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sall4 acts downstream of *tbx5* and is required for pectoral fin outgrowth

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Okihiro syndrome (OS) is defined by forelimb defects associated with the eye disorder Duane anomaly and results from mutations in the gene *SALL4*. Forelimb defects in individuals with OS range from subtle thumb abnormalities to truncated limbs. Mutations in the T-box transcription factor *TBX5* cause Holt-Oram syndrome (HOS), which results in forelimb and heart defects. Although mutations in *TBX5* result in HOS, it has been predicted that these mutations account for only ~30% of all individuals with HOS. Individuals with OS and HOS limb defects are very similar, in fact, individuals with mutations in *SALL4* have in some cases previously been diagnosed with HOS. Using zebrafish as a model, we have investigated the function of *sall4* and the relationship between *sall4* and *tbx5*, during forelimb development. We demonstrate that *sall4* and a related gene *sall1* act downstream of *tbx5* and are required for pectoral fin development. Our studies of Sall gene family redundancy and *tbx5* offer explanations for the similarity of individuals with OS and HOS limb defects.

KEY WORDS: *sall4, sall1, spalt, tbx5,* Pectoral fins, Zebrafish, Okihiro syndrome, Holt-Oram syndrome

INTRODUCTION

Mutations in the gene *SALL4* result in Okihiro syndrome [OS, also called Duane radial ray syndrome (DRRS), OMIM number 607323] (Al-Baradie et al., 2002; Kohlhase et al., 2002). OS is caused by *SALL4* haploinsufficiency (Borozdin et al., 2004) and is characterised by forelimb defects associated with the eye defect, Duane anomaly. The forelimb defects of individuals with OS range from subtle thumb abnormalities to severely truncated limbs (phocomelia) (Al-Baradie et al., 2002; Kohlhase et al., 2002). The thumb, which is the most anterior digit, is most commonly affected in OS (Borozdin et al., 2004). In addition to these defining features of OS, a range of less common abnormalities has been reported, including atrial septal defects (hole in the heart), ear problems, Hirschsprung's disease and pigmentation defects (Al-Baradie et al., 2002; Kohlhase et al., 2002). Another Sall gene family member associated with developmental defects in humans is *SALL1,* which when mutated results in Townes-Brocks syndrome (TBS, OMIM number 107480) (Kohlhase et al., 1998). Individuals with TBS have limb defects, abnormal ears, imperforate anus and kidney abnormalities (Kohlhase et al., 1998). The limb defects of individuals with TBS include preaxial polydactyly and triphalangeal thumb in the forelimbs, and syndactyly and club foot in the hindlimbs (Kohlhase et al., 1999). *Sall1*-null mice do not phenocopy TBS (Nishinakamura et al., 2001); however, mice expressing a truncated form of *Sall1* do have TBS-like defects (Kiefer et al., 2003). This suggests TBS results from mutations that produce a truncated, dominant-negative form of *SALL1* and is not due to *SALL1* haploinsufficiency. Consistent with such a model, mutations in *SALL1* associated with TBS are predicted to form truncated SALL1 protein products.

Sall1 and *Sall4* belong to a family of zinc finger transcription factors that share homology to the founding member of the gene family, the Drosophila *spalt* gene (Reuter et al., 1989). There are four known Sal-like (Sall) members in vertebrates (*Sall1-4*) that are defined by the presence of an N-terminal Cys2-His-Cys zinc finger. *Sall4* has a further seven zinc fingers of the Cys2-His2 type that are arranged into three double zinc-finger domains. An additional zinc finger is found in close proximity to the second double zinc finger (Al-Baradie et al., 2002; Kohlhase et al., 2002). The double zinc finger domains are characteristic of Sall gene family members. In *Drosophila*, *spalt* acts downstream of the T-box gene *optomotor-blind* (*omb*) and is required for correct patterning of the wing imaginal discs (de Celis et al., 1996; Del Alamo Rodriguez et al., 2004).

Members of the T-box transcription factor gene family are characterised by the presence of a conserved DNA-binding motif known as the T-domain. Mutations in several different T-box genes are associated with developmental disorders (for a review, see Packham and Brook, 2003), including *TBX5*, which, when mutated in humans, results in Holt-Oram syndrome (HOS, OMIM number 142900) (Basson et al., 1997; Li et al., 1997). HOS, which is caused by *TBX5* haploinsufficiency, is defined by heart and forelimb abnormalities (Packham and Brook, 2003). The limb deformities seen in individuals with HOS range from thumb defects to phocomelia (Basson et al., 1997; Li et al., 1997). There is an anterior bias to the limb defects of individuals with HOS such that the thumb and radius bones are predominantly affected (Packham and Brook, 2003). Less common defects reported in individuals with HOS include absent pectoral muscles and eye problems, such as Duane anomaly (Newbury-Ecob et al., 1996). Although *TBX5* mutations are associated with HOS it has been predicted that these mutations only account for ~30% of individuals with HOS (Cross et al., 2000).

Previous loss-of-function experiments in zebrafish and mouse, and misexpression of dominant-negative *Tbx5* constructs in chick, have demonstrated that *Tbx5* is required for the initiation and outgrowth of the forelimb (for a review, see Logan, 2003). Identifying genes that genetically interact with *Tbx5* could uncover genes with essential roles in normal limb development and which, when mutated in humans, may result in HOS-like phenotypes. The forelimb defects in individuals with OS and HOS are very similar. In both conditions there is an anterior bias to the limb defects and the left limb is more severely affected than the right. In addition to

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this phenotypic similarity, several individuals previously diagnosed with HOS, but lacking *TBX5* mutations, have subsequently been shown to have mutations in *SALL4* (Brassington et al., 2003). Zebrafish are a useful model species with which to study forelimb/pectoral fin development (Fischer et al., 2003; Garrity et al., 2002; van Eeden et al., 1996). We have used zebrafish to investigate the function of *sall4* during limb development and to explore the relationship between *sall4* and *tbx5*. We demonstrate an essential role for *sall4* during pectoral fin development and show redundant functions between sall gene family members. Our results offer explanations for the similar limb phenotypes of individuals with OS and HOS.

MATERIALS AND METHODS

Embryo staining

Whole-mount RNA in situ hybridisation was performed essentially as described (Thisse et al., 1993). The following probes have been described previously: *dlx2*, *fgf8*, *fgf10*, *fgf24*, *erm* (Fischer et al., 2003), *fgfr2* (Poss et al., 2000), *sp9* (Norton et al., 2005), *tbx5* (Begemann and Ingham, 2000) and *sall1a* (IMAGE consortium–accession number BI880033). A 1.6 kb fragment of *sall4* was isolated, with the primers 5′-CTACTAGTGTCTTACTATTCGCCCCTGAT-3′ and 5′-CAGAAGAAATCGATGCACCAT-3′ using RT-PCR on 24 hpf whole embryo RNA, and used as an in situ probe template. Section in situ analysis was performed by wax embedding and sectioning whole-mount preparations. Skeletal preparations were performed as previously described (Grandel and Schulte-Merker, 1998).

Morpholinos

To overcome problems with morpholino (MO) design, we cloned a fragment of *sall4* pre-mRNA that spans the boundary between exon 1 and intron 1, using RT-PCR with RNA from zebrafish lines that we intended to inject. Using the sequence of this clone and zebrafish genomic sequence, we designed a MO that is antisense to the boundary between exon 1 and intron 1 of *sall4* pre-mRNA: 5′-CGCTCCAAACTCACCATTTTCTGTC-3′. We used a 5 bp mismatch of this MO as a control: 5′-CGgTCgAAACTgACgATTTTCTgTC-3′ (lower case letters indicate altered bases).

To test the efficiency of the *sall4* MO, RT-PCR was performed using whole-embryo RNA from ~20 embryos at 24 hpf, using the primers 5′-TACAAAACTTCTCGAATTCAC-3′, 5′-GACATGCGCATTTCTACTCGAGGG-3′ and 5′-AGAATTCCGCAAACCCTTGTCTCCTCCG-3′ to detect spliced and un-spliced *sall4* mRNA transcripts.

The sequence of the *sall1a* MO, which is antisense to the 5′UTR, is 5′-GGCTCACGCATCAGCCACGAAAGAA-3′. The *tbx5* and *fgf24* MOs 5′-GAAAGGTGTCTTCACTGTCCGCCAT-3′ and 5′-GACGGCAGAACAGACATCTTGGTCA-3′, respectively, have previously been described previously (Ahn et al., 2002; Garrity et al., 2002; Fischer et al., 2003). All MOs were obtained from Gene Tools.

Embryo staging

Embryos were laid at 10 am and this time was taken as 0 hpf. Embryos were incubated at 28°C and were further staged using criteria previously established (Grandel and Schulte-Merker, 1998; Kimmel et al., 1995).

RESULTS

sall4 is required for pectoral fin outgrowth

To understand the role of *sall4* during limb development, we cloned the zebrafish *sall4* homologue and studied its expression during pectoral fin development. Using in situ hybridisation, *sall4* mRNA transcripts are first detectable in the mesenchyme and not the overlying ectoderm of the pectoral fin primordia at 22 hours post fertilisation (hpf, Fig. 1A). During early pectoral fin bud stages (32 hpf), *sall4* is expressed throughout the fin bud mesenchyme (Fig.

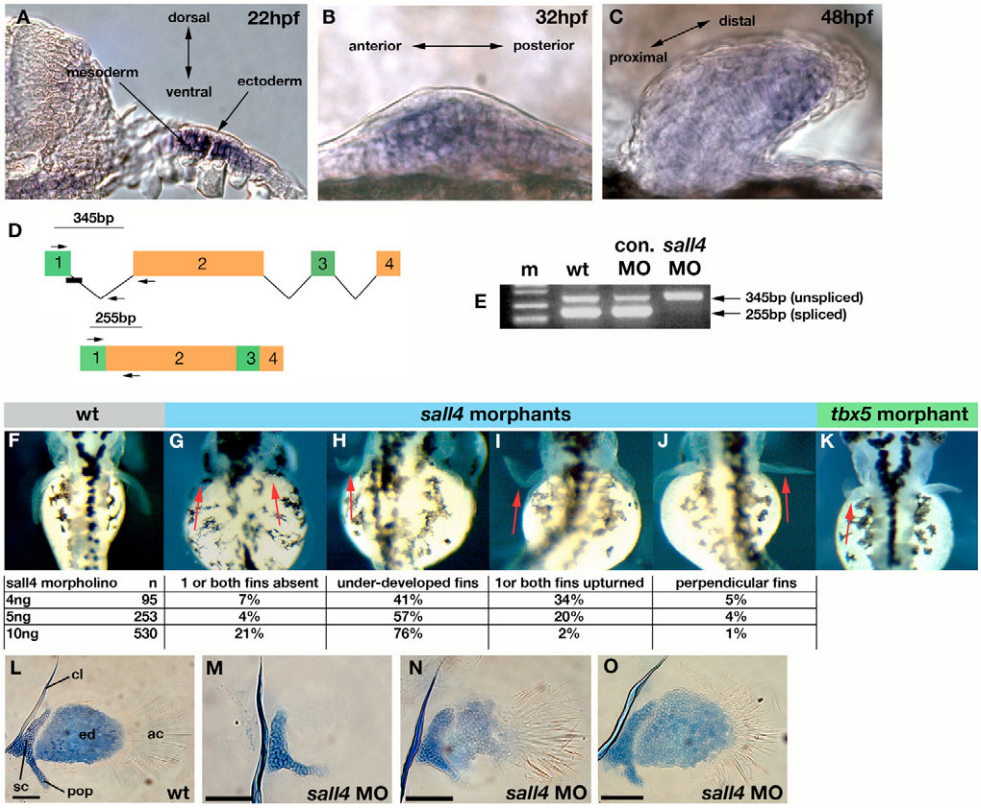


Fig. 1. *sall4* is required for pectoral fin outgrowth. (**A-C**) *sall4* expression during zebrafish pectoral fin development. (A) Section through the pectoral fins at 22 hpf. Lateral view of the fin buds at 32 hpf (B) and 48 hpf (C). (**D**) Schematic of the RT-PCR used to test the efficiency of a *sall4* splice-blocking MO. Bands of 255 bp and 345 bp represent spliced and unspliced *sall4* mRNA, respectively. Arrows represent primers used in the RT-PCR and the black box represents the *sall4* MO. (**E**) The RT-PCR was performed on wild-type embryos and embryos injected with 5 ng of the *sall4* MO. The *sall4* MO efficiently blocks *sall4* mRNA splicing. There was no inhibition of *sall4* splicing in embryos injected with 5 ng of a 5 bp mismatch control *sall4* MO (con. MO, control MO; M, DNA molecular weight marker). (F-K) Dorsal views of the pectoral fins of 3 dpf embryos. (**F**) Wild-type embryo. (**G-J**) Embryos injected with 5 ng of *sall4* MO. The percentages of phenotypes observed at 3 dpf following injection of different concentrations of *sall4* MO is listed below each picture. (**K**) Upturned pectoral fin phenotype produced following injection of 1 ng of *tbx5* MO. Arrows highlight the pectoral fin defects in G-K. (**L-O**) Pectoral fins (5 dpf) stained with Alcian Blue. (L) Wild type. (M-O) The pectoral fins of embryos injected with 4 ng of *sall4* MO. Scale bars: 75 μm. cl, cleithrum; sc, scapulocoracoid; pop, postcoracoid process; ed, endoskeletal disc; ac, actinotrichs.

1B). As the pectoral fins mature, transcripts remain detectable throughout the mesenchyme, with highest levels at the distal tip of the fin (Fig. 1C).

To investigate the function of *sall4* during pectoral fin development, we designed an antisense morpholino oligonucleotide (MO) that inhibits splicing of *sall4* pre-mRNA and subsequently leads to knockdown of *sall4* function. Using such a splice-blocking MO is advantageous as the efficiency of gene knockdown can be tested using RT-PCR (Draper et al., 2001). In wild-type embryos, we detect spliced and unspliced *sall4* mRNA, using RT-PCR (Fig. 1D,E). Only unspliced *sall4* transcripts are detectable in embryos injected with 5 ng of the *sall4* MO, whereas there is no effect on splicing in embryos injected with 5 ng of a 5 bp mismatch control MO (Fig. 1E). This demonstrates the *sall4* MO can efficiently block production of the mature *sall4* spliced transcript.

We allowed *sall4* morphant embryos to develop until 3 days post fertilisation (dpf) and compared their pectoral fins with those of wild-type embryos. *sall4* morphants have a range of pectoral fin defects, from a complete absence of both pectoral fins to those that develop to approximately wild-type size but are positioned perpendicular to the body (Fig. 1G-J). Injection of higher concentrations of *sall4* MO results in an increase in the severity of pectoral fin defects (Fig. 1). All embryos injected with 5 ng of the control MO are apparently wild type at 3 dpf (n=64, data not shown). Some *sall4* morphant embryos have pectoral fins that turn rostrally, towards the head of the embryo (Fig. 1I). We, and others (Garrity et al., 2002), have observed a similar pectoral fin phenotype when embryos are injected with low concentrations of a *tbx5* MO (Fig. 1K).

We stained 5 dpf *sall4* morphant embryos with Alcian Blue to study the individual elements that comprise their pectoral fin defects. At 5 dpf, wild-type zebrafish pectoral fins consists of a scapulocoracoid, postcoracoid process, endoskeletal disc and actinotrichs (Fig. 1L) (Grandel and Schulte-Merker, 1998). During the third week in development, these larval pectoral fins begin to be remodelled to form the adult pectoral fin. The scapulocoracoid and postcoracoid process will form the scapula, while the endoskeletal disc will form the proximal radials which articulate the lepidotrichia (fin rays), which form from the actinotrichs (Grandel and Schulte-Merker, 1998; Sordino et al., 1995). The most severely affected *sall4* morphant pectoral fins only possess proximal elements, the scapulocoracoid and postcoracoid process (Fig. 1M). Other *sall4* morphant pectoral fins retain a severely truncated endoskeletal disc (Fig. 1N). We also observe *sall4* morphant pectoral fins that have an endoskeletal disc that is decreased in size and truncated distally (Fig. 1O). Any actinotrichs that form in *sall4* morphant pectoral fins are truncated and scattered compared with wild-type embryos (Fig. 1O). These loss-of-function experiments demonstrate that although *sall4* is not required for the initiation of pectoral fin development, it is essential for outgrowth of the pectoral fins. These results also show that proximal pectoral fin elements, the scapulocoracoid and postcoracoid process, form independently of *sall4* function.

sall4 is downstream of *tbx5*, but not *fgf24*, in the pectoral fin primordia

Owing to the similar limb phenotypes of individuals with OS and HOS, and because *tbx5* expression precedes *sall4* in the pectoral fin primordia (Fig. 2A) (Begemann and Ingham, 2000; Ruvinsky et al., 2000), we investigated if *tbx5* is required for *sall4* expression during pectoral fin development. We used a *tbx5* MO of identical sequence to one previously demonstrated to phenocopy the ENU-induced *tbx5* mutation *heartstrings* (Ahn et al., 2002; Garrity et al., 2002). We

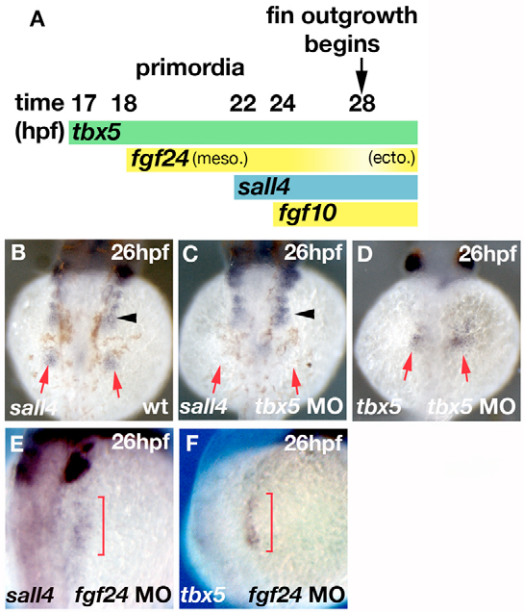


Fig. 2. The expression of *sall4* in the pectoral fins is regulated by *tbx5* but not *fgf24*. (**A**) Schematic representation of *tbx5, fgf24, sall4* and *fgf10* expression during early stages of pectoral fin development. Expression of *tbx5* initiates at 17 hpf, *fgf24* at 18 hpf, *sall4* at 22 hpf and *fgf10* at 24 hpf. The genes are all initially expressed throughout the mesenchyme of the pectoral fin primordia. At the time fin bud outgrowth is initiated, *fgf24* expression becomes downregulated in the mesenchyme and simultaneously upregulated in the overlying ectoderm. Dorsal (**B-D**) and dorsolateral (**E**,**F**) views of embryos, with red arrows and brackets highlighting the pectoral fin primordia. *sall4* is expressed in wild-type pectoral fin primordia at 26 hpf (B), but it is absent in the primordia of embryos injected with 4 ng of *tbx5* MO (C). Although *sall4* expression is absent in the pectoral fin primordia, it continues to be expressed normally in other regions of *tbx5* morphant embryos (black arrowheads in B,C). (D) *tbx5* expression in an embryo injected with 4 ng of *tbx5* MO at 26 hpf. (E) *sall4* is still expressed in the pectoral fin primordia of embryos injected with 9 ng of *fgf24* MO, although the domain of expression is more dispersed, consistent with a loss of *fgf24* function. This expression pattern is similar to that of *tbx5* in embryos injected with 9 ng of *fgf24* MO (F).

observe, as previously described, that embryos injected with 4 ng of *tbx5* MO have no pectoral fins at 3 dpf (data not shown). In *tbx5* morphant embryos, *sall4* expression is never detectable in the pectoral fin primordia (red arrows, compare Fig. 2B with 2C), although expression in other regions of the embryo is normal (black arrowheads, compare Fig. 2B and 2C). At the same stages, the pectoral fin primordia continues to express *tbx5* mRNA transcripts (Fig. 2D), demonstrating that the cells of the fin primordia are still present and that the loss of *sall4* expression is not simply due to apoptosis of these cells following MO knockdown of *tbx5*.

In the mesenchyme of the pectoral fin primordia, *tbx5* is required for *fgf24* expression (Fischer et al., 2003). The pectoral fins fail to form in *fgf24* mutant embryos, although the expression of *tbx5* is initiated normally (Fischer et al., 2003). *fgf24* is initially expressed in the fin bud mesenchyme and is required for the induction of *fgf10* expression at 24 hpf, also within the mesenchyme (Fischer et al., 2003). As *sall4* expression begins after *fgf24* (Fig. 1A, Fig. 2A) (Fischer et al., 2003), we tested the possibility that *fgf24* acts downstream of *tbx5* to initiate *sall4* expression in a linear fashion, using an *fgf24* MO demonstrated to phenocopy the *fgf24* mutant

ikarus (Fischer et al., 2003). Embryos injected with 6-9ng of *fgf24* MO have no pectoral fins at 3 dpf (data not shown), consistent with previously published results (Fischer et al., 2003). When *fgf24* morphant embryos were analysed at earlier stages (26hpf), *sall4* expression is maintained in the pectoral fin primordia but in a diffuse pattern (Fig. 2E). This expression pattern is similar to *tbx5* in *fgf24* morphant pectoral fin primordia (Fig. 2F), and is consistent with the reported disruption of cell migration following loss of *fgf24* function (Fischer et al., 2003). These results demonstrate that, although induction of *sall4* expression requires *tbx5*, it is independent of *fgf24*.

sall4 is required for FGF signalling during pectoral fin development

Zebrafish with mutations in *fgf10* lack pectoral fins, demonstrating that, like *sall4*, *fgf10* is required for pectoral fin development (Norton et al., 2005). As *fgf10* expression is first detected 2 hours after *sall4* in the pectoral fin primordia (Ng et al., 2002), we addressed the possibility that *sall4* is required for *fgf10* expression in the developing pectoral fins. For simplicity, we will now refer to embryos injected with 10 ng of *sall4* MO as *sall4* morphants. In *sall4* morphant pectoral fins, *fgf10* expression initiates but is reduced when compared with wild-type pectoral fins (compare Fig. 3A with 3B, reduced in 51% at 30 hpf, *n*=35). At later fin bud stages, *fgf10* expression is downregulated in anterior regions of *sall4* morphant pectoral fins (compare Fig. 3C with 3D, downregulated in 83%, *n*=29), demonstrating that *sall4* is required for correct *fgf10* expression during pectoral fin development.

During mouse and chick limb development, *Fgf10*, which is expressed in the mesenchyme, signals to the overlying ectoderm to activate the expression of *Fgf8* in cells that will form the apical ectodermal ridge (AER). In turn, FGF8 positively regulates the expression of *Fgf10* in the mesenchyme, thereby establishing a positive feedback loop in which ectodermal and mesenchymal FGFs maintain the expression of one another. This feedback loop is essential for limb outgrowth (for a review, see Martin, 1998). We therefore predicted that the downregulation of *fgf10* in the mesenchyme of *sall4* morphant pectoral fin buds would lead to the downregulation of ectodermal FGFs and a breakdown in FGF signalling in the fin bud. During normal pectoral fin development, *fgf24* is expressed in the mesenchyme from 18 hpf until ~28 hpf when it then becomes downregulated in the mesenchyme and begins to be expressed in the overlying ectoderm (Fig. 2A) (Fischer et al., 2003). In *sall4* morphant embryos, expression of ectodermal *fgf24* and *fgf8* are downregulated (*fgf24*: 36%, *n*=28; *fgf8*: 22%, *n*=58) or absent (*fgf24*: 36%; *fgf8*: 74%) from the fin ectoderm at 40 hpf but remains normal in other regions of the embryo (Fig. 3E,F; data not shown). *dlx2* and *sp9* are also expressed in the fin bud ectoderm and their expression is positively regulated by FGF signalling from the fin bud mesenchyme (Fischer et al., 2003; Norton et al., 2005). At early time points in pectoral fin development (32 hpf) *dlx2* and *sp9* expression is present in the ectoderm of all *sall4* morphant fin buds (*dlx2* *n*=24; *sp9* *n*=10). However, in more mature *sall4* morphant fin buds (40 hpf), *dlx2* and *sp9* expression is downregulated (*dlx2*: 75% *n*=16; *sp9*: 58% *n*=12)

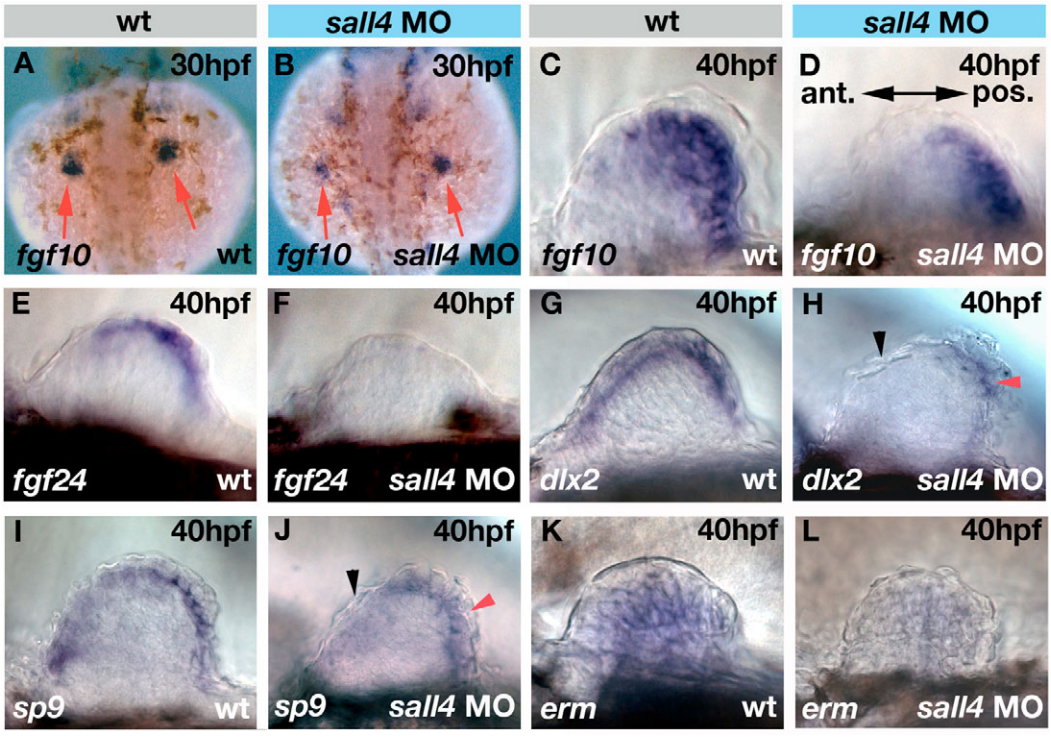


Fig. 3. *sall4* is required for FGF signalling in the developing pectoral fins. Comparison of gene expression patterns in wild-type (A,C,E,G,I,K) and *sall4* morphant pectoral fins (B,D,F,H,J,L). *fgf10* expression in the pectoral fins at 30 hpf (**A**) is reduced in *sall4* morphant embryos (**B**), as highlighted by the red arrows. *fgf10* expression at 40 hpf (**C**) is reduced in *sall4* morphant pectoral fins (**D**). This reduction is most pronounced in the anterior fin bud. At 40 hpf, *fgf24* is detected only in the ectoderm (**E**) but is absent in *sall4* morphant fin buds (**F**). *dlx2* is expressed in the fin bud ectoderm at 40 hpf (**G**). In *sall4* morphant embryos, *dlx2* expression is absent in the anterior fin bud ectoderm (**H**, black arrowhead) but retained in the posterior (red arrowhead). *sp9* is also expressed in the fin bud ectoderm at 40 hpf (**I**) but is downregulated in the anterior ectoderm of *sall4* morphant fin buds (**J**, black arrowhead) and retained in the posterior (red arrowhead). *erm* is expressed in wild-type pectoral fins (**K**) but is reduced in *sall4* morphant pectoral fins (**L**).

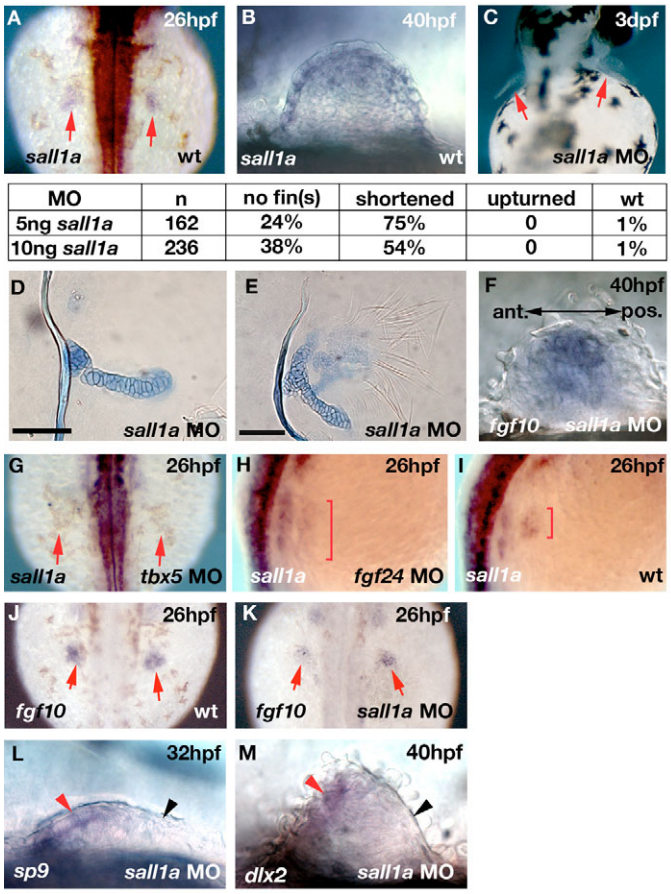
or absent (*dlx2* 25%; *sp9* 25%) (Fig. 3G-J). In those *sall4* morphant fin buds in which *dlx2* and *sp9* expression is downregulated, we observed that although transcripts remain detectable in the posterior fin bud ectoderm (Fig. 3H,J, red arrowheads) they are absent from the anterior ectoderm (black arrowheads), consistent with the loss of *fgf10* expression in the anterior of *sall4* morphant fin buds. The transcription factor *erm* is expressed throughout the fin bud mesenchyme and its expression is positively regulated by FGF signalling (Fischer et al., 2003; Roehl and Nusslein-Volhard, 2001). In *sall4* morphant pectoral fins, *erm* expression is initially unaffected but is downregulated at 40 hpf (compare Fig. 3K and 3L), while remaining normal in other regions of the embryo. These results show that *sall4* is required for *fgf10* expression in the developing pectoral fins and the downregulation of *fgf10* expression in *sall4* morphant pectoral fins results in a breakdown in FGF signalling in the fin bud.

sall1a is expressed in the developing pectoral fins

During mouse limb development, *Sall1* and *Sall3* are expressed in overlapping domains and deletion of either gene individually does not produce a limb phenotype (Nishinakamura et al., 2001; Parrish et al., 2004). This suggests Sall genes have redundant functions during mouse limb development. In *sall4* morphant embryos, *fgf10* expression is downregulated only in the anterior fin bud (Fig. 3D), suggesting that another Sall gene family member may perform a similar function to *sall4* in the posterior fin bud. The expression patterns of *sall1a*, *sall1b* and *sall3* during zebrafish embryonic development have previously been described (Camp et al., 2003). Of these three genes, only *sall1a* is expressed during pectoral fin development (Camp et al., 2003). *sall1a* is weakly expressed in the pectoral fin primordia at 24 hpf and becomes more visible at 26 hpf (Fig. 4A). At later fin bud stages, *sall1a* expression is seen in both the mesenchyme and ectoderm, and at greatest levels in the distal fin bud (Fig. 4B). This expression pattern is comparable with that of mouse and chick *Sall1* during limb development (Buck et al., 2001; Farrell and Munsterberg, 2000).

sall1a is required for pectoral fin development

To address whether *sall1a* plays a role in pectoral fin development, we used a MO to knockdown *sall1a* mRNA translation and compared the pectoral fins of 3dpf *sall1a* morphants with those of wild-type embryos. Embryos injected with the *sall1a* MO have truncated and often absent pectoral fins, demonstrating *sall1a* is required for pectoral fin outgrowth (Fig. 4C). *sall1a* morphant pectoral fin defects differ from those of *sall4* morphants, as we never observe upturned pectoral fins in *sall1a* morphants (see table in Fig. 4). We stained 5 dpf embryos injected with 2 ng of *sall1a* MO with Alcian Blue to study the skeletal defects. Proximal skeletal elements such as the postcoracoid process always form in *sall1a* morphant embryos (Fig. 4D). We also observed *sall1a* morphant pectoral fins in which the endoskeletal disc and actinotrichs are severely abnormal (Fig. 4E). The *sall1a* morphant pectoral fin defects observed are comparable with those seen in embryos injected with the *sall4* MO. To understand the regulation of *sall1a* during pectoral fin development, we studied *sall1a* expression in *tbx5* and *fgf24* morphant embryos. *sall1a* is not expressed in *tbx5* morphant pectoral fin primordia (Fig. 4G), but is expressed in the pectoral fins of *fgf24* morphant embryos (Fig. 4H). *sall1a* is expressed in a diffuse pattern in *fgf24* morphant pectoral fin primordia when compared with *sall1a* expression in wild-type pectoral fins (Fig. 4I). This expression pattern is consistent with a disruption in cell migration following loss of *fgf24* function (Fischer et al., 2003) and is comparable with *sall4* and *tbx5* expression in *fgf24* morphant embryos (Fig. 2E,F). These results demonstrate that like *sall4*, *sall1a* expression in the developing pectoral fins is dependant on *tbx5* but independent of *fgf24*.



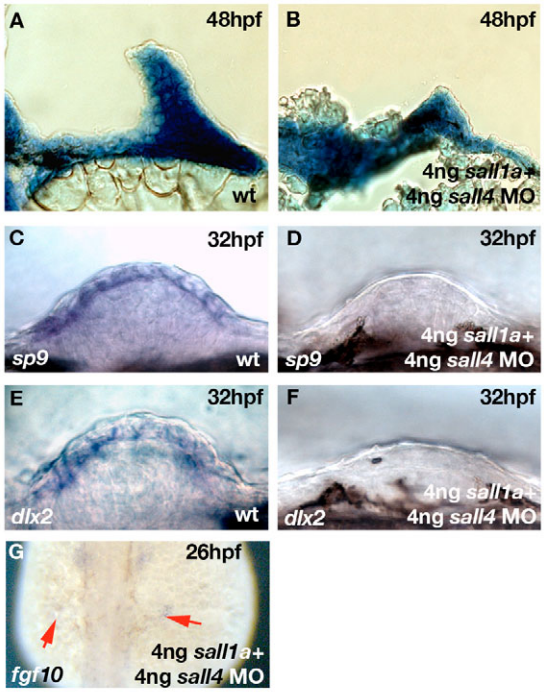
MO	n	no fin(s)	shortened	upturned	wt
5ng *sall1a*	162	24%	75%	0	1%
10ng *sall1a*	236	38%	54%	0	1%

Fig. 4. *sall1a* is essential for pectoral fin development. (A,B) *sall1a* expression during wild type pectoral fin development. (**A**) Dorsal view of a 26 hpf embryo with red arrows indicating the pectoral fin primordia. (**B**) Lateral view of a 40 hpf pectoral fin showing *sall1a* expression in the fin bud. (**C**) Dorsal view of a 3 dpf embryo injected with 5 ng of *sall1a* MO. The pectoral fins are truncated as highlighted by red arrows (compare with wild type in Fig. 1F). The percentages of phenotypes observed at 3 dpf following injection of 5 ng and 10 ng of *sall1a* MO are shown in the table. (**D**,**E**) Alcian Blue stained pectoral fins of 5 dpf embryos injected with 2 ng of *sall1a* MO show that pectoral fin development is disrupted in *sall1a* morphant embryos (compare with wild-type pectoral fin in Fig. 1L). (**F**) A lateral view of *fgf10* expression at 40 hpf in an embryo injected with 5 ng of *sall1a* MO. The expression of *fgf10* is most profoundly affected in the posterior fin bud (compare with Fig. 3C). (**G**) *sall1a* is not expressed in the pectoral fin primordia (red arrows) of embryos injected with 4 ng of *tbx5* MO. (H,I) Dorsolateral views show that *sall1a* (red brackets) continues to be expressed in the pectoral fin primordia of embryos injected 9 ng of *fgf24* MO (**H**), but is in a diffuse pattern when compared with the expression of *sall1a* in wild-type primordia (**I**). (J,K) Dorsal views of *fgf10* expression at 26 hpf in the fin primordia (as indicated with red arrows) of a wild-type embryo (**J**) and an embryo injected with 5 ng of *sall1a* MO (**K**). (L,M) Expression of the ectodermal fin bud markers *sp9* (**L**) and *dlx2* (**M**) are downregulated in the posterior fin bud ectoderm of embryos injected with 5 ng of *sall1a* MO (black arrowheads) but continue to be expressed in the anterior fin bud (red arrowheads; compare L with wild-type expression in Fig. 5C, and M with Fig. 3G).

Sall gene family members have redundant functions during pectoral fin development

As *sall1a* could be responsible for the maintenance of the posterior domain of *fgf10* expression in the pectoral fin bud of *sall4* morphants, we studied *fgf10* expression in *sall1a* morphant embryos (embryos injected with 5 ng of *sall1a* MO). *fgf10* expression initiates in *sall1a* morphant pectoral fin primordia but at reduced levels compared with wild-type embryos (Fig. 4J,K). At later fin bud stages, *fgf10* expression is downregulated but most profoundly in the posterior of *sall1a* morphant fin buds (Fig. 4F, compare with Fig. 3C). We also studied the expression of the ectodermal fin bud markers *dlx2* and *sp9* in *sall1a* morphant embryos. At 32 hpf *sp9* expression is downregulated in the posterior of *sall1a* morphant fin buds (Fig. 4L, black arrowhead, 13% *n*=24), but continues to be expressed in the anterior (red arrowhead). *dlx2* is also expressed in all *sall1a* morphant fin buds at 32 hpf (*n*=25), but at 40 hpf becomes downregulated (37%, *n*=16) or is absent (63%). In those embryos displaying a downregulation of *dlx2* expression, transcripts are detectable in the anterior fin bud ectoderm but are absent in the posterior (Fig. 4M). The preferential downregulation of *dlx2* and *sp9* expression in the posterior fin bud ectoderm is consistent with the downregulation of *fgf10* in the posterior mesenchyme of *sall1a* morphant pectoral fins.

As *sall1a* and *sall4* appear to perform similar roles in positively regulating the expression of *fgf10* during pectoral fin development, we studied the phenotype of *sall1a/sall4* double morphant embryos. The pectoral fins fail to form in the majority of embryos injected with 4 ng of *sall1a* and 4 ng of *sall4* MO (Fig. 5, table). Methylene Blue stained sections of 48 hpf *sall1a/sall4* double morphant embryos shows that, similar to *fgf10*$^{-/-}$ zebrafish (Norton et al., 2005), a fin bud initially forms in these embryos (Fig. 5A-B). At 26 hpf, *fgf10* expression is lost in *sall1a/sall4* double morphant pectoral fin primordia, although it is expressed normally in other regions of the embryo (Fig. 5G). At 32 hpf expression domains of both *dlx2* and *sp9* are absent in the fin bud ectoderm of *sall1a/sall4* double morphant embryos (Fig. 5C-F; *dlx2* 90% *n*=52; *sp9* 81% *n*=27). These results demonstrate that *sall1a* and *sall4* perform common, semi-redundant roles in initiating the expression of *fgf10* in the pectoral fin primordia. Furthermore, in the absence of *sall4* function, *sall1a* is able to maintain the posterior domain of *fgf10* expression, while following knockdown of *sall1a* function, *sall4* can maintain the anterior domain of *fgf10* expression.



4ng *sall1a*+	n	no fin(s)	shortened	upturned	wt
4ng *sall4* MO	64	77%	23%	0	0

Fig. 5. Pectoral fin development is severely disrupted in embryos injected with both *sall1a* and *sall4* MO. As shown in the table, co-injection of *sall1a* and *sall4* MOs leads to a significant increase in the most severe phenotype (no fins). Methylene Blue stained sections of the pectoral fin forming region in a wild-type embryo (**A**) and an embryo injected with 4 ng *sall1a* + 4 ng *sall4* MO (**B**) at 48 hpf shows that a fin bud initially forms in *sall1a/sall4* double morphant embryos. (**C-F**) Lateral views of 32 hpf pectoral fin buds in wild-type embryos (C,E) and embryos injected with 4 ng of *sall1a* + 4 ng of *sall4* MO (D,F). At this time point, *sp9* is expressed in the ectoderm of wild-type fin buds (C) but is absent in *sall1a/sall4* double morphant fin buds (D). Likewise, *dlx2* is present in wild-type fin bud ectoderm (E) but is absent in *sall1a/sall4* double morphant fins (F). At 26 hpf (**G**), *fgf10* expression fails to initiate in the pectoral fin primordia of *sall1a/sall4* double morphant embryos (compare G with Fig. 4J).

sall1a and *sall4* are required for the expression of *fgfr2* in the developing pectoral fins

Our results, together with those of others, have demonstrated that *fgf10* expression in the developing pectoral fins is dependant on *sall1a*, *sall4* and *fgf24* (Fig. 5G) (Fischer et al., 2003), although *sall1a* and *sall4* expression is not dependant on *fgf24*. For *fgf24* to activate the expression of *fgf10* it must signal via an FGF receptor. We therefore investigated if the expression of an FGF receptor is regulated by *sall1a* and *sall4*. As *fgf10* expression initiates in the pectoral fins of both *sall1a* (Fig. 4K) and *sall4* (Fig. 3B) morphant embryos, but is not expressed in embryos injected with both *sall1* and *sall4* MO (Fig. 5G), we predicted that expression of this receptor will not initiate in embryos injected with both *sall1a* and *sall4* MO. Limb outgrowth fails to occur in mice lacking *Fgfr2* (De Moerlooze et al., 2000; Xu et al., 1998) and therefore we studied the expression of *fgfr2* during zebrafish embryonic development. *fgfr2* expression is first detectable in the pectoral fin primordia mesenchyme at 23 hpf and is not expressed in the overlying ectoderm (Fig. 6A). *fgfr2* expression therefore initiates after *sall1a* and *sall4* transcripts are first detected in the fin bud mesenchyme. At 24 hpf, *fgfr2* is expressed in the pectoral fin primordia of wild-type (Fig. 6B) and *fgf24* morphant (Fig. 6D) embryos, but is absent from the pectoral fins of embryos injected with 4 ng of *sall1a* and 4 ng of *sall4* MO (Fig. 6C). This demonstrates that *fgfr2* expression in the pectoral fin primordia is dependant on *sall1a/sall4*, but not on *fgf24*.

DISCUSSION

Pectoral fin development is disrupted in *sall4* morphant embryos

During vertebrate limb development, a cascade of signals are required to initiate and maintain limb outgrowth (for a review, see Logan, 2003). Our studies of *sall4*, the gene mutated in Okihiro syndrome, add another factor to the series of events that control limb outgrowth. Expression of *Sall4* in the developing limb has been conserved in several species (Barembaum and Bronner-Fraser, 2004; Neff et al., 2005; Kohlhase et al., 2002) (S.A.H. and M.P.O.L., this study and unpublished). We have shown that *sall4* is required for outgrowth of the pectoral fins, but not the initiation of pectoral fin development, as *tbx5* and *fgf24* are induced normally and proximal

skeletal elements form in *sall4* morphants. The upturned fin phenotype found in some *sall4* morphant embryos (Fig. 1I) and those injected with low concentrations of *tbx5* MO (Fig. 1K) demonstrate that reduction of *sall4* and *tbx5* function produces similar fin defects in zebrafish. This is consistent with the similarity of limb phenotypes seen in individuals with OS and HOS, which are caused by haploinsufficiency of *SALL4* and *TBX5,* respectively. From fish to mammals, *Fgf10* has an evolutionary conserved function that is essential for limb outgrowth (Min et al., 1998; Sekine et al., 1999; Norton et al., 2005). Disruption of *Fgf10* signalling is the common cause of the similar abnormalities that arise from fish to humans, following perturbation of either *Tbx5* or *Sall4* function.

tbx5 regulates the expression of *fgf10* in the developing pectoral fins using a feed-forward method of gene regulation

In the pectoral fin primordia, *sall1a*, *sall4* and *fgf24* expression is dependant on *tbx5* (Fig. 2) (Fischer et al., 2003); however, expression of either *sall1a/sall4* or *fgf24* can occur independently of one another (Figs 2 and 4). Therefore, *tbx5* activates the expression of two different sets of target genes, both of which are required for pectoral fin outgrowth (Fig. 6E) (Fischer et al., 2003). *sall1a/sall4* and *fgf24* are required for the initiation of *fgf10* expression and we have addressed how this interaction occurs. *fgf24* must signal via a receptor to activate the expression of *fgf10* in the pectoral fin primordia. In zebrafish, *fgfr2* is first expressed in the pectoral fin primordia at 23 hpf, just after *sall1a/sall4* expression is first detected and just prior to the initiation *fgf10.* The temporal and spatial expression pattern of *fgfr2* therefore makes it a good candidate receptor to mediate the activation of *fgf10* expression by *fgf24.* As *sall1a* and *sall4* are zinc-finger transcription factors they are good candidates to directly positively regulate the expression of *fgfr2,* although conflicting data exists regarding whether Sall genes act as transcriptional activators or repressors (Kiefer et al., 2002; Li et al., 2004; Netzer et al., 2001; Onai et al., 2004). Our results support a model (Fig. 6E) in which *sall1a/sall4* act as transcriptional activators to positively regulate *fgfr2* transcription, and that *fgf24* signals via *fgfr2* to initiate *fgf10* expression in the fin bud mesenchyme.

Collectively, these results show that *tbx5* regulates the expression of *fgf10* in the pectoral fin primordia using a feed-forward model of transcriptional regulation (Fig. 6E). Feedforward transcriptional motifs have been most comprehensively characterised in studies in *E. coli* (Shen-Orr et al., 2002) and *S. cerevisiae* (Lee et al., 2002). In one branch of the pathway *tbx5* activates the expression of *sall1a/sall4*, which in turn induce *fgfr2* expression, and in the other branch *tbx5* activates the expression of *fgf24* (Fig. 6E). The delay between the initiation of *tbx5* and *sall1a/sall4* expression suggests that this regulation may be indirect or that *tbx5* requires a co-factor to activate *sall1a/sall4* expression. A third possibility is that higher threshold levels of tbx5 protein are required to activate different target genes. *tbx5* is likely to directly activate the expression of *fgf24* as expression of *fgf24* is detected only 1 hour after *tbx5* (Begemann and Ingham, 2000; Fischer et al., 2003). In the mouse, *Tbx5* has been shown to regulate the expression of FGFs directly (Agarwal et al., 2003). The expression of *fgf24* in the pectoral fin primordia begins at 18 hpf, ~6 hours before *fgf10* expression commences at 24 hpf. *fgfr2* expression is detected at 23 hpf (Fig. 6E). During the interval between the initiation of *fgf24* and *fgfr2* expression, we predict that fgf24 protein levels accumulate in the absence of receptor. Presumably when *fgfr2* expression initiates, fgfr2 proteins are rapidly occupied by ligand, owing to the presence of a reservoir of fgf24. Although our results do not provide an explanation for the apparent 'priming' of FGF signalling, we predict that in the pectoral fin mesenchyme the dynamics of this regulation would favour a paracrine rather than an autocrine mode of signalling, and would produce rapid, robust and uniform signalling via the FGF receptor.

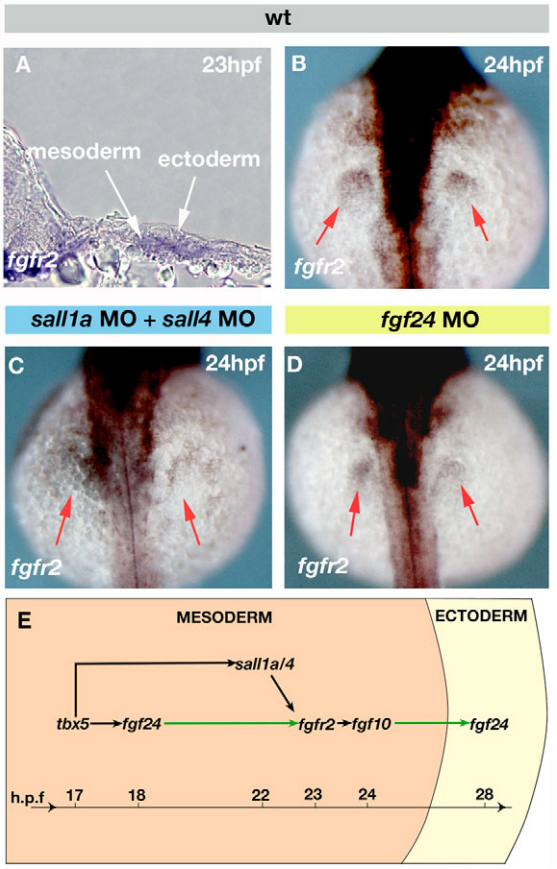


Fig. 6. *sall1a* and *sall4* are required for *fgfr2* expression in the pectoral fin primordia. A section of a 23 hpf embryo shows that *fgfr2* is expressed in the pectoral fin primordia mesenchyme but is excluded from the ectoderm (**A**). Dorsal views highlight *fgfr2* expression in a wild-type embryo at 24 hpf (**B**). At the same time point, *fgfr2* expression is absent in the pectoral fin primordia of embryos injected with 4 ng *sall1a* MO + 4 ng *sall4* MO (**C**), but continues to be expressed in the primordia of embryos injected with 9 ng of *fgf24* MO (**D**). At this time point, *fgfr2* is also downregulated in the trunk of *sall1a/sall4* double morphant embryos (C and data not shown). The red arrows indicate the pectoral fin primordia in B-D. (**E**) A schematic representation of the regulatory relationships between genes required for pectoral fin development. Green arrows signify relationships between FGF receptors and ligands, while black arrows represent transcriptional regulatory relationships.

Mesenchymal FGFs are required to induce the expression of FGFs in the pectoral fin ectoderm

Studies in mouse and chick have shown that *Fgf10*, which is expressed in the mesenchyme of the developing limb buds, signals to the overlying ectoderm to induce *Fgf8* expression. These ectodermal and mesenchymal FGFs form a positive-feedback loop that is essential for outgrowth of the developing limbs (for a review, see Martin, 1998). Although this positive-feedback loop has been described in mouse and chick limb buds, it has been studied less in zebrafish pectoral fins. In zebrafish, the situation is

different from chick and mouse, owing to the presence of *fgf24*. In chick, mouse and humans, no *fgf24* gene has been found, and it appears *fgf24* has been lost in the terrestrial vertebrate lineage (Draper et al., 2003). In zebrafish pectoral fin primordia mesenchyme, *fgf24* is required for the expression of *fgf10* (Fischer et al., 2003). Ectodermal FGFs fail to be expressed in *fgf24* mutant fin buds (Fischer et al., 2003), demonstrating that, similar to limb development in higher vertebrates, mesenchymal FGFs are required for the induction of ectodermal FGF gene expression. However, previously it has been unclear whether *fgf24* or *fgf10* were required for induction of ectodermal FGFs. The downregulation of *fgf10* in *sall4* morphant fin buds, and the subsequent loss of ectodermal FGF expression, suggests that it is *fgf10*, rather than *fgf24,* that is required for the induction of FGF expression in the overlying ectoderm (Fig. 3). These results are also supported by observations of zebrafish *fgf10* mutants (Norton et al., 2005). The induction of ectodermal FGFs only after the time point at which *fgf10* is first expressed, and long after the induction of *fgf24* expression (Fig. 6E), also supports a model in which *fgf10* is the crucial mesenchymal signal.

Specification of proximal limb skeletal elements is *tbx5* dependant, but *sall4* independent

The scapulocoracoid, a proximal pectoral fin skeletal element that is equivalent to the scapula in higher vertebrates, is always present in *sall4* morphant embryos (Fig. 1M) and therefore forms independently of *sall4* function. This differs from *tbx5* and *fgf24* mutant embryos, which lack all pectoral fin structures, including the scapulocoracoid (Ahn et al., 2002; Garrity et al., 2002). These experiments suggest that specification of proximal pectoral fin structures is dependant on *tbx5* and *fgf24* function and may occur at stages prior to the initiation of *sall4* expression. A parallel situation occurs during mouse limb development as *Tbx5* conditional knockouts lack all forelimb structures including the scapula and clavicle (Rallis et al., 2003), while *Fgf10*-null mice possess a scapula rudiment (Min et al., 1998; Sekine et al., 1999). The formation of these proximal skeletal elements also suggest that *fgf24* performs functions other than just the induction of *fgf10* expression. Although the limb defects of individuals with OS and HOS are very similar, there are some clear differences. Defects affecting the proximal forelimb, such as hypoplastic clavicles, have been reported in individuals with HOS (Newbury-Ecob et al., 1996) but never in individuals with OS. Our data suggest that these proximal forelimb defects are not observed in individuals with OS, as these structures are specified independently of *SALL4* function. Defects affecting proximal limbs elements such as the clavicle should therefore be specific to HOS and not OS.

sall1a and *sall4* perform similar roles during pectoral fin development

The preferential downregulation of *fgf10* in the anterior of *sall4* morphant fin buds (Fig. 3) led us to investigate whether a *sall4*-related gene is required to maintain the posterior domain of *fgf10* expression. Although the expression of *sall2* is yet to be described during zebrafish development, it appears that the only other Sall gene expressed in the developing pectoral fins is *sall1a* (Fig. 4A,B). Interestingly, although *sall4* is required for the anterior domain of *fgf10* expression in the fin bud (Fig. 3D), *sall1a* is required for the posterior domain (Fig. 4F). In *sall1a* (Fig. 4K) or *sall4* (Fig. 3B) morphant pectoral fin primordia, *fgf10* expression initiates; however, it fails to commence in *sall1a/sall4* double morphant embryos (Fig. 5G). This suggests that the functions of *sall1a* and *sall4* are partially redundant, such that *fgf10* expression initiates in the primordia in the absence of either gene individually, but at later stages is absent in either the anterior or posterior fin bud.

The pectoral fin defects observed following loss of *sall1a* function are different from other vertebrates, as *Sall1*-null mouse embryos do not have a limb phenotype (Nishinakamura et al., 2001). This difference in phenotype can be explained by the variation in expression of a related gene, *Sall3*, that is expressed in an almost identical pattern to *Sall1* during mouse limb development (Nishinakamura et al., 2001; Ott et al., 2001) but is not expressed in the developing zebrafish pectoral fins (Camp et al., 2003). *Sall1* is most closely related to *Sall3*, suggesting that *Sall1*-null mice do not have a limb phenotype because *Sall3* can compensate for the loss of *Sall1*. As *sall3* is not expressed during zebrafish pectoral fin development, it cannot substitute for *sall1a* and as a result *sall1a* morphant embryos have truncated pectoral fins.

Individuals with Holt-Oram and Okihiro syndromes have similar limb phenotypes

Our studies of *tbx5* and *sall4* function during zebrafish pectoral fin development offer explanations to the similar limb defects seen in individuals with HOS and OS. We have shown that *sall4* is a target of *tbx5* and that *tbx5* and *sall4* act in a pathway required to establish an FGF signalling loop that signals between the mesenchyme and ectoderm of the fin bud. During normal limb development, FGFs expressed in the AER are an essential component of a feedback loop between the ectoderm and underlying distal mesenchyme that is required to maintain FGF signalling (for a review, see Martin, 1998). The result of disrupting this positive feedback loop is demonstrated in classical embryological experiments in the chick in which the AER is surgically removed. When anterior regions of the AER are removed, limbs develop that lack anterior skeletal elements (Saunders, 1948). Similarly, alteration of either *Tbx5* or *Sall4* function preferentially leads to a disruption of *Fgf10* in the anterior of the limb bud (Rallis et al., 2003) (this study) and it is loss of FGF signalling in this region that ultimately causes the anterior bias of the deletion deformities characteristic of both HOS and OS. An unresolved issue that remains is why the anterior fin bud is sensitive to the loss of *sall4* function and *tbx5* haploinsufficiency, as both genes are expressed uniformly throughout the early fin bud. A contributing factor could be that partial redundancy of Sall-related genes leads to the maintenance of *fgf10* expression in the posterior limb. Another, not mutually exclusive, explanation is that *sall4* is more susceptible to *tbx5* levels than other Sall-related genes expressed in the limbs.

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