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The Polycomb group protein Ring1b is essential for pectoral fin development

Yme U. van der Velden, Liqin Wang, Maarten van Lohuizen and Anna-Pavlina G. Haramis^{*,†}

SUMMARY

Polycomb group (PcG) proteins are transcriptional repressors that mediate epigenetic gene silencing by chromatin modification. PcG-mediated gene repression is implicated in development, cell differentiation, stem-cell fate maintenance and cancer. However, analysis of the roles of PcG proteins in orchestrating vertebrate developmental programs *in vivo* has been hampered by the early embryonic lethality of several PcG gene knockouts in mice. Here, we demonstrate that zebrafish Ring1b, the E3 ligase in Polycomb Repressive Complex 1 (PRC1), is essential for pectoral fin development. We show that differentiation of lateral plate mesoderm (LPM) cells into presumptive pectoral fin precursors is initiated normally in *ring1b* mutants, but fin bud outgrowth is impaired. Fgf signaling, which is essential for migration, proliferation and cell-fate maintenance during fin development, is not sufficiently activated in *ring1b* mutants. Exogenous application of FGF4, as well as enhanced stimulation of Fgf signaling by overactivated Wnt signaling in *apc* mutants, partially restores the fin developmental program. These results reveal that, in the absence of functional Ring1b, fin bud cells fail to execute the pectoral fin developmental program. Together, our results demonstrate that PcG-mediated gene regulation is essential for sustained Fgf signaling in vertebrate limb development.

KEY WORDS: Ring1b, Zebrafish, FGF signaling, Fin, Polycomb

INTRODUCTION

Polycomb group (PcG) proteins are transcriptional repressors that act as crucial regulators of differentiation, proliferation, DNA repair and cell-fate maintenance during embryonic development and in adult tissue homeostasis (Bracken and Helin, 2009; Gieni and Hendzel, 2009; Sauvageau and Sauvageau, 2010; Sparmann and van Lohuizen, 2006; Surface et al., 2010). PcG proteins form multimeric protein complexes that mediate epigenetic gene silencing through multiple mechanisms, including the organization of higher-order chromatin structure, post-translational modifications on nucleosomes and interference with the transcription machinery (Eskeland et al., 2010; Sparmann and van Lohuizen, 2006; Stock et al., 2007; Surface et al., 2010; Vire et al., 2006; Zhou et al., 2008). PcG protein-mediated epigenetic modification of histones is probably the best characterized PcG function. Polycomb repressive complex 2 (PRC2) mediates trimethylation of histone H3 at lysine 27 (H3K27) through the action of the histone methyltransferases EZH1 and EZH2 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002). This epigenetic mark is recognized by the chromodomain of Polycomb (Camarata et al., 2010) in the Polycomb repressive complex 1 (PRC1). Recruitment of PRC1 results in the mono-ubiquitylation of histone H2A at lysine 119 through the E3 ligase activity of RING-domain-containing proteins (de Napoles et al., 2004; Wang et al., 2004). In addition, Ring1b can participate in several PRC1-like complexes (Gao et al., 2012) and PRC1 can be found at chromatin independently of PRC2 (Trojer et al., 2011). Two orthologs of the *Drosophila* E3 ligase dRing, Ring1a and Ring1b,

are found in mammals and amphibians, whereas only a single gene that is most homologous to Ring1b has been identified in zebrafish (Le Faou et al., 2011; Vidal, 2009).

Analysis of the roles of Ring1b in orchestrating differentiation programs during vertebrate development has been hampered because disruption of *Ring1b* (*Rnf2* – Mouse Genome Informatics) in mice leads to an arrest at gastrulation (Voncken et al., 2003). To address the function of Ring1b in vertebrate development, we turned to zebrafish. In zebrafish, owing to external fertilization and optical clarity of the embryos, development can be followed from very early stages and, thus, even an early phenotype is informative. Furthermore, in zebrafish, maternal contribution of several crucial factors enables the completion of gastrulation, despite harboring mutations in embryonic essential genes. This provides the unique opportunity to investigate gene regulation mechanisms in early and late developmental processes in an unbiased manner.

In this study, we generated Ring1b-deficient zebrafish and uncovered an essential function for Ring1b (*Rnf2* – Zebrafish Information Network) in pectoral fin development. The development of the vertebrate limb bud is a tightly regulated developmental program that is well conserved from fish to tetrapods. Pectoral fin bud outgrowth depends on epithelial-mesenchymal communication; proliferation and differentiation need to be coordinated as the limb grows, and fin morphogenesis involves the orchestrated action of several intertwined molecular networks.

Establishment of the fin field by axial signals is controlled by retinoic acid (RA) signaling. RA is synthesized mainly by aldehyde dehydrogenase 1 family member a2 (*Aldh1a2*) in the anterior somites (Begemann et al., 2001; Grandel et al., 2002). In response to RA signaling, *wnt2ba* expression is initiated in the intermediate mesoderm (Ng et al., 2002). In turn, Wnt2ba is required for expression of the T-box transcription factor *tbx5* in the lateral plate mesoderm (LPM) (Neto et al., 2012). Between the 6- and 15-somite stages (ss; 12–16 hours post-fertilization, hpf) (Kimmel et al., 1995), *tbx5*-positive cells comprise two bilateral stripes that contain both heart and fin precursors (Ahn et al., 2002; Begemann

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and Ingham, 2000; Furthauer et al., 2001). From 15 ss onwards, heart precursors migrate medially to form the heart tube at the 20 ss (19 hpf). The more posteriorly located fin precursors condense into a compact fin field. Notably, *tbx5* is the earliest known marker of prospective pectoral fin mesenchyme and is essential for the migration of these precursors (Ahn et al., 2002).

Fin-mesenchyme compaction proceeds through Tbx5-mediated activation of fibroblast growth factor 24 (Fgf24), a teleost-specific Fgf and the first family member to be expressed in the pectoral fin mesenchyme (Fischer et al., 2003). Fgf24 signaling is required for both maintaining *tbx5* expression and inducing *fgf10* expression in the LPM cells, possibly through binding to Fgf receptor 2 (Fgfr2) (Fischer et al., 2003; Harvey and Logan, 2006). In turn, Fgf10 maintains *fgf24* expression and contributes to the induction of the apical ectodermal ridge (AER), a signaling center that promotes outgrowth of the pectoral fin, starting at 28 hpf (Norton et al., 2005). Fgf10 signaling is then uniquely required for maintenance of AER function. Notably, *fgf24* expression in the fin mesenchyme is downregulated at around 32 hpf, and ectodermal expression commences (Fischer et al., 2003). AER-derived Fgfs signal back to the pectoral fin mesenchyme to maintain *fgf10* expression, thereby creating a positive-feedback loop in order to sustain *tbx5* expression and further fin outgrowth (Fischer et al., 2003; Nomura et al., 2006; Norton et al., 2005).

Here, we show that pectoral fin development is initiated normally in Ring1b-deficient zebrafish embryos. Pectoral fin precursors express *tbx5* and are located at the correct position during somitogenesis in *ring1b* mutants. However, RA signaling is upregulated after somitogenesis and Fgf signaling is never fully activated. Indeed, we show that enhanced Fgf signaling partially rescues the defects in pectoral fin development. This demonstrates that the PcG protein Ring1b coordinates the evolutionary conserved pectoral fin program via regulation of the Fgf-signaling pathway.

MATERIALS AND METHODS

Zebrafish strains and genotyping methods

Zebrafish were maintained as previously described (Westerfield, 2000). Fish were cared for in accordance with institutional guidelines and as approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences. *Ring1b* founder fish were out-crossed to AB and TL genetic backgrounds. Genotype analysis was performed by PCR using the primer set *ring1b_F* (AGGAGTGTCCAACATGCAGAAAG) and *ring1b_R* (GAGGATTGTGAACAAAGCCGC), followed by sequence analysis for the *ring1b*^{Δ4} allele or digestion of the PCR product with restriction enzyme TaqI to identify the *ring1b*^{Δ14} allele.

Sample preparation and western blot analysis

Histone extracts were prepared by lysis of 60 embryos per tube in 5% perchloric acid containing Complete protease inhibitor cocktail tablets (PIC, Roche), 1 mM PMSF and 10 mM iodoacetamide. To extract core histones, the pellet was resuspended in 0.4 N HCl containing PIC, 1 mM PMSF and 10 mM iodoacetamide, and incubated overnight at 4°C while rotating. Samples were centrifuged and the pellet discarded. Core histones were precipitated by the addition of one volume of ice-cold trichloroacetic acid and washed with ice-cold acetone containing 0.006% HCl. The pellet was washed in ice-cold acetone and vacuum dried, then solubilized in 50 mM Tris-HCl (pH 7.5).

To detect endogenous Ring1b, 30 embryos per tube were lysed in 100 mM PIPES (pH 6.8) containing 1 mM EGTA, 1 mM MgCl₂, PIC, 1 mM PMSF and 1 mM DTT. TritonX-100 (3.5%) was added after 5 minutes. Samples were centrifuged and pellets resuspended in RIPA lysis buffer containing PIC, 1 mM PMSF and 1 mM DTT. Samples were sonicated for 5 minutes (210 W, 30-second pulse) and after centrifugation, supernatant was collected. To detect myc-tagged Ring1b, 5 embryos per tube were lysed in RIPA lysis buffer containing PIC, 1 mM PMSF and 1 mM DTT. Samples were centrifuged and supernatant collected.

Protein extracts were separated on 4-12% bis-Tris precast gels (NuPAGE) and transferred to Immobilon-P membranes (Amersham Biosciences). Primary antibodies used were: mouse anti-β-actin (1:5000; ab6276, Abcam), rabbit anti-c-myc (1:1000; SC789, Santa Cruz), rabbit anti-H2A (1:1000; 07-146, Millipore) and rabbit anti-H3 (1:1000; ab1791, Abcam). Ring1b rabbit polyclonal antibodies were obtained from M. Dyers. Secondary antibodies used were goat anti-mouse IgG (1:10,000; Zymed) and goat anti-rabbit IgG (1:10,000; BioSource).

Generation of expression vectors

ZFNs were generated essentially as described previously (Carroll et al., 2006). The DNA sequence encoding both zinc fingers was obtained from Geneart and cloned into pENTR-NLS-G-FN using *NdeI* and *SpeI* restriction sites. This construct was shuttled into the pCS2-DEST expression vector using the Gateway cloning system (Invitrogen). To generate the *ring1b* expression construct, the open reading frame of *ring1b*, excluding the 5' and 3' untranslated regions, was cloned into pCS2+-Myc using *BamHI* restriction sites.

mRNA and morpholino injections

Vectors were linearized with *NotI*. Capped mRNA was synthesized using the SP6 mMessage mMachine kit (Ambion). mRNA (100 pg) encoding each ZFN and 25-500 pg *ring1b*-myc mRNA was injected into one-cell stage zebrafish embryos. Morpholinos against *ring1b* were obtained from Gene Tools (Oregon, USA). ATGMO1 (5 ng) (ACACCACGTCTTTTATCTCAATGTT) and 20 ng of splice-blocking MO2 (TTAATAACTCAAACAAACCCTGATC) were injected into fertilized oocytes.

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were carried out according to a standard protocol (Westerfield, 2000). BM purple and INT/BCIP (Roche) were used as alkaline phosphatase substrates. Probes for *axin2*, *fgf8*, *myca* and *shh* have been described previously (Haramis et al., 2006; Krauss et al., 1993; Reifers et al., 1998). Antisense riboprobes amplified from cDNA were *dhxs3*, *dusp6*, *eng1a*, *hoxa9b*, *hoxb5b*, *hoxc6a*, *hoxc8a*, *hoxd9a*, *meis3*, *msxc*, *pea3*, *spry4* and *wnt7a*. Primer sequences can be found in supplementary material Table S1. The Ring1b riboprobe was directed against the C-terminal 500 bp of the cDNA.

Bead implantation

Bead implantations were carried out essentially as described by Picker et al. (Picker et al., 2009). Recombinant human FGF4 (R&D Systems) was dissolved in PBS containing 0.1% BSA at a concentration of 250 μg/ml. The bead solution was washed in methanol and air-dried. Beads were overnight soaked with FGF4 at 4°C while rotating. Dechorionated embryos were embedded into 1.5% low melting agarose. After solidification, the gel was fenestrated to expose the epidermis. The epidermis was digested away by repetitive placing of light white mineral oil drops (Sigma) on the flank of the embryo, ventral to somites 5-7. A fire-polished tungsten needle was used to open the epidermis further, in order to create a tunnel below the epidermis. FGF4-soaked beads were inserted below the epidermis and pushed anteriorly through the tunnel to the level of somite boundary 2-3.

Immunohistochemistry

Embryos were fixed in 40% ethanol, 5% acetic acid and 10% formalin for 2 hours at room temperature, embedded in 1.5% low melting agarose and processed into paraffin. Primary antibody was rabbit anti-Tbx5 (1:50; 55866, Eurogentec) and secondary was biotinylated goat anti-rabbit IgG (1:800; DakoCytomation). For whole-mount immunohistochemistry, embryos were fixed overnight at 4°C in Dent's fixative, digested in PBS containing 10 μg/ml proteinase K, 0.1% Tween20 and blocked in PBS containing 10% normal goat serum, 0.5% DMSO and 0.3% Triton X-100. Primary antibody was rabbit anti-ph3 (1:750; sc8656R, Santa Cruz) and secondary goat anti-rabbit IgG (1:300, DakoCytomation).

TUNEL

To detect apoptotic cells, whole-mount TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's recommendations.

RESULTS

Generation of *ring1b* mutants

To study the function of Ring1b in vertebrate development, we generated *ring1b* knockout zebrafish using zinc finger nuclease (ZFN)-mediated targeted gene inactivation (Meng et al., 2008). We identified potential ZFN-target sites in the coding sequence of zebrafish *ring1b* (BC164137.1) using ZiFit 3.0 (<http://zifit.partners.org/ZiFit/>). A suitable ZFN-target site (exon 4, bp 480-503) was recovered by using the 'OPEN' strategy (Fig. 1A,B) (Maeder et al., 2008). ZFN recognizing the 9 bp that flanked the target site were generated and 100 pg mRNA encoding each ZFN was injected into one- or two-cell stage eggs. Functionality of the ZFN was verified in vivo and injected embryos were raised to adulthood. Out of 25 potential founders, we identified two fish in which *ring1b* was mutated at the ZFN cleavage site (Fig. 1B). As reported for other ZFNs, the mutated alleles were of insertion/deletion origin (Doyon et al., 2008; Foley et al., 2009; Meng et al., 2008). One mutation leads to insertion of 4 bp within the ZFN target site, whereas the second mutation causes deletion of 14 bp ($\Delta 14$ mutation). Both mutations result in an open reading frame-shift that leads to a premature stop codon.

We next assayed for *ring1b* mRNA and protein expression. Whole-mount in situ hybridization showed strong *ring1b* expression in the brain and pectoral fins in wild-type embryos at 72 hpf. By contrast, *ring1b* mRNA was not detectable in homozygous *ring1b* mutants (Fig. 1D,D'), suggesting that the mutant *ring1b* mRNA was degraded via nonsense-mediated decay. In line with these results, Ring1b protein (Fig. 1E) and mono-ubiquitylation of H2A (Fig. 1F) were not detected in 72 hpf *ring1b* mutants. These results indicate that both mutant alleles are functional nulls and confirm that Ring1b is the sole H2A E3 ligase in the zebrafish PRC1 complex.

Because mRNA and/or protein are often maternally deposited in zebrafish, we extended the expression analysis to stages before the onset of zygotic transcription. *ring1b* mRNA was indeed maternally deposited (supplementary material Fig. S1B). Moreover, Ring1b protein was detected in embryos at 2.5 hpf, i.e. before onset of zygotic gene expression (supplementary material Fig. S1C). Maternal Ring1b protein persisted up to 15 ss and was hardly, if at all, detectable in *ring1b* mutants at 24 hpf (supplementary material Fig. S1D).

As the Hox genes are among the best-characterized targets of Polycomb repression (Paro, 1995; Pirrotta, 1997), we investigated axial Hox gene expression in *ring1b* mutants over time. This analysis showed that, up to 24 hpf, axial Hox expression is largely normal in *ring1b* mutants (supplementary material Fig. S2A-H). However, at later stages, there was a progressive anterior expansion of the expression domain for the Hox genes examined (supplementary material Fig. S2I-X).

Homozygous, as well as transheterozygous deletion of both alleles led to identical phenotypes, which further established that the mutations indeed disrupt Ring1b. Heterozygous mutants did not display any abnormalities. Homozygous mutants were phenotypically indistinguishable from wild-type siblings up to 24 hpf. During organogenesis, however, *ring1b* mutants displayed several defects, including jaw malformations, pericardial edema and diminished blood circulation (supplementary material Fig. S3). *ring1b* mutants died at around 4-5 dpf. We were able to obtain the same developmental phenotype by injecting two independent morpholinos against *ring1b* (supplementary material Fig. S4). Here, we focus our analysis on the striking absence of pectoral fins in *ring1b* mutants (Fig. 1H).

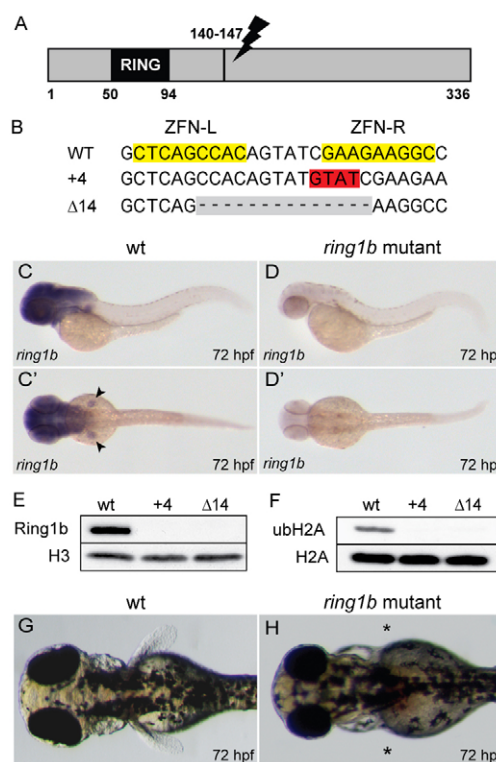


Fig. 1. Generation of *ring1b* mutants. (A) Schematic representation of the zebrafish *ring1b* gene depicting the location of the ZFN target site. (B) Wild-type *ring1b* sequence is shown at the top; the ZFN target sites are highlighted in yellow. ZFN-induced bp insertions are highlighted in red, and deletions in gray. (C,C') *ring1b* mRNA staining in brain and pectoral fins in wild-type larvae. (D,D') Expression is absent in *ring1b* mutants. (E,F) Ring1b (E) and mono-ubiquitylated histone H2A (F) are not detected in 72 hpf *ring1b* mutants by western blot analysis. (G,H) Dorsal view of wild-type (G) and *ring1b* (H) larvae at 72 hpf. Asterisks indicate the lack of pectoral fins in *ring1b* mutants.

Rescue of the *ring1b* phenotype by wild-type *ring1b* mRNA

To validate that the observed developmental phenotype correlated with loss of Ring1b, we injected wild-type myc-tagged-*ring1b* mRNA into one- to two-cell stage eggs derived from heterozygote *ring1b* crosses. We assayed injected *ring1b* mutants for expression of *tbx5* and *hoxd9a*, a reported direct target of Ring1b-mediated silencing (Li et al., 2011) that is also expressed in the fin bud. Injection of wild-type *ring1b* mRNA restricts the anterior boundary of axial *hoxd9a* expression in *ring1b* mutants, although not to the extent in wild type (Fig. 2A-E). Moreover, exogenous wild-type *ring1b* mRNA restored *tbx5* and *hoxd9a* expression in the fin bud in a dose-dependent manner and partially rescued fin bud outgrowth in *ring1b* homozygotes at 72 hpf (Fig. 2L). Western blot analysis confirmed that myc-tagged Ring1b protein was expressed in a dose-dependent manner at 24 hpf (Fig. 2M). Of note, exogenous Ring1b protein levels were greatly diminished at 48 hpf (Fig. 2N), which likely explains the partial rescue and indicates that Ring1b activity is also required for later stages of fin bud outgrowth. These results, when taken together, confirm that we have induced null mutations in *ring1b* and that Ring1b is essential for pectoral fin development in zebrafish.

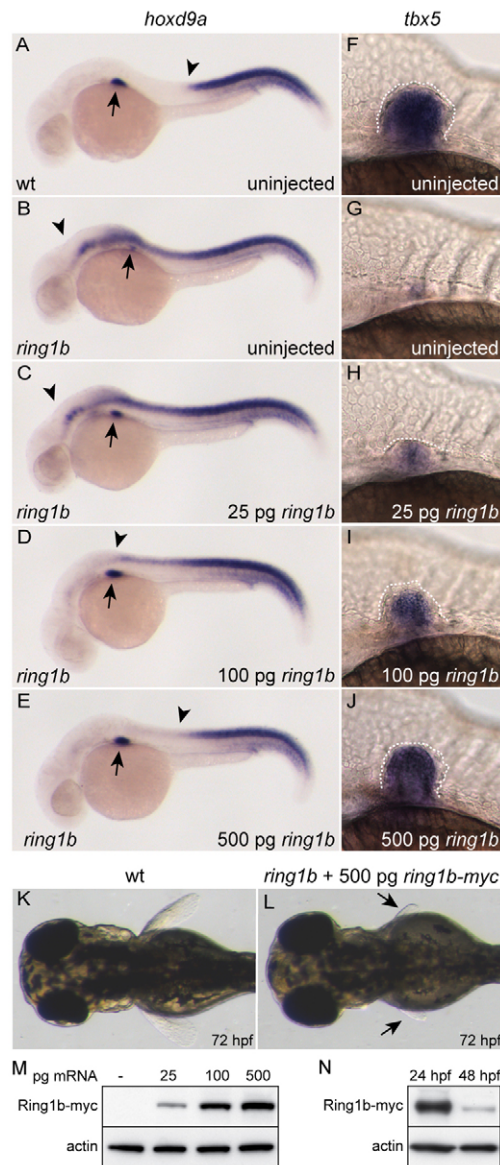


Fig. 2. Injection of *ring1b* mRNA rescues pectoral fin outgrowth and restores *hoxd9a* and *tbx5* expression in *ring1b* mutants.

(A–J) Lateral views of 32 hpf embryos stained for *hoxd9a* (A–E) and 48 hpf fin buds stained for *tbx5* (F–J). Anterior expansion of axial *hoxd9a* expression in *ring1b* mutants is suppressed by exogenous wild-type *ring1b* mRNA in a dose-dependent manner (C–E, arrowheads). (K,L) Fin-bud *hoxd9a* (A–E, arrows) and *tbx5* expression (F–J) is restored (arrows) in injected *ring1b* mutants and fin bud outgrowth is partially rescued at 72 hpf (compare K with L). (M) Western-blot analysis for myc-tagged Ring1b shows dose-dependent expression in 24 hpf embryos. (N) Exogenous Ring1b protein is markedly reduced in 48 hpf embryos.

Gene expression defects in the pectoral fin mesenchyme of *ring1b* mutants

To address at which point during pectoral fin development the defect arises in *ring1b* mutants, we assayed expression of three genes that are expressed in the fin field mesenchyme and are important for fin development. In addition to *tbx5*, the earliest known marker for fin mesenchyme, we examined the expression

of the bHLH transcription factor *hand2* and of the RA-synthesizing enzyme *aldh1a2* (Ahn et al., 2002; Begemann and Ingham, 2000; Begemann et al., 2001; Grandel et al., 2002; Yelon et al., 2000).

In situ hybridization experiments showed that *tbx5* was expressed at levels comparable to wild type in the pectoral fin field of *ring1b* mutants at 18 ss, albeit the expression domain appeared diffuse (Fig. 3A,B). Migration and compaction of the LPM was slightly delayed, resulting in a fuzzy *tbx5* expression domain in the *ring1b* mutants at 32 hpf. At 40 hpf, *tbx5* expression was greatly reduced in the mutant pectoral fin mesenchyme, indicating a defect in maintenance of *tbx5* expression. We also addressed the localization of Tbx5 protein in the LPM of *ring1b* mutants, as it has been reported that the transcription factor Tbx5 shuttles between the nucleus and cytoplasm, providing an additional layer of Tbx5 regulation (Camarata et al., 2006). Tbx5 was detectable in the *ring1b* pectoral fin mesenchyme, and was correctly localized in the nucleus (supplementary material Fig. S5), indicating that the regulation of Tbx5 localization is intact.

We found that *hand2* expression was indistinguishable from wild-type siblings up to 24 hpf. However, at 32 hpf, *hand2* expression was diminished in *ring1b* mutants and was subsequently lost by 40 hpf (Fig. 3R,T). In contrast to *tbx5* and *hand2* expression, we found that *aldh1a2* was already overexpressed at 18 ss and not restricted to the posterior margin of the fin field, as observed in wild-type siblings (Fig. 3U–DD).

Based on the early expression patterns of both *tbx5* and *hand2*, we conclude that specification of LPM into pectoral fin mesenchyme is initiated in *ring1b* mutants. However, maintenance of gene expression is impaired.

Normal LPM patterning in *ring1b* mutants

As *aldh1a2* expression has been shown to be feedback controlled by RA during somitogenesis (Begemann et al., 2001), the altered expression of *aldh1a2* in *ring1b* mutants at 18 ss (18 hpf) may reflect aberrant RA signaling at even earlier stages. We addressed the possibility that the LPM is not fully specified or correctly patterned owing to deregulation of RA signaling.

To investigate LPM patterning and the response to RA signaling, we carried out double stainings at 10 ss and 15 ss, the time point at which the LPM separates into the heart and fin fields. We examined expression of the heart marker *nkx2.5*, the LPM marker *tbx5* and the RA target genes *dhrs3* and *hoxb5b*, which are expressed in the pectoral fin mesenchyme (Waxman et al., 2008). This showed that the heart precursors are located correctly and express *nkx2.5* at normal levels at 10–15 ss in *ring1b* mutants. Moreover, *tbx5* and *hoxb5b* were normally expressed at these stages, indicating that both the heart and fin fields are correctly specified in *ring1b* mutants (Fig. 4A–L). We noticed an upregulation of *dhrs3* expression in some *ring1b* embryos at 15 ss (Fig. 4H), which could reflect increased RA signaling or increased response to RA signaling.

To address a possible deregulation of RA signaling further, we stained for *dhrs3*, *hoxb5b* and *meis3*, another RA-target gene expressed in the pectoral fin field, at 20 ss and 32 hpf (Gongal and Waskiewicz, 2008; Kudoh et al., 2002; Manfroid et al., 2007). The expression of *hoxb5b*, *meis3* and *dhrs3* was indistinguishable from wild-type embryos at 20 ss (Fig. 4M,N,Q,R,U,V). At 32 hpf, when a fin bud is visible in wild-type embryos, *hoxb5b* and *meis3* were expressed at normal levels in *ring1b* mutants. By contrast, expression of *dhrs3* was reproducibly upregulated in the *ring1b* fin field (Fig. 4P). Because of the observed upregulation of *dhrs3* at 32 hpf, we next

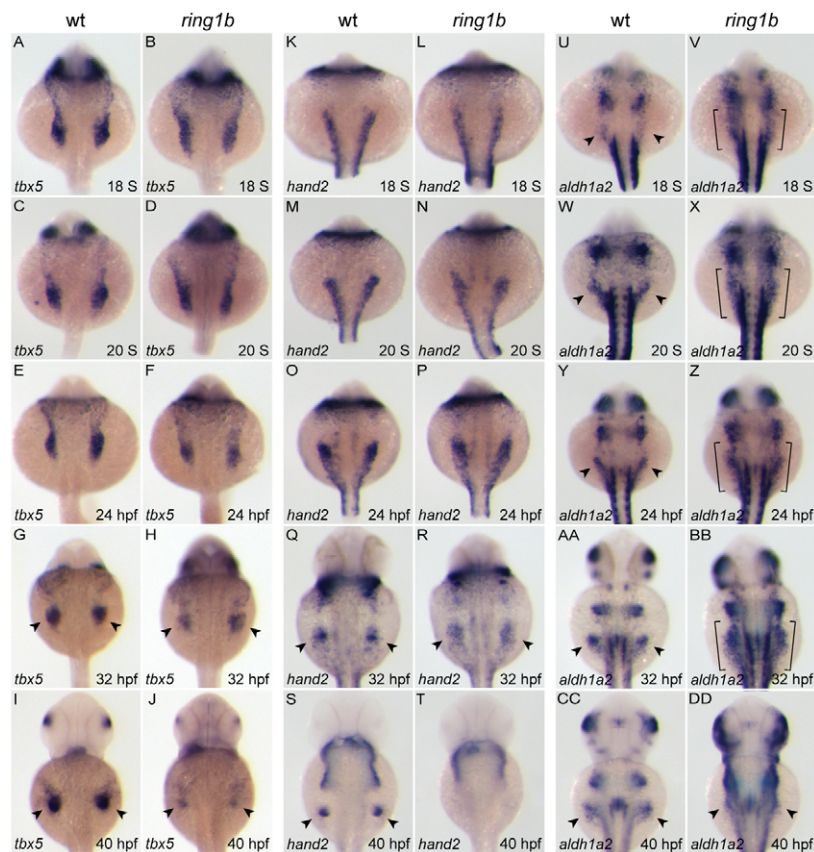


Fig. 3. Gene expression defects in the *ring1b* pectoral fin mesenchyme. (A-DD) Dorsal views of embryos at the indicated stages stained for *tbx5* (A-J), *hand2* (K-T) and *aldh1a2* (U-DD). Migration and compaction of *tbx5*-positive pectoral fin precursors is slightly delayed in *ring1b* mutants (A-F), whereas *hand2* expression is identical to wild-type siblings up to 24 hpf (K-P). Expression of *tbx5* (G-J) and *hand2* (Q-T) is not maintained in the *ring1b* fin mesenchyme. *aldh1a2* is overexpressed and not restricted to the posterior LPM in *ring1b* mutants (U-DD, brackets). Arrowheads indicate staining at the fin bud region.

examined whether timed inhibition of RA signaling could rescue aspects of the *ring1b* fin phenotype. RA signaling was chemically inhibited by application of DEAB at two developmental time points: 15 ss and 24 hpf. We confirmed that 10 μ M and 100 μ M DEAB efficiently inhibited RA signaling, as demonstrated by inhibition of the RA-responsive genes *dhsr3*, *hoxc6a*, *hoxc8a* (supplementary material Fig. S6A). DEAB treatment, when initiated at 15 ss, led to partial inhibition of fin formation in wild-type embryos accompanied by dose-dependent downregulation of gene expression (supplementary material Fig. S6B). DEAB treatment of 15s *ring1b* embryos led to even weaker *tbx5* expression in the fin mesenchyme. DEAB treatment of wild-type embryos at 24 hpf had little impact on fin formation, as has been reported previously (Gibert et al., 2006). By contrast, DEAB treatment of *ring1b* mutants at 24 hpf led to partial restoration of gene expression. Both *tbx5* and *hand2* expression levels were increased when compared with untreated mutants, albeit not reaching wild-type levels (supplementary material Fig. S6C). However, the partial rescue of mesenchymal gene expression was not sufficient to restore ectodermal *fgf24* expression and fin outgrowth. Overall, these data suggest that there is an increase in RA signaling or in the response to RA signaling in *ring1b* mutants after 24 hpf that may contribute to the pectoral fin phenotype.

Impaired Fgf-signaling in *ring1b* mutants

Tbx5 promotes expression of *fgf24*, which, in turn, maintains *tbx5* and induces *fgf10* in the pectoral fin mesenchyme to promote fin bud outgrowth (Fischer et al., 2003). As *tbx5* expression is initiated, but not maintained, in *ring1b* mutants, we addressed whether processes directly downstream of *tbx5* were deregulated. We performed an expression time-course analysis for *fgf24* and

fgf10. *fgf24* expression was initiated in both wild type and *ring1b* mutants at 18 ss (Fig. 5A,B). However, *fgf24* was expressed at lower levels and in a smaller domain in the mutants. Low levels of *fgf24* persisted at later developmental stages, although expression was progressively restricted to a very small domain. Expression of *fgf10* was also properly initiated at 20 ss in *ring1b* mutants, albeit at greatly reduced levels. At later stages, *fgf10* expression remained markedly decreased, similar to *fgf24* (Fig. 5N,P). We also examined expression of the Fgf receptors *fgfr1a*, *fgfr2* and *fgfr3* in the developing fin bud (supplementary material Fig. S7). *fgfr1a* was not expressed in either wild-type or *ring1b* embryos at 18 ss. At 24 hpf, *fgfr1a* expression was reduced in *ring1b* mutants and expression was diminished further at 32 hpf. The mesenchymal *fgfr2* expression domain was slightly broader in *ring1b* mutants at 18 ss. However, expression was not maintained. We detected a slight expansion of the *fgfr3* expression domain in *ring1b* mutants at 18 ss. At both 24 and 32 hpf, *fgfr3* was overexpressed and the expression domain was expanded. Interestingly, a correlation between lack of Fgf8 signaling and expansion of the *fgfr3* expression domain has been previously reported (Sleptsova-Friedrich et al., 2001). These results suggest that Fgf signaling is never fully activated in *ring1b* mutants.

To test this further, we examined whether activation of Fgf target genes was disrupted in the pectoral fin field of *ring1b* mutants. Analysis of *dusp6*, *pea3* and *spry4* (Furthauer et al., 2001; Kawakami et al., 2001; Roehl et al., 2001) showed that expression of all three genes was impaired in *ring1b* mutants at 24 hpf and 32 hpf (Fig. 6). *pea3* levels were severely reduced, whereas *dusp6* and *spry4* expression was undetectable. This confirmed that Fgf signaling, which is essential for pectoral fin mesenchyme compaction and fin bud outgrowth, is disrupted in *ring1b* mutants. Importantly, all examined processes downstream of Fgf signaling

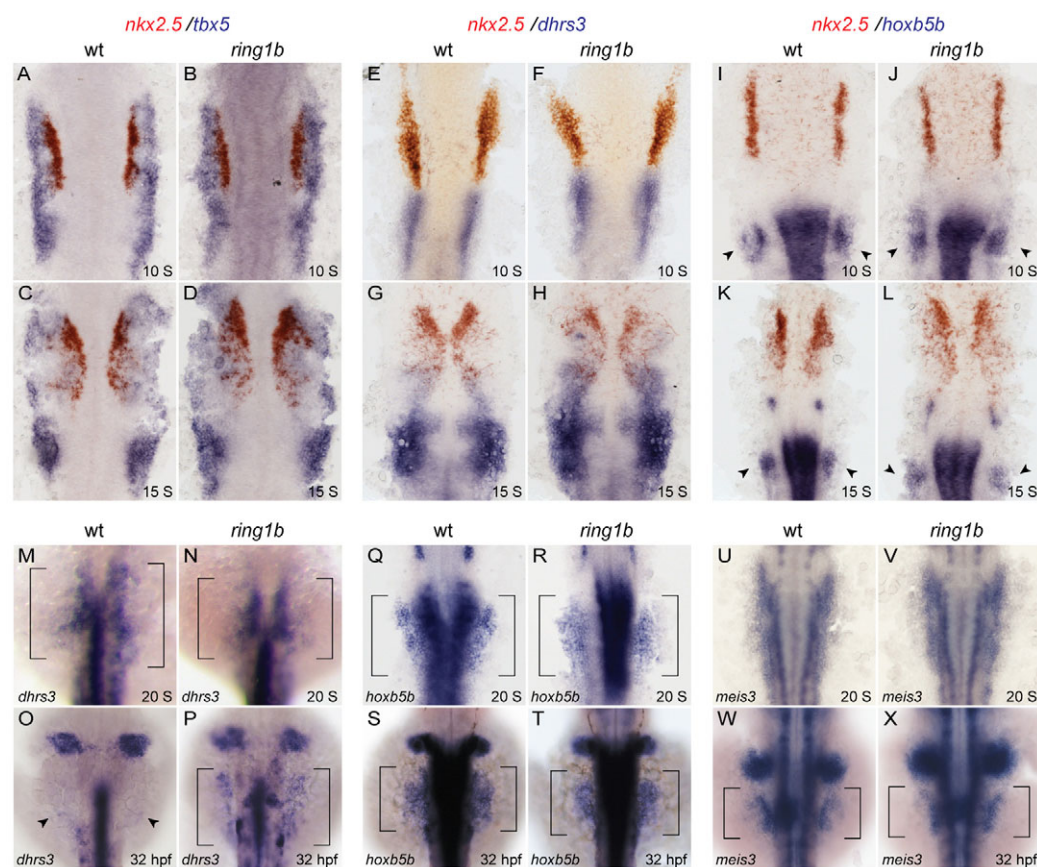


Fig. 4. LPM patterning and the RA-signaling response in *ring1b* mutants. (A-L) Double in situ hybridization for *nkx2.5* in red and *tbx5* (A-D), *dhars3* (E-H) and *hoxb5b* (I-L) in blue at 10 ss and 15 ss shows similar staining patterns in wild-type and *ring1b* mutants. Arrowheads indicate staining at the pectoral fin mesenchyme.

(M-X) Dorsal views of embryos stained for the RA targets *dhars3* (M-P), *hoxb5b* (Q-T) and *meis3* (U-X) at 20 ss and 32 hpf. Expression of *hoxb5b* and *meis3* is normal in the *ring1b* LPM at 20 ss and 32 hpf, whereas *dhars3* expression is upregulated in the *ring1b* LPM at 32 hpf (P). Arrowheads indicate staining at the fin bud region.

were severely impaired in *ring1b* mutants. This included reduced or absent expression of genes involved in anterior-posterior (AP) and dorsal-ventral (DV) patterning, as well as the absence of ectodermal gene expression (supplementary material Fig. S8). The latter finding indicated that the AER, an Fgf-dependent signaling center essential for proximal-distal outgrowth of the pectoral fin bud (Kawakami et al., 2001), was not established in *ring1b* mutants.

We also assayed for proliferation by pH3 staining and found that it was impaired only from 32 hpf onwards (supplementary material Fig. S9A). Because Fgf signaling is greatly reduced at earlier time points, this reduction in proliferation reflects a secondary effect, which is in agreement with the previously reported role of Fgf signaling in cell-cycle progression (Prykhodzhiy and Neumann, 2008). Finally, only very few apoptotic cells were detected in the *ring1b* fin field (supplementary material Fig. S9B).

Exogenous FGF restores gene expression but is not sufficient to promote fin bud outgrowth in *ring1b* mutants

It has been shown that exogenously provided FGF, by means of FGF-coated bead implantation into the flank of zebrafish embryos, can replace AER function and rescue gene expression in the pectoral fin bud (Grandel et al., 2000; Norton et al., 2005). As mesodermal Fgf signaling is impaired in *ring1b* mutants, we explored whether exogenously provided FGF would be sufficient to restore the positive Fgf signaling feedback loop and promote fin bud outgrowth in *ring1b* mutants.

To test successful loading of the beads, we first confirmed that implantation of FGF-coated beads at the 1000-cell stage promoted ectopic expression of the FGF target genes *dusp6*, *pea3* and *spyr4* at 90% epiboly (supplementary material Fig. S10).

We next implanted FGF4-coated beads in the flank of embryos at 15–20 ss because of the early defects in Fgf signaling in *ring1b* mutants. Exogenously provided FGF4 promoted maintenance of *tbx5* expression in the *ring1b* pectoral fin field (Fig. 7B). Importantly, the *tbx5* expression domain was increased upon bead implantation and was always directly adjacent to the FGF4-coated implanted bead. Thus, exogenously provided FGF4 enabled a domain of *tbx5* expression that more closely resembled that of wild-type embryos. However, *fgf24* and *fgf10* expression were not detectable in the pectoral fin field of bead-implanted *ring1b* embryos at 40 hpf, and fin bud outgrowth was not restored (Fig. 7D,F,H). Taken together, we conclude that the *Ring1b*-deficient fin precursors are partially responsive to FGF signaling, as illustrated by maintained *tbx5* expression.

Genetic activation of Fgf signaling stimulates fin bud outgrowth in *ring1b* mutants

We postulated that a stronger or different Fgf stimulus might be required to promote fin outgrowth in *ring1b* mutants. To test this hypothesis, we sought to stimulate mesodermal Fgf signaling by genetic means. Zebrafish mutants with locally increased pectoral fin mesenchymal Fgf signaling have not been described to our knowledge. However, Wnt signaling has been shown to cooperate with FGF signaling during limb initiation and outgrowth in several studies in chick, mouse and zebrafish (Agarwal et al., 2003; Galceran et al., 1999; Hill et al., 2006; Kawakami et al., 2001; Nagayoshi et al., 2008; Narita et al., 2005; Ng et al., 2002; ten Berge et al., 2008). In chick, implantation of WNT2b- or β -catenin-expressing cells induces ectopic *fgf10* expression and limb bud outgrowth (Kawakami et al., 2001). Therefore, we hypothesized that the *apc* mutants that exhibit hyperactivated Wnt signaling

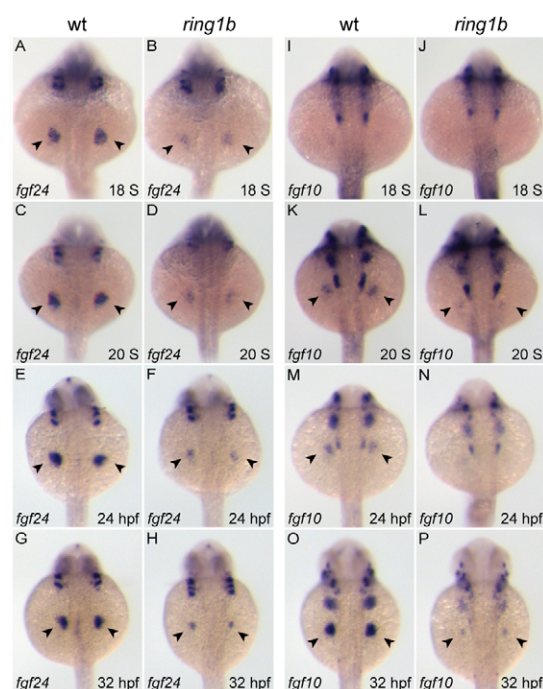


Fig. 5. Reduced *fgf24* and *fgf10* expression in *ring1b* fin mesenchyme. (A–P) Dorsal views of embryos stained for *fgf24* (A–H) and *fgf10* (I–P). Expression of both genes is initiated at the correct developmental stage, but the levels are reduced in the *ring1b* pectoral fin mesenchyme. *fgf24* and *fgf10* expression is restricted to a very small domain in the *ring1b* fin mesenchyme at 32 hpf (H,P). Arrowheads indicate staining at the pectoral fin mesenchyme and fin bud region.

would provide a good candidate for increased Fgf signaling. In *apc* mutants, canonical Wnt signaling is hyperactivated owing to destabilization of the axin-containing degradation complex, of which Apc is an essential component (Clevers, 2006; Fodde et al., 2001; Hurlstone et al., 2003). Consequently, β -catenin is stabilized, accumulates in the nucleus and, together with TCF, activates Wnt-target gene transcription (Korinek et al., 1997). Indeed, the Wnt-target genes *myca* (*c-myc*) and *axin2* (He et al., 1998; Jho et al., 2002) were overexpressed in the *apc* pectoral fin mesenchyme (supplementary material Fig. S11B,F). *myca* and *axin2* were downregulated in the *ring1b* fin field at 56 hpf (supplementary material Fig. S11C,G); however, this probably represents a secondary effect resulting from disruption of the pectoral fin program.

To determine whether mesodermal Fgf-signaling was increased in *apc* mutants, we assayed expression of *tbx5*, *fgf24* and *fgf10* at 32 hpf and 72 hpf. Indeed, all three genes were upregulated and the expression domains were expanded in *apc* fin buds at 32 hpf; this was exacerbated at 72 hpf (Fig. 8B,F,I,N,R,V). At 72 hpf, fin elongation was impaired in *apc* mutants, despite a large *tbx5* expression domain. The pectoral fin ectoderm displayed a ruffled morphology and *fgf24* and *fgf10* were expressed at high levels in the mesenchyme (Fig. 8R,V,Z). Furthermore, expression of the Fgf target genes *dusp6*, *spry* and *pea3* was highly increased in *apc* mutants (supplementary material Fig. S12). These data showed that *apc* mutants exhibit increased activation of the *tbx5*-*fgf24*-*fgf10* signaling cascade, although *fgf24* expression remains confined to the mesenchyme and is not expressed in the ectoderm.

To test whether this level of activation could rescue the pectoral fin developmental program, we generated *apc/ring1b* mutants. As expected, we found that fin outgrowth is initiated in the *apc/ring1b* animals, as a small fin bud visible at 40 hpf, continued to grow and gave rise to a small, albeit misshapen, fin at 72 hpf (Fig. 8BB). We analyzed expression of the *tbx5*-*fgf24*-*fgf10* axis in the *apc/ring1b* fin buds at different stages of development. At 32 hpf, *tbx5* expression in *apc/ring1b* mutants is similar to that of *ring1b* mutants: compaction of pectoral fin mesenchyme occurs, but *tbx5* is poorly expressed and the domain is not well demarcated. Expression of *fgf24* and *fgf10*, although increased in *apc/ring1b* embryos when compared with *ring1b* mutants, did not reach wild-type levels. Interestingly, *tbx5* expression is well maintained in *apc/ring1b* mutants at 72 hpf (Fig. 8P). This is in striking contrast to *ring1b* mutants, in which *tbx5* expression was not detectable at this stage. Expression of both *fgf24* and *fgf10* in 72 hpf *apc/ring1b* fins resembles that of *apc* mutants, but the expression domains are smaller (Fig. 8T,X). Similarly, Fgf-target genes are expressed in *apc/ring1b* mutants, but not in *ring1b* mutants (supplementary material Fig. S12). Taken together, genetic activation of Fgf-signaling restores the pectoral fin program of *Ring1b*-deficient embryos and is sufficient to promote fin bud outgrowth.

DISCUSSION

In this study, we implemented ZFN-mediated targeted gene inactivation to generate the first zebrafish mutant in a PcG gene. We show that, in contrast to mice, *ring1b* homozygote zebrafish mutants are embryonically viable and exhibit developmental defects that enable the study of *Ring1b* in vertebrate development. One striking feature of the *ring1b* phenotype is the lack of pectoral fins, whereas the lateral plate mesoderm (LPM) is specified appropriately and the fin program initiates correctly with the expression of *tbx5*. In the absence of *Ring1b*, upregulation of RA

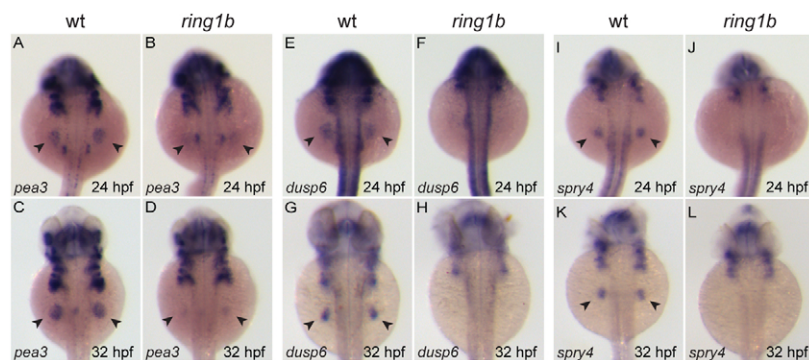


Fig. 6. Loss of Fgf target gene expression in *ring1b* mutants. (A–L) Expression analysis of the Fgf target genes *pea3* (A–D), *dusp6* (E–H) and *spry4* (I–L) at 24 hpf and 32 hpf. *pea3* is greatly reduced and *dusp6* and *spry4* are undetectable in the *ring1b* pectoral fin mesenchyme. Arrowheads indicate staining at the fin bud region.

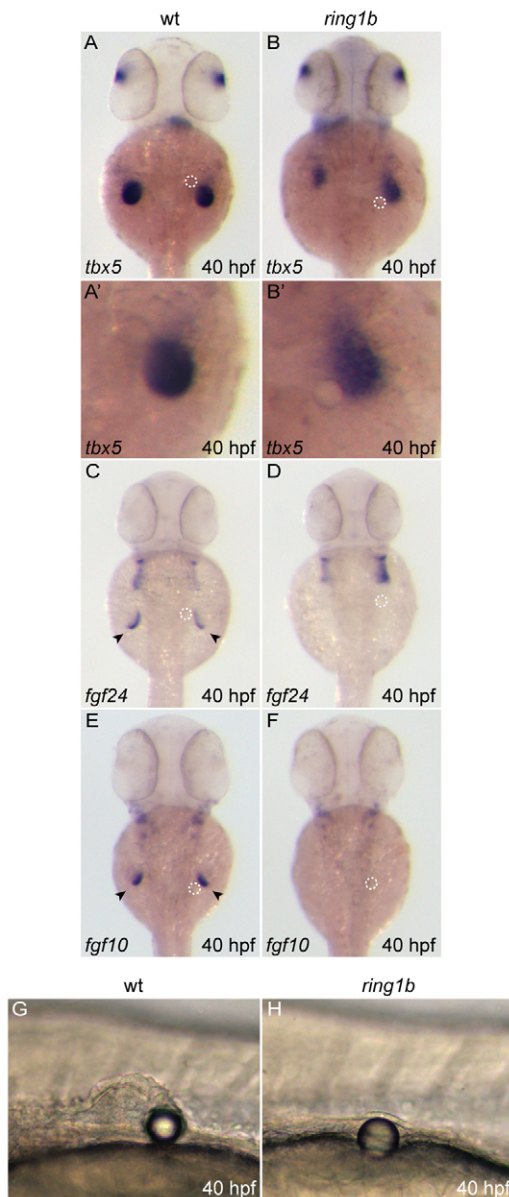


Fig. 7. FGF4-soaked bead implantation restores *tbx5* expression but not fin bud outgrowth. (A–F) Dorsal views of bead-implanted embryos stained for *tbx5* (A–B'), *fgf24* (C,D) and *fgf10* (E,F) at 40 hpf. (G,H) Exogenous FGF4 enhances *tbx5* expression maintenance but is not sufficient to initiate *fgf24* or *fgf10* expression in *ring1b* mutants. Bead location is indicated by the white dashed circle. Pectoral fin bud outgrowth is not restored in *ring1b* mutants. Arrowheads indicate staining at the fin bud region.

signaling occurs and Fgf signaling is not sufficiently activated in the pectoral fin mesenchyme, culminating in loss of fin bud outgrowth.

Hox genes in pectoral fin development

We observed that axial Hox gene expression is only mildly affected until 24 hpf in *ring1b* mutants, indicating that initiation of axis specification is largely correct. *ring1b* mRNA and protein are maternally deposited, which could explain the relatively late onset of Hox gene deregulation in *ring1b* mutants.

Correct axial Hox gene expression is also essential for proper induction and positioning of the forelimb along the axis in vertebrates (Burke et al., 1995; Cohn et al., 1997). In zebrafish, it has been shown that regulation of axial Hox gene function by *pbx4* is essential for the establishment of the pectoral fin field (Popperl et al., 2000). *Pbx4* deficiency in the *lazarus* mutant results in a distinct lack of *tbx5* expression at 24 hpf and suggests that the LPM is never specified as pectoral fin mesenchyme (Popperl et al., 2000). By contrast, in *ring1b* mutants, *tbx5* expression in the LPM at 24 hpf is fairly normal, which strengthens the conclusion that Hox-mediated induction of the forelimb field is unaffected.

Interestingly, the forelimb field is positioned just anteriorly of axial *hoxc6* and *hoxc8* expression (Bejder and Hall, 2002). It has been demonstrated that anterior extension of *hoxc6* and *hoxc8* expression in pythons correlates with lack of forelimbs (Cohn and Tickle, 1999). In *ring1b* mutants, anterior expansion of the *hoxc6* and *hoxc8* expression domains occurs only after the fin field has been established, at 24 hpf. Indeed, several pectoral fin markers, including *tbx5*, *hoxb5b* and *meis3* were expressed at the proper location along the AP axis in *ring1b* mutants. Thus, axial Hox gene function is sufficient to mediate correct specification and positioning of the pectoral fin field in *ring1b* mutants.

RA signaling in *ring1b* embryos

Aldh1a2 is the only gene from the genes involved in pectoral fin development we examined that is robustly overexpressed in the *ring1b* LPM. This enzyme catalyzes the last step in RA synthesis (Begemann et al., 2001; Grandel et al., 2002). Axial *aldh1a2* expression is essential for *tbx5* expression and initiation of the pectoral fin field, whereas *aldh1a2* expression in the LPM is less crucial, as chemical inhibition of RA signaling after 16–22 hpf does not abrogate pectoral fin emergence (Gibert et al., 2006). Despite the high *aldh1a2* expression levels, we did not detect general upregulation of RA target genes in the LPM of *ring1b* mutants. However, the RA-target gene *dhars3* was reproducibly upregulated in *ring1b* mutants at 32 hpf and potent inhibition of RA signaling from 24 hpf onwards led to partial restoration of mesenchymal gene expression. It is plausible that increased RA signaling after 24 hpf contributes to the developmental defect of fin formation in *ring1b* mutants. However, fin outgrowth is not initiated upon inhibition of RA signaling at 24 hpf, and inhibition at 15 ss in fact enhances the defect in fin mesenchyme compaction. Thus, our data indicate that deregulation of RA signaling may contribute to, but is not primarily involved in, the *ring1b* fin phenotype.

The interplay of Wnt and Fgf-signaling in pectoral fin development

Pectoral fin development is partially rescued in *apc/ring1b* mutants through potentiation of the *tbx5*-*fgf24*-*fgf10* axis, most probably owing to increased activation of Wnt signaling. In support of this, the Wnt target genes *myca* and *axin2* are overexpressed in the *apc* fin mesoderm, indicating an increased Wnt-signaling response. We postulate that this augmented Wnt-signaling response in the mesoderm stimulates, directly or indirectly, *tbx5*, *fgf24* and *fgf10* expression. Interestingly, in *apc* mutants, *fgf24* and *fgf10* are overexpressed in other tissues besides the pectoral fin, including the pharyngeal arches. The overexpression in the pharyngeal arches is *Tbx5* independent, as *tbx5* is not expressed there. We postulate that the increased *tbx5* expression in the *apc* fin buds is attributed to the increased Fgf signaling, as *tbx5* expression is feedback controlled by Fgf

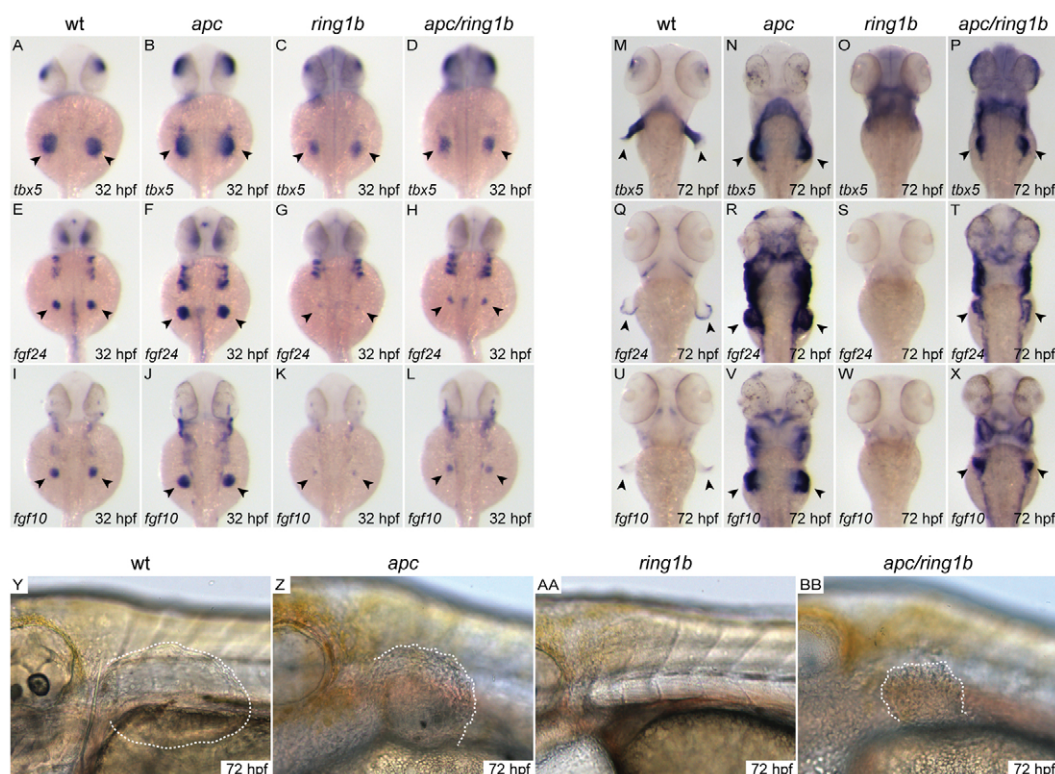


Fig. 8. Restoration of mesenchymal gene expression and fin bud outgrowth in *apc/ring1b* mutants. (A–X) Dorsal views of embryos of the indicated genotypes stained for *tbx5*, *fgf24* and *fgf10* at 32 hpf (A–L) and 72 hpf (M–X). *tbx5* (B,N), *fgf24* (F,R) and *fgf10* (J,V) are overexpressed in the *apc* pectoral fin mesenchyme at both 32 and 72 hpf. Expression of *fgf24* (H) and *fgf10* (L) is increased in *apc/ring1b* mutants compared with *ring1b* mutants (G,K) at 32 hpf. Expression of *tbx5* (P), *fgf24* (T) and *fgf10* (X) is maintained in *apc/ring1b* mutants at 72 hpf. Arrowheads indicate staining at the fin bud region. (Y–BB) Lateral views of 72 hpf embryos of the indicated genotypes at the level of the pectoral fin. A rudimentary fin bud (outlined) is formed in *apc/ring1b* mutants at 72 hpf (BB).

signaling. This raises the possibility that the overexpression of *fgf24* and *fgf10* in both pharyngeal arches and pectoral fin mesenchyme of *apc* mutants is caused by a common mechanism, which is then *Tbx5* independent.

Importantly, mesodermal *fgf24* expression is downregulated at 30 hpf (Fischer et al., 2003) as ectodermal *fgf24* commences. This ‘switch’ does not occur in *apc* and *apc/ring1b* mutants, and, instead, high amounts of *fgf24* remain mesodermal. Ectodermal Fgf signaling is required for elongation of the growing fin bud. Therefore, the deregulation in *fgf24* distribution may account for the presence of small fins with ruffled morphology in *apc* and *apc/ring1b* mutants.

Epigenetic regulation of pectoral fin development

We have shown that pectoral fin development is disrupted due to loss of *Ring1b* and that impaired Fgf-signaling is causally linked to this phenotype. Although we cannot exclude the possibility that *Ring1b* directly represses a single negative regulator of Fgf signaling, we propose that *Ring1b* deficiency causes a broader deregulation of gene expression based on several observations.

Zebrafish mutants that are deficient for globally acting chromatin-associated proteins show surprising tissue-specific defects, such as loss of pectoral fins. These mutants include the *lazarus/pbx4* (Popper et al., 2000), *colgate/histone deacetylase 1* (HDAC1) (Nambiar et al., 2007) and mediator component thyroid hormone receptor-associated protein (TRAP)230/MED12 (Hong et al., 2005; Rau et al., 2006).

Mechanistically, the lack of fins in *ring1b* mutants could possibly be ascribed to tissue-specific interactions between the PRC1 repressive pathway and single master regulators of tissue development, as previously shown in some instances (Yu et al., 2012). Alternatively, the genetic disruption of an essential epigenetic pathway may have a broader impact, resulting in profound alterations of temporal and spatial controls of zebrafish fin development. For example, it is conceivable that loss of *Ring1b* alters the chromatin landscape and may allow the redistribution of activators and/or silencers at the expense of their normal targets. Thus, the consequences of *Ring1b* loss may involve activation and silencing of gene expression through altering the chromatin landscape, in addition to de-repression of direct targets.

Indeed, loss of *Ring1b* may not be seen as an activation switch for single genes in isolation. *Ring1b* inactivation in mouse embryonic stem cells causes aberrant activation of several key developmental genes and deregulation of signaling pathways involved in cellular differentiation (Leeb and Wutz, 2007; van der Stoep et al., 2008). Furthermore, PRC1 ablation lowers the threshold for cellular response to hormones during mammary development (Pietersen et al., 2008), highlighting the role of non-cell autonomous effects in determining the Polycomb phenotype.

In line with this complex scenario of *Ring1b* function, we found that fin-specific expression of *hoxa9b*, *hoxc8a* and *hoxd9a*, reported direct targets of PcG/*Ring1b*-mediated repression, was impaired in *ring1b* mutants, whereas their axial expression domains were expanded. This illustrates that *Ring1b* loss can lead to distinct

aberrations in gene expression, in a context-dependent fashion, and highlights the importance of PcG in the coordinated control of gene expression during development. Future work will aim to elucidate the exact mechanisms of the role of PcG epigenetic gene regulation on the limb developmental program.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077156/-/DC1>

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