrast to these results, mouse embryos mutant for Left1 and Tcf1, two nuclear transducers of Wnt signaling, have normal limb bud initiation and show no effects on Tbx5 activation (Agarwal et al., 2003; Galceran et al., 1999). This could either reflect a species-specific role of tbx5 signaling in limb induction, or additional Tcf genes may compensate for the loss of these genes in the mouse (Logan, 2003).

The zebrafish has recently gained popularity as a model to study limb development, as its paired fins are homologous to tetrapod limbs (Grandel and Schulte-Merker, 1998). Several large-scale mutagenesis screens have led to the isolation of zebrafish mutants affecting fin development (van Eeden et al., 1996). The fin primordium in zebrafish larvae is composed of a very thin layer of LPM cells. In order to form a bud, the appropriate organ size of the fin buds is generated not only through proliferation, but also by migration of LPM cells towards the limb field (Ahn et al., 2002). Fgf124, a member of the Fgf8/17/18 family of Fgf molecules, is the earliest fgf gene known to be expressed in the zebrafish forelimb bud, and one of its functions is to promote migration of tbx5-positive cells towards the fin field (Fischer et al., 2003). In the fgf24 mutant ikarus (ika), the tbx5-expressing LPM population does not compact and eventually disappears at later stages of development, indicating that fgf24 is needed on one hand for migration of tbx5-expressing cells to the limb primordium, and on the other for the activation of fgf10, which then relays the limb-inducing signal to the overlying ectoderm (Fischer et al., 2003; Norton et al., 2005).

A recent study showed that activity of the prdm1 gene is also required for pectoral fin development, as knock down of Prdm1 leads to an absence of pectoral fins (Wilm and Solnica-Krezel, 2005). prdm1, also called bimpl1 (B-lymphocyte induced maturation protein 1), encodes a transcriptional repressor. Its N-terminal PR-domain possesses methyltransferase activity, which is shared with other members of the SET domain protein family (Kouzarides, 2005). Prdm1 contains five Krüppel-like zinc finger domains through which it binds to target promoters and, together with Groucho corepressors and Histone deacetylases, causes transcriptional repression (Makar and Wilson, 2004; Ren et al., 1999; Yu et al., 2000).

Prdm1 has been shown to play an essential role during the development of several tissues. Analysis of null mutant mice has revealed a function for Prdm1 in specification of the germ cell lineage (Ohnata et al., 2005; Vincent et al., 2005). In zebrafish, Prdm1 regulates Bmp2 activity during gastrulation through the repression of chordin (Wilm and Solnica-Krezel, 2005), and is involved in neural crest cell differentiation (Hernandez-Lagunas et al., 2005; Roy and Ng, 2004). Zebrafish Prdm1 has also been shown to act downstream of sonic hedgehog signaling during slow muscle specification (Baxendale et al., 2004).

While it is clear that prdm1 is crucial for limb formation in zebrafish (Wilm and Solnica-Krezel, 2005), its relationship to other genes in the limb induction cascade has not been analysed in detail. We therefore systematically examined the role of prdm1 in the regulatory hierarchy triggering limb development. Second, because the regulatory relationship between RA, Wnt and Fgf signaling in the axial mesoderm has not been fully determined, nor how this cascade regulates Tbx5 and Fgf activity in the limb bud, we made use of the availability of zebrafish raldh2, tbx5 and fgf24 mutants, and the Fgf-pathway inhibitor SU5402, to systematically examine the regulatory hierarchy controlling zebrafish limb induction.

Our analysis reveals that prdm1 activation is downstream of RA, Wnt2b and Tbx5 activity in the limb primordium. Activation of prdm1 expression is also downstream of an early Fgf signaling event downstream of tbx5, directed in part by Fgf24. Following its activation in the limb bud, Prdm1 acts in a feedback loop to maintain fgf24 expression, and is required for further progression of the limb initiation cascade leading to fgf10 activation. We also find that RA signaling is necessary for wnt2b expression in the IM, whereas Fgf signaling activity is not necessary for this event. Likewise, Fgf signaling is not required for the activation of tbx5 expression in the LPM. These results indicate that Fgf signaling does not participate in the transfer of the limb-inducing signal from the axial mesoderm to the LPM, and instead plays a local role within the limb primordium downstream of tbx5. We propose a model in which RA signaling from the somitic mesoderm leads to activation of wnt2b expression in the IM, which then signals to the LPM to trigger tbx5 expression. tbx5 in turn is required for an Fgf signaling event in the limb bud leading to the activation of prdm1 expression, which then triggers activation of fgf10.

**MATERIALS AND METHODS**

**Zebrafish lines**

WIK and Tübingen were used as wild-type strains. Mutant strains used were: the fgf24 mutant ikarus (ika), the fgf10 mutant daedalus (dae) (Norton et al., 2005), the tbx5 mutant heartstrings (hst) (Garrity et al., 2002), and the raldh2 mutant neckless (nls) (Begemann et al., 2001).

**Morpholino injection**

Antisense morpholino oligonucleotides against prdm1 (5'-TGTGGATCCTCCCCTGAGTGTGT-3') (Wilm et al., 2005) and raldh2 (5'-GCAGTCTAACACTCAGGAGCTCAT-3') (Begemann et al., 2001) start codon regions were purchased from Gene Tools (Corvallis, OR). Morpholinos were diluted in distilled water and injected into one-cell stage embryos at a concentration of 0.1 mM for MOprdm1 and 0.2 mM for MOraldh2. Because there was some variation in the phenotypic penetrance of prdm1 morphants, ranging from reduced fins (stumps) and unilateral fin stumps to no fins, for all injection experiments, 10% of injected embryos were allowed to develop until 72 hpf (hours postfertilization) to monitor the effectiveness of prdm1 knock down. Only batches with >75% larvae showing complete absence of fin development were further processed for in situ hybridization.

**Mosaic experiments**

Bpe-GFP transgenic embryos at sphere stage were used as donors (Higashijima et al., 1997). Between 20 and 30 cells were transplanted into the lateral marginal zone of sphere to dome-stage host embryos, which had previously been injected with either MOprdm1 or MORaldh2. Rescue of pectoral fin development and contribution of wild-type cells to anterior somites or pectoral fin mesenchyme was monitored three days later under a fluorescent light binocular (Leica, Cambridge, UK).

**Treatment with DEAB, SU5402**

Diethylaminobenzoic acid (DEAB) (Sigma) and the FGF receptor inhibitor SU5402 (Calbiochem) were dissolved in dimethylsulfoxide (DMSO) and used at a concentration of 10 mM and 16 μM, respectively. Incubations were carried out in the dark at 28°C.
RESULTS

Knock down of Prdm1 by antisense morpholino oligonucleotide injection has recently been shown to lead to absence of pectoral fins (Wilm and Solnica-Krezel, 2005). As this study did not address the pectoral fin phenotype in detail, we decided to further examine the effect of Prdm1 knock down on pectoral fin development. Consistent with the report of Wilm and Solnica-Krezel (Wilm and Solnica-Krezel, 2005), we find that knock down of Prdm1 causes an absence of pectoral fins (Fig. 1A,B). Furthermore, Alcian Blue cartilage staining reveals that all skeletal elements of the pectoral fins are absent in prdm1 morphants at four days postfertilization (Fig. 1C,D). We also failed to detect any morphological signs of pectoral fin buds at earlier stages in prdm1 morphants (Fig. 1E,F). As these results indicate that Prdm1 acts at an early stage in limb induction, we examined the expression pattern of prdm1 during the time window when pectoral fin development commences in zebrafish. Prior to fin bud formation, at the 15 to 17-somite stage, prdm1 expression is detectable in the somites and in the posterior LPM (Fig. 2A-C). At the 18-somite stage, which corresponds to 18 hours postfertilization (hpf), we first detect prdm1 expression in the LPM regions close to somite 2, the region where the tbx5-positive pectoral fin mesenchyme starts to condense (Fig. 2D-F). This expression domain overlaps with the fgf24 expression domain. During the next few hours, prdm1 expression increases in the fin bud mesenchyme and, at the 23-somite stage (20.5 hpf), is clearly visible in the fin primordia (Fig. 2G), overlapping with fgf24 and tbx5 expression (Fig. 2H,I).

Prdm1 acts downstream of retinoic acid signaling during pectoral fin induction

As the prdm1 morphant phenotype indicates that Prdm1 is crucial for an early stage in pectoral fin induction, we examined the relationship between prdm1 and raldh2, the earliest gene known to be required for fin induction. Since prdm1, like raldh2, is also expressed in somitic mesoderm at the level of the forelimbs, we examined the possibility that Prdm1 is required for raldh2 expression in the somites. However, knock down of Prdm1 activity does not affect expression of raldh2 in the somites, nor in the LPM (Fig. 3A-B’). Furthermore, activation of cyp26a1, a target of retinoic acid signaling in the anterior somites (Doobs-McAuliffe et al., 2002), leads to absence of pectoral fins at four days postfertilization (Fig. 1C,D). In contrast, activation of cyp26a1 in the LPM, using the following probes: wnt2b (~22 to ~1366 bp) (Ng et al., 2002), prdm1 (Wilm et al., 2005), tbx5 (Begemann and Ingham, 2000), fgf24 (Fischer et al., 2003), pea3 (Roehl and Nüsslein-Volhard, 2001), fgf10 (Ng et al., 2002), bmp2b (Kishimoto et al., 1997), raldh2 (Grandel et al., 2001) and cyp26a1 (ch24 EST clone, Zebrafish International Resource Center). BM purple (Roche) was used as a substrate. For in situ hybridization at embryonic stages prior to limb bud initiation, lateral views of a 12-somite (A) and a 15-somite (B) stage embryo revealing prdm1 (blue) and myod (red) expression. Note prdm1 expression overlapping with myod in the somites. (C) Lateral view of a 17-somite stage embryo. Arrows in A-C reveal the most anterior limit of prdm1 expression within the lateral plate mesoderm. (D-F) Dorsal views of 18-somite stage embryos hybridized with prdm1 (D), fgf24 (E) or tbx5+myod (F) riboprobes. Arrows in D point towards the onset of prdm1 expression in the pectoral fin primordia. Note that the prdm1 and fgf24 expression domains are very similar, and that the tbx5 expression domain is broader than the prdm1 domain. (G-I) Dorsal views of 23-somite stage embryos hybridized with prdm1 (G), fgf24 (H) or tbx5+myod (I) riboprobes. Arrows in G point towards the expanded prdm1 expression domain in the pectoral fin.

In situ hybridization and histochemical methods

Whole-mount in situ hybridization was performed as described (Kishimoto et al., 1997), using the following probes: wnt2b (~22 to ~1366 bp) (Ng et al., 2002), prdm1 (Wilm et al., 2005), tbx5 (Begemann and Ingham, 2000), fgf24 (Fischer et al., 2003), pea3 (Roehl and Nüsslein-Volhard, 2001), fgf10 (Ng et al., 2002), bmp2b (Kishimoto et al., 1997), raldh2 (Grandel et al., 2001) and cyp26a1 (ch24 EST clone, Zebrafish International Resource Center). BM purple (Roche) was used as a substrate. For in situ hybridization and histochemical methods, performed according to Grandel and Schulte-Merkel (Grandel and Schulte-Merkel, 1998).

Fig. 1. prdm1 morphants lack pectoral fins. (A, B) Dorsal views of wild-type (WT, A) and prdm1 morphant (MO prdm1, B) four-day-old larvae. (C, D) Cartilage stainings of wild-type and prdm1 morphant pectoral fins at four days postfertilization. Note that prdm1 morphants only develop a cleithrum. (E, F) Methylene Blue-stained transverse cryosections of 48 hpf embryos. Arrows point towards pectoral fins in the wild type and asterisks indicate the absence of pectoral fins in the morphant. cl, cleithrum; ed, endochondral disc; sc, scapulocoracoid; pc, postcoracoid process.

Fig. 2. Expression of prdm1 compared with fgf24 and tbx5 during limb bud initiation. (A-C) prdm1 whole-mount in situ hybridization at embryonic stages prior to limb bud initiation. Lateral views of a 12-somite (A) and a 15-somite (B) stage embryo revealing prdm1 (blue) and myod (red) expression. Note prdm1 expression overlapping with myod in the somites. (C) Lateral view of a 17-somite stage embryo. Arrows in A-C reveal the most anterior limit of prdm1 expression within the lateral plate mesoderm. (D-F) Dorsal views of 18-somite stage embryos hybridized with prdm1 (D), fgf24 (E) or tbx5+myod (F) riboprobes. Arrows in D point towards the onset of prdm1 expression in the pectoral fin primordia. Note that the prdm1 and fgf24 expression domains are very similar, and that the tbx5 expression domain is broader than the prdm1 domain. (G-I) Dorsal views of 23-somite stage embryos hybridized with prdm1 (G), fgf24 (H) or tbx5+myod (I) riboprobes. Arrows in G point towards the expanded prdm1 expression domain in the pectoral fin.
2004), is unperturbed in prdm1 morphants (Fig. 3C,D). Consistent with this observation, pectoral fin induction in prdm1 morphants is not rescued by the administration of exogenous RA (data not shown); this is in contrast to raldh2 mutants, which can be rescued by RA administration (Begemann et al., 2004; Grandel et al., 2002). Therefore Prdm1 does not seem to act upstream of RA signaling.

Furthermore, loss of Raldh2 activity in neckless mutants, or in embryos treated with the chemical inhibitor DEAB (Mahmoud et al., 1993) leads to an absence of prdm1 expression in several tissues, including the anterior somites (Fig. 3E-F) and the pectoral fin buds (Fig. 3G,H). Taken together, these results indicate that prdm1 activation is downstream of RA signaling during somite formation and limb induction.

**Activation of wnt2b depends on Raldh2 activity, but not on Fgf signaling or Prdm1**

Because Wnt2b is also necessary for forelimb induction, we next examined the regulatory interactions between Wnt2b, Prdm1, RA and Fgf signaling. First, to determine whether Prdm1 activity is needed for wnt2b expression in the IM, we examined wnt2b expression in prdm1 morphants. No difference in wnt2b expression could be detected in prdm1 morphants when compared with wild-type siblings (Fig. 3I,J). We also examined whether Fgf signaling is required for wnt2b expression, by blocking the Fgf pathway with the Fgf receptor inhibitor SU5402 (Mohammadi et al., 1997). Treatment of zebrafish embryos with SU5402 has been shown to block the expression of Fgf target genes (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). As described, we find that treatment of embryos with 16 μM SU5402 from 11.5 hpf onwards leads to an absence of expression of the Fgf target gene pea3 at 24 hpf (data not shown). By contrast, wnt2b is expressed normally in the same batch of SU5402-treated embryos at 24 hpf (Fig. 3K). However, wnt2b expression in the IM is absent in neckless (nls) mutants, which disrupts Raldh2 activity (Fig. 3L), indicating that wnt2b transcription is dependent on RA signaling. Loss of wnt2b was not caused by general disruption of IM formation, as the IM molecular marker pax2a was still expressed in nls embryos (not shown). Taken together, these results show that wnt2b activation in the IM depends on Raldh2 activity, but not on Fgf signaling, nor on Prdm1 activity.

**Prdm1 activity is not required in the somites during pectoral fin induction**

Transplantation experiments have shown that Raldh2 activity is sufficient within the somitic mesoderm at the level of the first three somites to direct pectoral fin induction (Linville et al., 2004). As prdm1, like raldh2, is expressed in the anterior somites,

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**Fig. 3.** Prdm1 acts downstream of retinoic acid and Wnt2b signaling during limb bud initiation. (A,B) Lateral views of the retinaldehyde dehydrogenase 2 (raldh2) expression pattern in 12-somite stage wild-type (A) or prdm1 MO-injected (B) embryos. Anterior is to the left. A’ and B’ depict close-up views of lateral plate mesoderm (arrows) and somite expression at the axial level of pectoral fin formation. (C,D) Expression of the retinoic acid degrading enzyme cyp26a1 in wild-type and prdm1 morphant embryos at the 12-somite stage. Arrow indicates cyp26a1 expression within somites 1 and 2. Note that there is no difference in raldh2 and cyp26a1 expression between prdm1 morphants and wild types. (E-F’) prdm1 in situ hybridization of control DMSO-treated 17-somite stage embryos (E,E’) and DEAB-treated embryos (F,F’). Note that DEAB treatment leads to a reduction of prdm1 expression within branchial arches (arrow in E) and anterior somites (compare straight and dotted lines in E’ and F’). (G,H) prdm1 in situ hybridization on 24 hpf wild-type (G) and neckless mutant (H) pectoral fin bud regions. Arrows in G indicate fin buds; asterisks in F and H indicate a absence of prdm1 expression within the corresponding structure. (I-L) Expression pattern of wnt2b mRNA in wild-type (I), prdm1 morphant (J), 16 μM SU5402-treated (K) and neckless mutant (L) embryos at 24 hpf. Panels show dorsal views with anterior to the top. Note that wnt2b expression is normal in MOprdm1 and SU5402-treated embryos, but is lost in the raldh2 mutant nls (asterisks). DEAB, diethylaminobenzoic acid; DMSO, dimethylsulfoxide; nls, neckless; MOprdm1, prdm1 morphant; WT, wild type.
expression of prdm1 in this tissue depends on Raldh2 activity, we considered the possibility that prdm1 acts in the somitic mesoderm to direct pectoral fin induction. However, because prdm1 is also expressed in the nascent pectoral fin buds, an alternative is that this latter expression domain might be necessary for fin induction. To distinguish between these two possibilities, we performed mosaic experiments in which we transplanted wild-type cells into prdm1 morphants. As a control, we compared this experiment to the effect of transplanting wild-type cells into raldh2 morphants. As previously described, pectoral fin induction can be rescued in raldh2 morphants by wild-type cells located in anterior somites (Fig. 4A-B'). In some cases, we observed rescue when wild-type cells were found both in the somites and in the fins (n=6), but in other cases we observed rescue when cells were found only in the somites (n=3; total number of chimeric embryos with wild-type cells in anterior somites, n=7; Fig. 4A-B'). We did not observe cases in which wild-type cells exclusively contributed to the rescued limb. These results indicate that Raldh2 activity in the somites is sufficient to direct fin induction. In the case of prdm1 morphants, we never observed rescue in cases where wild-type cells were located only in the somites (n=26). Even in cases were GFP expression in the anterior somites was very strong, fin outgrowth was not restored in MOprdm1 embryos (n=19) (Fig. 4C-D'). This result indicates that Prdm1 activity, unlike Raldh2, is not required in the somites for pectoral fin induction, but instead suggests that it is required in the fin bud primordium.

**prdm1 is downstream of tbx5 and fgf24 during fin induction**

As our results indicate that Prdm1 acts in the nascent fin primordium to mediate limb induction, and because Tbx5 and Fgf24 activity is also required within the fin primordium during limb induction, we next examined whether Prdm1 activity is necessary for the activation of fgf24 and tbx5 expression. We find that fgf24 expression is activated in prdm1 morphants at 18 hpf (Fig. 5A,B), but is subsequently downregulated and lost (Fig. 5C,D,F,G). Similarly, fgf24 expression is activated in fgf24 mutants, and is lost later on (Fig. 5A,E,H).

We also find that tbx5 expression is activated normally in the LPM of prdm1 morphants (Fig. 5I,J). This is similar to previously reported data showing that tbx5 is activated normally in tbx5 and fgf24 mutants (Fig. 5K) (Ahn et al., 2002; Fischer et al., 2003). At a slightly later stage (24 hpf), we find that tbx5 expression in prdm1 morphants fails to form a compact domain in the fin bud, and the tbx5-expressing cells instead remain spread throughout the LPM (Fig. 5L,M). The same effect is observed in tbx5 mutants, although tbx5 downregulation is more severe in that case (data not shown) (Ahn et al., 2002). The stronger downregulation of tbx5 expression in tbx5 mutants compared with prdm1 morphants is consistent with tbx5 being upstream of prdm1 in the fin initiation cascade and suggests that Tbx5 activates other genes necessary for fin initiation, such as sall4 (Harvey and Logan, 2006). Tbx5 activity is necessary for activation of fgf24 expression in the fin bud, and fgf24 mutants also fail to form a compact tbx5-expressing domain (Fig. 5N) (Fischer et al., 2003). Like fgf24, prdm1 also fails to be activated in the fin buds of the tbx5 mutant heartstrings (hst) (Fig. 6D,H). Taken together, these results indicate that Tbx5 acts upstream of Prdm1, consistent with the observation that tbx5 expression is activated in the fin primordium earlier than prdm1 (Fig. 2F,I).

Because both Prdm1 and Fgf24 act downstream of Tbx5, we investigated whether prdm1 expression is regulated by Fgf24. We find that initiation of prdm1 transcription in the fin buds of the fgf24 mutant ikarus (ika) is both delayed and reduced. At the 23-somite stage (20.5 hpf), prdm1 expression is present in wild-type embryos,

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**Fig. 4. Mosaic analysis in raldh2 and prdm1 morphants.** (A) Dorsal view of a three-day-old raldh2 morphant embryo revealing rescued pectoral fin outgrowth on the right side (arrow). (A') Dark-field image of the same embryo, showing transplanted GFP-positive cells labeled in green. (B-B') Merged bright-field and dark-field images showing green wild-type cells localizing to the anterior somite region. (C-C') Lateral views of the same MOraldh2 mosaic embryo as in A. Dotted lines in B' indicate somite boundaries. Note strong GFP-expression in somites 1 to 3. (D-D') Dorsal and lateral views of an MOpadm1 embryo, where transplanted wild-type cells contribute to anterior somites but do not rescue fin outgrowth. (C',D') Merged bright and dark field pictures showing GFP-positive wild-type cells incorporated into the left fin. Dotted lines in D' indicate somite boundaries. Asterisks mark the missing pectoral fin. s, somite.
but not in ika mutants (Fig. 6A,C), but at 24 hpf, faint expression of prdm1 is present (Fig. 6E,G). However, at later stages, prdm1 expression is lost again in ika mutants (Fig. 6I,K). Loss of prdm1 expression is not due to increased cell death in the fin mesenchyme of ika mutants (Fig. 7). This indicates that activation and maintenance of prdm1 expression depends on fgf24, but that there is also fgf24-independent prdm1 expression.

Taken together, these results indicate that Prdm1 acts downstream of Tbx5 and Fgf24 during limb induction, and that it forms part of a feedback loop to maintain fgf24 expression.

Prdm1 acts upstream of Fgf10 during fin induction

A further Fgf signaling event in the early limb bud is mediated by Fgf10 signaling from the limb mesenchyme to the overlying ectoderm. Fgf24 has been shown to act upstream of fgf10 during limb initiation (Fischer et al., 2003). As Prdm1 acts downstream of fgf24, we analyzed the regulatory relationship between prdm1 and fgf10 by making use of the zebrafish fgf10 mutant daedalus (Norton et al., 2005). We find that onset of prdm1 expression and maintenance in the fin mesenchyme of fgf10 mutants is identical to wild-type siblings (Fig. 6A,B,E,F,I,J). However, at 36 hpf prdm1 expression is not activated in the AER of fgf10 mutants, although it is expressed in the wild-type AER (Fig. 6L,M). Because fgf10 is required for the establishment of the AER, it is likely that the loss of prdm1 expression in daedalus mutants is due to lack of AER formation, rather than due to a specific role of Fgf10 in prdm1 activation. Conversely, loss of Prdm1 activity leads to a loss of fgf10 expression (Fig. 8A,D), as has been reported for fgf24 and tbx5 mutants (Fig. 8B,C) (Fischer et al., 2003; Ng et al., 2002). Taken together, these results indicate that Prdm1 acts upstream of fgf10 activation in the fin bud mesenchyme.

As Fgf24 is not required for the activation of several early genes expressed in the fin mesenchyme, including pea3 and bmp2b (Fig. 8E,F,I,J) (Fischer et al., 2003), we examined whether Prdm1 activity is necessary for these genes. We find that both pea3 and bmp2b fail to be activated in prdm1 morphants, and observe the same absence of expression in tbx5 mutants (Fig. 8G,H,K,L). Taken together, these results indicate that Prdm1 is required for the activation of fgf10, pea3 and bmp2b transcription.

The earliest Fgf signaling required for fin induction is downstream of Tbx5

To address the question whether Fgf signaling is involved in relaying the limb-inducing signal from the axial mesoderm to the LPM, or whether it acts at a later stage during limb induction, we used the inhibitor SU5402 to assay the effect of Fgf pathway inhibition on the activation of early limb genes. Treatment of embryos with 16 μM SU5402 from the one-somite stage (10.7 hpf) onwards leads to complete downregulation of the Fgf target gene erm throughout the embryo at 20.5 hpf and at 24 hpf (Fig. 9A-D), indicating that Fgf signaling is severely inhibited in these embryos. prdm1 is not activated in the fin bud at any stage in SU5402-treated embryos (Fig. 9E-H). By contrast, we find that fgf24 transcription in the LPM is activated in the same batch of SU5402-treated embryos but becomes strongly downregulated at 24 hpf (Fig. 9I-L). At 20.5 hpf, the tbx5 expression domain is not altered upon SU5402 treatment (Fig. 9M,N), but at the 24 hpf stage, we observe a failure of tbx5-expressing cells to congregate towards the fin bud (Fig. 9O,P). As this defect is also observed in fgf24 mutants (see Fig. 5N) (Fischer et al., 2003), it is likely to be due to the absence of Fgf24 activity in SU5402-treated embryos. Also, the fact that prdm1 activation is completely blocked in SU5402-treated embryos, but is only delayed and is partially reduced in fgf24 mutant embryos, suggests there is an additional Fgf protein acting downstream of Tbx5 to activate prdm1 expression, which is semi-redundant with Fgf24. This proposal is further supported by the observation that activation of the Fgf target pea3 is not completely blocked in fgf24 mutants, indicating there is still Fgf signaling present in fgf24 mutants (Fig. 8F).

DISCUSSION

The role of Prdm1 during zebrafish forelimb induction

We have shown here that zebrafish prdm1 is crucial for an early step during forelimb induction. Together with tbx5 and fgf24, prdm1 is among the earliest genes expressed in the zebrafish forelimb
primordium. Of these three genes, tbx5 is the first to be expressed in the forelimb-forming region of the LPM (Begemann and Ingham, 2000; Chapman et al., 1996; Gibson-Brown et al., 1996; Simon et al., 1997), followed by fgf24, and then prdm1 a few hours later. Interestingly, tbx5 is expressed more broadly in the LPM than are the other two genes. Both prdm1 and fgf24 are expressed in a small patch of cells corresponding to the nascent fin primordium, whereas tbx5 is also expressed in surrounding cells that later migrate to the fin bud (Ahn et al., 2002; Fischer et al., 2003).

Our transplantation data indicate that Prdm1 activity is required within the fin bud itself during forelimb initiation. This excludes a role for Prdm1 in the anterior somites during forelimb initiation, even though prdm1 is expressed in this tissue under the control of Raldh2 activity. In contrast to Prdm1, Raldh2 functions in the anterior somites to direct forelimb initiation (Linville et al., 2004) (this study).

During plasma cell differentiation, Prdm1 has been shown to act as a repressor, directly repressing the transcription of cmyc (previously known as c-myc), PAX5 and CIITA (Lin et al., 2002; Lin et al., 1997; Piskurich et al., 2000). This suggests that the activation of limb genes downstream of prdm1 would have to be indirect, via repression of another repressor. We have observed that prdm1 morphants have elevated levels of prdm1 transcripts (data not shown), suggesting that during zebrafish development, Prdm1 can act as a repressor of its own transcription. However, it has also been proposed that Prdm1 could also act as a transcriptional activator (Baxendale et al., 2004), and we therefore cannot exclude that it might directly activate target gene expression during fin initiation. To discriminate between these options, further work needs to be carried out.

A cascade of inductive events originating in the anterior somites leads to initiation of forelimb development in the LPM

We have systematically analyzed the hierarchical relationship between the genes and signaling pathways required for zebrafish pectoral fin induction. This group of genes includes raldh2, wnt2b, tbx5, prdm1, fgf24 and fgf10, and our results support a model in
which these genes form a linear hierarchy controlling the transfer of the limb-inducing signal from the anterior somites to the LPM (Fig. 10). The earliest gene known to function in pectoral fin induction is \textit{raldh2} (Begemann et al., 2001; Grandel et al., 2002). In the absence of Raldh2 activity, all other limb genes fail to be expressed, including \textit{wnt2b} (this study) and \textit{tbx5} (Begemann et al., 2001). This is consistent with the observation that Raldh2 activity is necessary for limb induction at early segmentation stages (Grandel et al., 2002), which is well before the earliest fin bud marker, \textit{tbx5}, is expressed in the LPM. Furthermore, as Raldh2 activity is required in the first three somites (Linville et al., 2004) (this study), this indicates that the signaling cascade leading to pectoral fin induction originates in the somitic mesoderm during early segmentation stages.

The early requirement of \textit{raldh2} for limb development raises the possibility that the effect of RA signaling is mediated via a second signal. Indeed, our results suggest that \textit{wnt2b} performs this role in the pectoral fin. \textit{wnt2b} is expressed in the IM adjacent to the forelimbs before \textit{tbx5} is activated in the LPM, and our data show that \textit{wnt2b} expression in the IM depends on Raldh2 activity. The simplest

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Comparison of \textit{fgf10}, \textit{pea3} and \textit{bmp2} expression in MO\textit{prdm1}-injected embryos, and in \textit{hst} and \textit{ika} mutants. (A-L) Dorsal views of 30 hpf embryos stained for \textit{fgf10} (A-D), \textit{pea3} (E-H) or \textit{bmp2b} (I-L) expression. While \textit{fgf10} expression is absent in \textit{ika}, \textit{hst} and MO\textit{prdm1} embryos (B,C,D), \textit{pea3} and \textit{bmp2b} are transiently expressed in \textit{ika} (F,I) but not \textit{hst} or MO\textit{prdm1} embryos (G,H,K,L). Asterisks indicate a lack of marker gene expression within the pectoral fin mesenchyme. \textit{hst}, heartstrings; \textit{ika}, ikarus; MO\textit{prdm1}, \textit{prdm1} morphant embryo; WT, wild type.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Effect of early Fgf inhibitor treatment on \textit{erm}, \textit{prdm1}, \textit{fgf24} and \textit{tbx5} expression. Whole-mount in situ hybridization on 20.5 hpf (23-somite stage) (A,B,E,F,I,J,M,N) and 24 hpf (C,D,G,H,K,L,O,P) control DMSO and SU5402-treated embryos. Probes are as indicated in each panel. (A-D) \textit{erm} expression is abolished in SU5402-treated embryos. (E-H) \textit{prdm1} expression is downregulated in SU5402-treated embryos. (I-L) Upon SU5402 treatment, \textit{fgf24} is still present at 20.5 hpf but becomes strongly reduced at 24 hpf. (M-P) \textit{tbx5} expression is not affected at the 23-somite stage (M,N) but is reduced in 24 hpf embryos upon SU5402 treatment (O,P).}
\end{figure}
interpretation of this result is that Wnt2b triggers activation of tbx5 in the LPM, thus mediating the inductive signaling cascade between Raldh2 and Tbx5 (Fig. 10). This scenario is further supported by the observation that Wnt2b activity is required for tbx5 expression, and that loss of Wnt2b activity can be rescued by tbx5 mRNA injection, but not vice versa (Ng et al., 2002).

In the fin mesenchyme, Tbx5 triggers an early Fgf signalling event leading to prdm1 activation. Although activation of prdm1 expression is strongly dependent on Fgf24, a reduced level of prdm1 is still detectable in the absence of fgf24 activity. Because treatment with the Fgf pathway inhibitor SU5402 completely blocks prdm1 activation, this Fgf24-independent expression of prdm1 is most likely directed by an additional Fgf acting at a similar position in the limb induction cascade (termed "FgfX" in our model in Fig. 10), which is partially redundant with Fgf24. This proposal is further supported by the observation that expression of the Fgf target pea3 is not completely blocked in fgf24 mutants, reflecting activation of the Fgf pathway independent of Fgf24 in the early fin bud. The delay in onset of prdm1 expression in fgf24 mutants could thus be due to the fact that a minimal Fgf signaling threshold must be reached to initiate prdm1 expression. In the absence of fgf24, more time is required to accomplish this threshold. There are a number of examples described in the literature of fgf genes co-expressed and partially redundant, as in the example of fgf24 and fgf8 during posterior mesoderm development (Draper et al., 2003). Further work will be necessary to identify the complete set of zebrafish fgf genes acting during limb initiation.

Maintenance of fgf24 expression becomes dependent on Prdm1 activity soon after its initial activation, indicating that Prdm1 operates in a feedback loop to regulate fgf24 maintenance. The failure of tbx5-expressing LPM cells to congregate towards the fin bud in the absence of Prdm1 activity is most likely due to the failure of fgf24 maintenance, as Fgf24 is required for this migratory event (Fischer et al., 2003).

Finally, Prdm1 activity is required for the downstream activation of fgf10 expression, which then relays the limb initiation signal from the mesenchyme to the ectoderm, to direct AER development and limb outgrowth.

**The earliest requirement for Fgf signaling during forelimb induction is downstream of tbx5 activation**

An important issue remaining unresolved so far is whether Fgf signaling is required for the transfer of the limb-inducing signal from the axial mesoderm to the LPM. We addressed this question by using the Fgf pathway inhibitor SU5402 to define the earliest step at which Fgf signaling is required for forelimb induction. Our results reveal that Fgf signaling is necessary neither for expression of wnt2b in the IM, nor for the activation of tbx5 expression in the LPM, suggesting that the transfer of the limb-inducing signal from the axial mesoderm to the LPM is independent of Fgf signaling. This is consistent with the observation that conditional removal of Fgf8 activity from the IM in the mouse has no effect on limb development (Boulet et al., 2004; Perantoni et al., 2005). Similarly, the zebrafish fgf8 mutant acerebellar does not show impaired pectoral fin development (Reifers et al., 1998). Our results indicate that the earliest requirement for Fgf signaling during limb induction is for the activation of prdm1 but not for onset of tbx5 expression. Taken together, these data suggest that Fgf signaling is not required for the transfer of positional information from the somites or IM to the LPM during limb induction, and instead plays a local role within the limb primordium. They also show that the Fgf signaling cascade is established downstream of Tbx5 activity.

**Conservation of the limb induction cascade among vertebrate species**

The expression pattern of prdm1 during limb development is conserved between zebrafish and tetrapods. In chick and mouse, Prdm1 is also initially expressed within the limb mesenchyme, and later becomes activated in the overlying AER (Ha and Riddle, 2003; Vincent et al., 2005). In contrast to prdm1 knock down in zebrafish, Prdm1 null mutant mice do not display any defects in limb bud initiation (Vincent et al., 2005). This difference may be due to redundancy of Prdm1 function with a related gene in the mouse, or it may reflect a species-specific role of prdm1 in zebrafish limb induction. It will be interesting to analyze the effect of Prdm1 loss-of-function and gain-of-function in the chick, to determine whether Prdm1 activity plays a role during limb induction in this species.

In contrast to Prdm1 mutants, mouse mutants for Raldh2 (Mic et al., 2004; Niederreither et al., 1999), Tbx5 (Agarwal et al., 2003; Rallis et al., 2003) or Fgf10 (Min et al., 1998; Sekine et al., 1999) all display failure of limb induction similar to the corresponding zebrafish mutants (Begemann et al., 2001; Garrity et al., 2002; Grandel et al., 2002; Norton et al., 2005), thus indicating that the limb induction cascade is broadly conserved between tetrapods and teleost fish. However, there are clearly also differences. For example, mouse Wnt2b does not play a role in limb induction (Ng et al., 2002), and no gene corresponding to zebrafish fgf24 is present in tetrapod genomes (Draper et al., 2003). In both cases, other members of their respective gene families may fulfill their role in the mouse. Alternatively, specific steps in the limb induction cascade may have changed during evolution. To answer this question, it will be important to understand the regulation of early limb induction genes in several vertebrate species at the level of their promoter activity, as changes in signals regulating limb induction should be reflected in altered regulation of the promoters of their target genes.

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