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

**Nadia Mercader, Sabine Fischer and Carl J. Neumann** *Development* **133**, 2805-2815.

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 (Berggren 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 forelimb 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.

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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  $\beta$ -*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 *Lef1* 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 Wnt 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). *Fgf24*, 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 *blimp1* (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, 2002). In addition, *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 (Ohinata 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*<sup>hx118</sup>), 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'-TGTGTGATCTCTCCCCTGAGTGTGT-3') (Wilm et al., 2005) and *raldh2* (5'-GCAGTTCAACTTCACTGGAGGTCAT-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 *MOaldh2*. 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 *MOaldh2*. 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

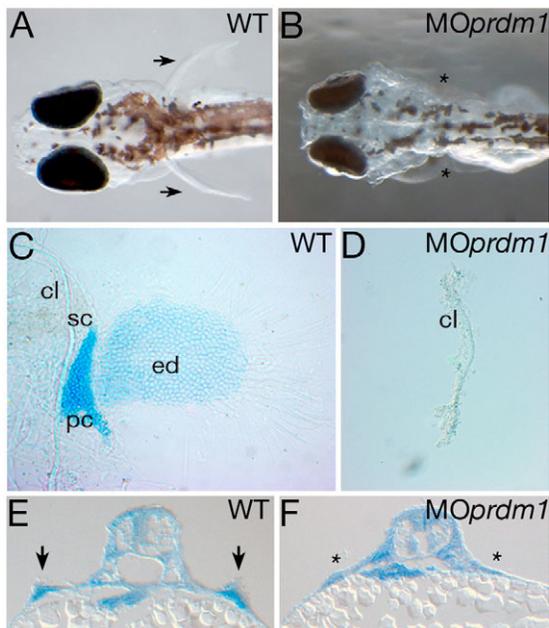
Diethylaminobenzoic acid (DEAB) (Sigma) and the FGF receptor inhibitor SU5402 (Calbiochem) were dissolved in dimethylsulfoxide (DMSO) and used at a concentration of 10 mM and 16  $\mu$ M, respectively. Incubations were carried out in the dark at 28°C.

### 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* (cb24 EST clone, Zebrafish International Resource Center). BM purple (Roche) was used as a substrate. For *prdm1* and *fgf24*, staining reactions were performed overnight at 37°C. Alcian Blue staining was performed according to Grandel and Schulte-Merkel (Grandel and Schulte-Merkel, 1998).

### 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

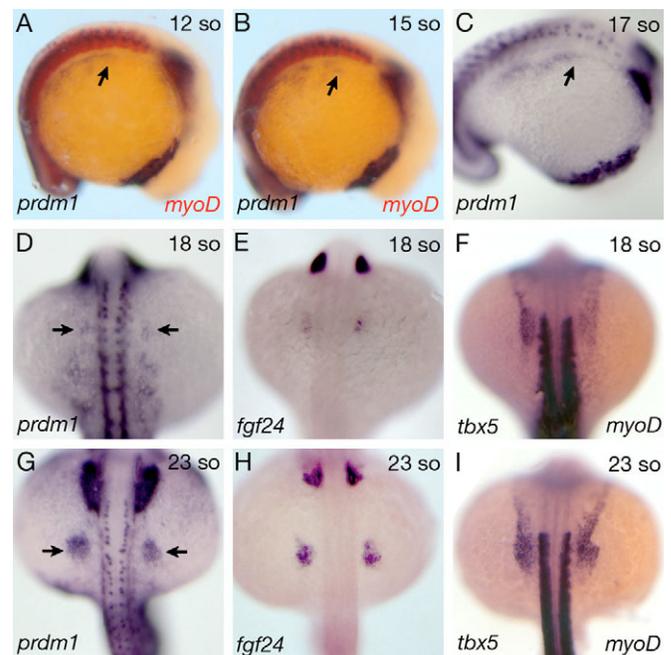


**Fig. 1. *prdm1* morphants lack pectoral fins.** (A,B) Dorsal views of wild-type (WT, A) and *prdm1* morphant (MO *prdm1*, B) four-day-old larvae. (C,D) Cartilage stainings of wild-type and *prdm1* morphant pectoral fins at four days postfertilization. Note that *prdm1* morphants only develop a cleithrum. (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.

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 (Dobbs-McAuliffe et al.,



**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.

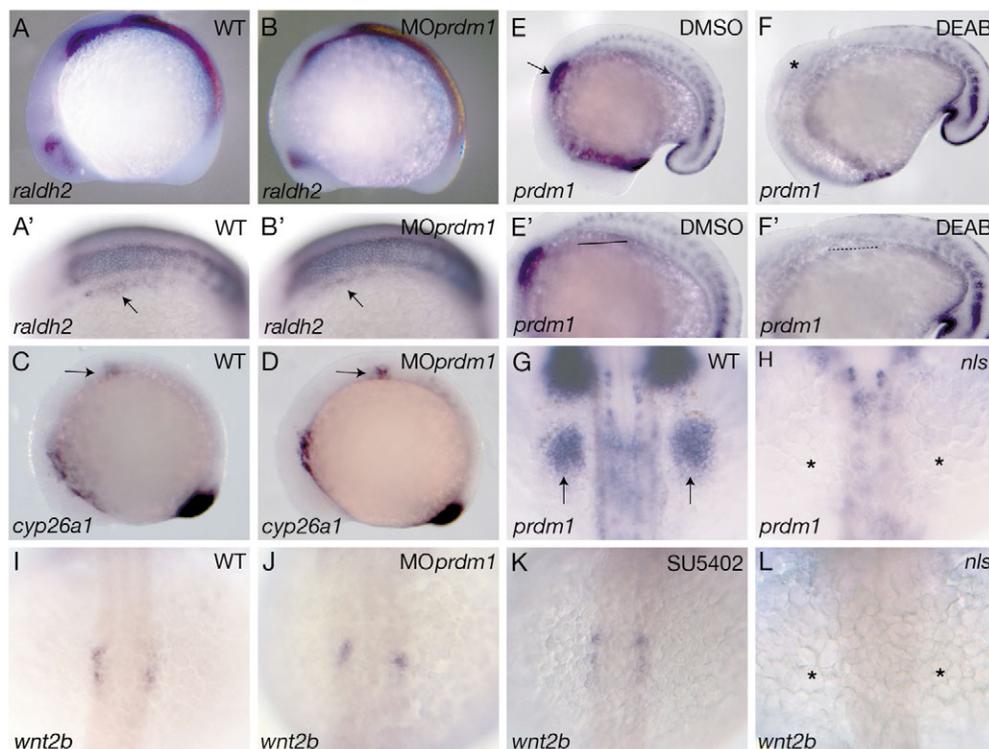
### Activation of *wnt2b* depends on Raldh2 activity, but not on Fgf signaling or Prdm1

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  $\mu$ M 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, and



**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  $\mu$ M 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 MO*prdm1* and SU5402-treated embryos, but is lost in the *raldh2* mutant *nls* (asterisks). DEAB, diethylaminobenzoic acid; DMSO, dimethylsulfoxide; *nls*, *neckless*; MO*prdm1*, *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 where GFP expression in the anterior somites was very strong, fin outgrowth was not restored in MO*prdm1* 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.

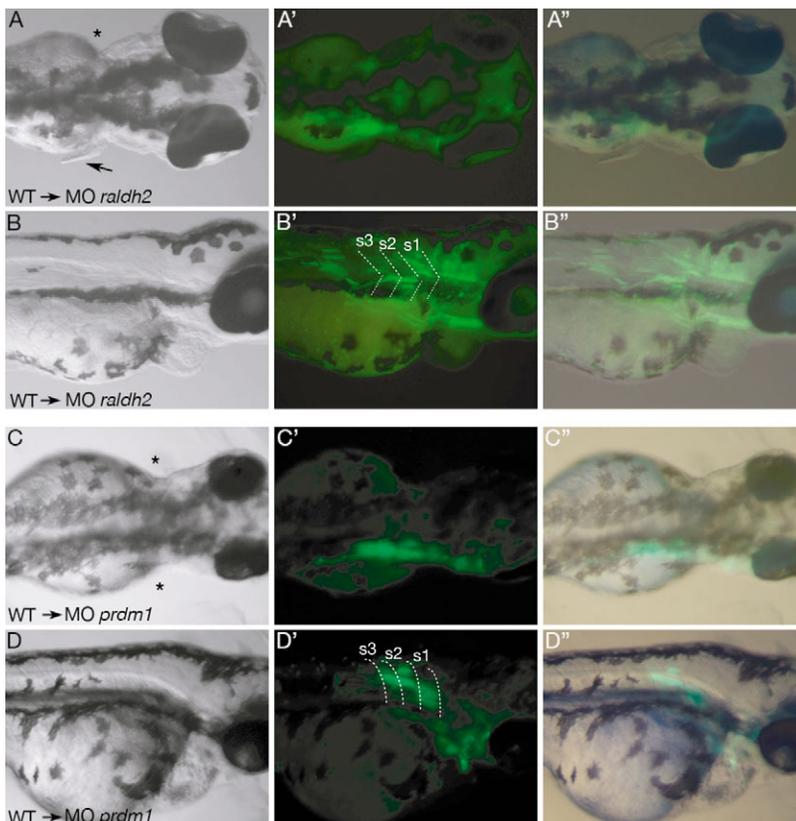
#### ***prdm1* is downstream of *tbx5* and *fgf24* during fin induction**

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,



**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. (A'') Merged bright-field and dark-field images showing green wild-type cells localizing to the anterior somite region. (B-B'') Lateral views of the same MO*raldh2* mosaic embryo as in A. Dotted lines in B' indicate somite boundaries. Note strong GFP-expression in somites 1 to 3. (C-D'') Dorsal and lateral views of an MO*prdm1* 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.

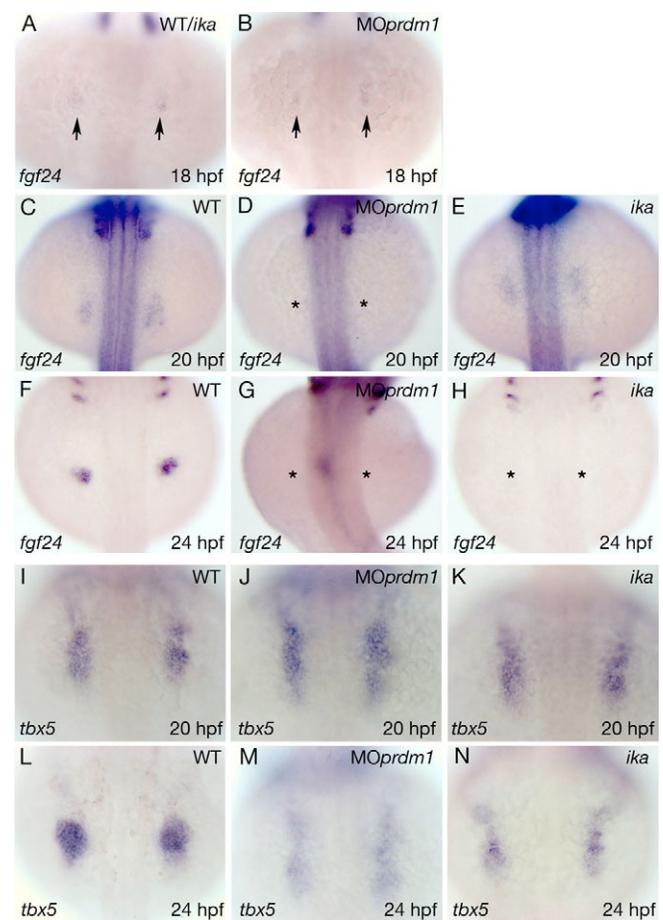
### Prdm1 acts upstream of Fgf10 during fin induction

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  $\mu$ M 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



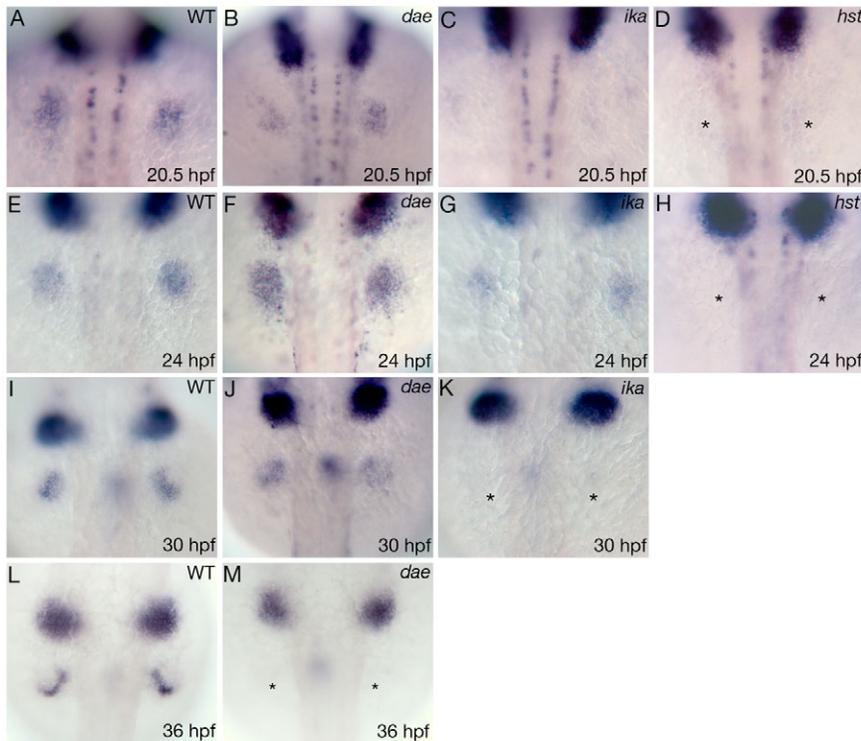
**Fig. 5. Expression of *fgf24* and *tbx5* in MO*prdm1*-injected embryos compared with *ika* mutants.** (A-H) Dorsal views of *fgf24* whole-mount in situ hybridization on wild-type, *prdm1* morphant or *ikarus* mutant embryos, as indicated in each panel. Anterior is to the top. *Fgf24* expression is reduced at 18 hpf (B) and is not visible in pectoral fin regions of 20 hpf (D) or 24 hpf (G) MO*prdm1*-injected embryos. Although the onset of *fgf24* expression is normal in *fgf24* mutant *ikarus* embryos (A,E), its expression is not maintained at later stages (H). Asterisks indicate missing *fgf24* expression. (I-N) Dorsal views of *tbx5* in situ hybridizations on 20 hpf (I-K) and 24 hpf (L-N) wild-type, *ika* and MO*prdm1* embryos. At 20 hpf, no difference can be detected between wild-type, MO*prdm1* and *ika* embryos. At 24 hpf, *tbx5* expression is strongly reduced in MO*prdm1* and *ika* fin buds. *ika*, *ikarus*; MO*prdm1*, *prdm1* morphant embryo; WT, wild type.

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



**Fig. 6. *prdm1* expression in *dae*, *ika* and *hst* mutant embryos.** (A-M) Dorsal views of *prdm1* expression in pectoral fin buds at 20.5 hpf (A-D), 24 hpf (E-H), 30 hpf (I-K) and 36 hpf (L,M) of wild-type, *hst*, *ika* and *dae* mutant embryos, as indicated in each panel. In *dae* embryos, activation of *prdm1* expression is normal (B,F,J) but its expression disappears at 36 hpf (asterisks, M). In *ika*, weak *prdm1* staining can be detected within the fin field at 24 hpf (G). This expression disappears in 30 hpf embryos (asterisks, K). In *hst*, *prdm1* cannot be detected at any stage analysed (marked by asterisks in D and H). *dae*, *daedalu*; *hst*, *heartstrings*; *ika*, *ikarus*; WT, wild type.

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).

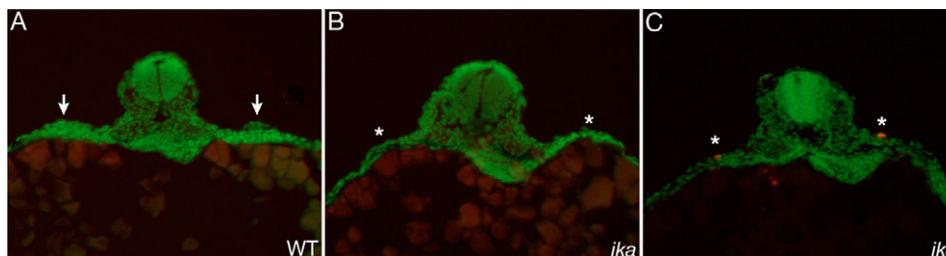
Our transplantation data indicate that Prdm1 activity is required within the fin bud itself during forelimb initiation. This excludes a role for Prdm1 in the anterior somites during forelimb initiation, even though *prdm1* is expressed in this tissue under the control of Raldh2 activity. In contrast 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

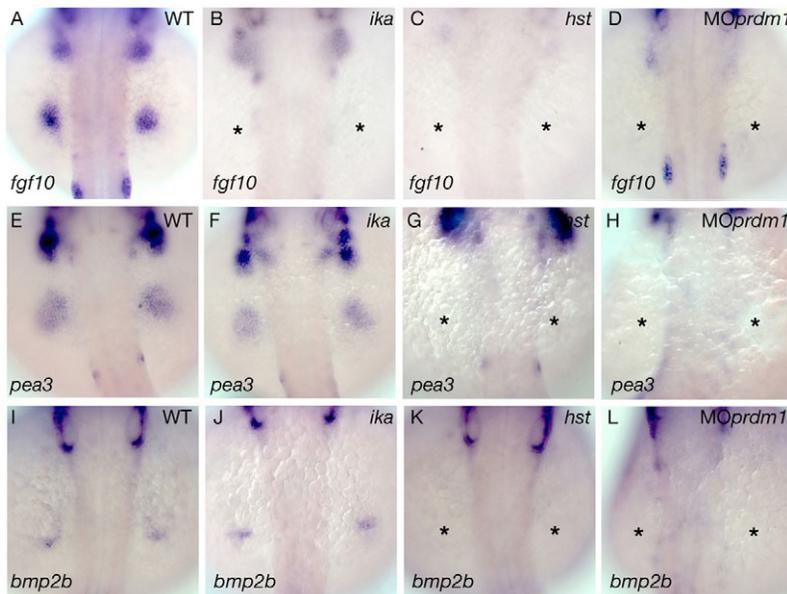
et al., 1997; Piskurich et al., 2000). This suggests that the activation of limb genes downstream of *prdm1* would have to be indirect, via repression of another repressor. We have observed that *prdm1* morphants have elevated levels of *prdm1* transcripts (data not shown), suggesting that during zebrafish 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



**Fig. 7. Analysis of cell death in *ika* mutant pectoral fin mesenchyme.** (A-C) TUNEL staining on 30 hpf wild-type (WT, A) and *ikarus* (*ika*) mutant (B,C) fin level cryosections. Anterior is to the top. No apoptotic cells could be detected in the fin mesenchyme of *ika* mutants ( $n=16$ , B). In one specimen, a few apoptotic cells could be detected in the apical fin ectoderm but not in the fin mesenchyme (C). Arrows indicate fin bud; asterisks indicate the absence of bud formation in *ika* mutants.

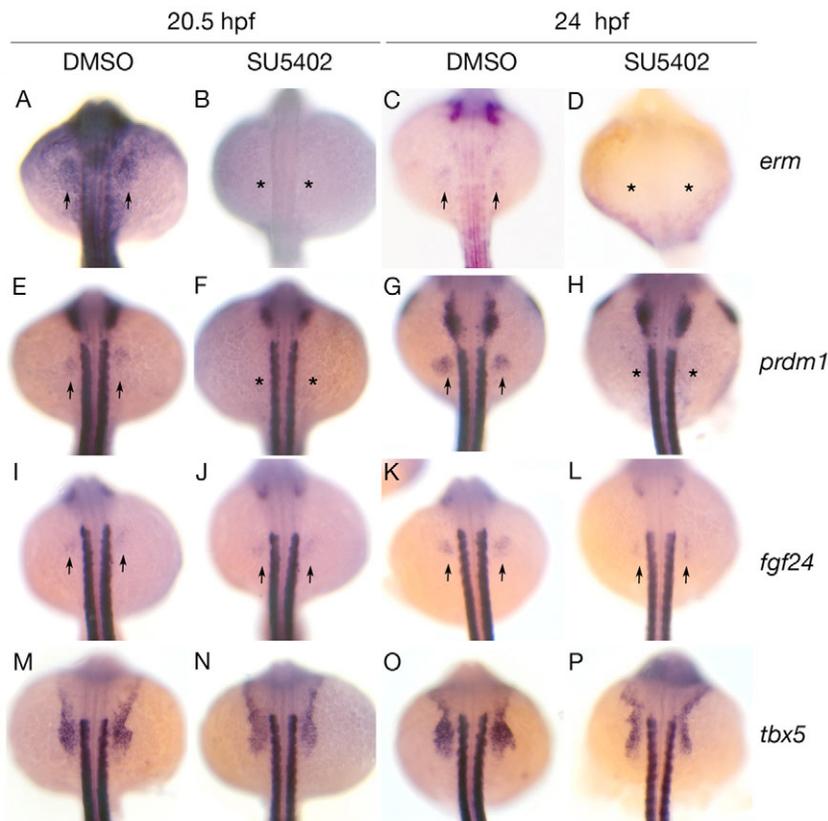


**Fig. 8. Comparison of *fgf10*, *pea3* and *bmp2* expression in *MOprdm1*-injected embryos, and in *hst* and *ika* mutants. (A-L)** Dorsal views of 30 hpf embryos stained for *fgf10* (A-D), *pea3* (E-H) or *bmp2b* (I-L) expression. While *fgf10* expression is absent in *ika*, *hst* and *MOprdm1* embryos (B,C,D), *pea3* and *bmp2b* are transiently expressed in *ika* (F,J) but not *hst* or *MOprdm1* embryos (G,H,K,L). Asterisks indicate a lack of marker gene expression within the pectoral fin mesenchyme. *hst*, *heartstrings*; *ika*, *ikarus*; *MOprdm1*, *prdm1* morphant embryo; WT, wild type.

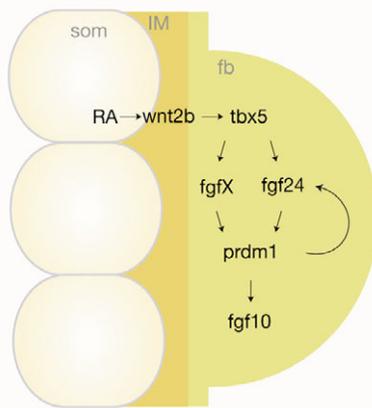
which these genes form a linear hierarchy controlling the transfer of the limb-inducing signal from the anterior somites to the LPM (Fig. 10). The earliest gene known to function in pectoral fin induction is *raldh2* (Begemann et al., 2001; Grandel et al., 2002). In the absence of Raldh2 activity, all other limb genes fail to be expressed, including *wnt2b* (this study) and *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, *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 *raldh2* for limb development raises the possibility that the effect of RA signaling is mediated via a second signal. Indeed, our results suggest that *wnt2b* performs this role in the pectoral fin. *wnt2b* is expressed in the IM adjacent to the forelimbs before *tbx5* is activated in the LPM, and our data show that *wnt2b* expression in the IM depends on Raldh2 activity. The simplest



**Fig. 9. Effect of early Fgf inhibitor treatment on *erm*, *prdm1*, *fgf24* and *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) *erm* expression is abolished in SU5402-treated embryos. (E-H) *prdm1* expression is downregulated in SU5402-treated embryos. (I-L) Upon SU5402 treatment, Fgf24 is still present at 20.5 hpf but becomes strongly reduced at 24 hpf. (M-P) *tbx5* expression is not affected at the 23-somite stage (M,N) but is reduced in 24 hpf embryos upon SU5402 treatment (O,P).



**Fig. 10. Schematic model of the genetic hierarchy leading to limb initiation in the zebrafish.** Arrows indicate position within the hierarchy. FgfX refers to an Fgf signaling molecule acting in parallel to Fgf24 that is required for *prdm1* expression. Bent arrow from *prdm1* to *fgf24* indicates a feedback mechanism operating between these two genes. fb, fin bud; IM, intermediate mesoderm; RA, retinoic acid; som, somites.

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 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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