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Chapter 2

The Polycomb group protein Ring1b is essential for pectoral fin development

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The Polycomb group protein Ring1b is essential for pectoral fin development

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Polycomb group (PcG) proteins are transcriptional repressors that mediate epigenetic gene silencing by chromatin modification. PcG protein-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 the 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 protein-mediated gene regulation is essential for sustained Fgf signaling in vertebrate limb development.

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, posttranslational 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 in the Polycomb repressive complex 1 (PRC1). Recruitment of PRC1 results in the mono-ubiquitination 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 is 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 for several critical factors enables 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 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 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: AGGAGTGTC CAACATGCAGAAAG and ring1b_R: GAGGATTTGTAACAAAGCCGC, followed by sequence analysis for the ring1b\(^{+4}\) allele or digestion of the PCR product with restriction enzyme TaqI to identify the ring1b\(^{14}\) 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 1 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. Pellet was solubilized in 50 mM Tris-HCl, pH 7.5.

To detect endogenous Ring1b, 30 embryos per tube were lyzed in 100 mM PIPES (pH 6.8) containing 1 mM EGTA, 1 mM MgCl\(_2\), 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 lyzed 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 Ndel 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) (ACACCCACGTCTTTATCTCAATGTT) and 20 ng of splice-blocking MO2 (TTATAACTCAAACAAACCCTGATC) 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 *dhrs3*, *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. 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 of 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% Tween 20 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 manufacturers' 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://zifi.t.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 base pairs (bp) flanking the target-site were generated as described in Materials and Methods and 100 pg mRNA encoding each ZFN was injected into one-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 (Δ14 mutation). Both mutations result in an open reading frame-shift leading 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 non-sense mediated decay. In line with these results, Ring1b protein (Fig. 1E) and ubiquitination 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 also detected in embryos at 2.5 hpf, i.e.
The Polycomb group protein Ring1b is essential for pectoral fin development before onset of zygotic gene expression (supplementary material Fig. S1C). Maternal Ring1b protein persisted up to the 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 gene 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 examined Hox genes (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).

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 in one- to two-cell stage eggs derived from heterozygote ring1b crosses.

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). Fin bud hoxd9a (A-E, arrows) and tbx5 expression (F-J) is restored in injected ring1b mutants and (K, L) fin bud outgrowth is rescued at 72 hpf. (M) Western blot analysis for myc-tagged Ring1b shows dose-dependent expression in 24 hpf embryos. (N) Exogenous Ring1b protein is hardly detectable in 48 hpf embryos.
We assayed injected ring1b mutants for expression of tbx5 and hoxd9u, 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 hoxd9u expression in ring1b mutants, although not to the wild-type extent (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 was 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.

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 (supplemental 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 overexpressed already 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
The Polycomb group protein Ring1b is essential for pectoral fin development (et al., 2008). This showed that the heart precursors were 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

![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.

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 were 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 hoxb5, 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 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; at 15 ss and at 24 hpf. We confirmed that 10 µM and 100 µM DEAB efficiently inhibited RA signaling as demonstrated.
by inhibition of the RA responsive genes *dhrs3*, *hoxc6a* and *hoxc8a* (supplementary material Fig. S6C).

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 15 ss *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 to untreated mutants, albeit not reaching wild-type levels (supplementary materials 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 the response to RA signaling in *ring1b* mutants after 24 hpf that may contribute to the pectoral fin phenotype.

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.
**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 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 was 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 and Nusslein-Volhard, 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 were severely impaired.

![Image of figure 6](image-url)
The Polycomb group protein Ring1b is essential for pectoral fin development

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 (supplemental 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 (Prykhozhij 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 spry4 at 90% epiboly (supplementary material Fig. S10). We next implanted FGF4-coated beads in

![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. Exogenous FGF4 enhances tbx5 expression maintenance but is not sufficient to initiate mesenchymal fgf24 and fgf10 expression in ring1b mutants. Bead location is indicated by the white dashed circle. (G,H) Pectoral fin bud outgrowth is not restored in ring1b mutants.](image)
the flank of embryos at the 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 would provide a good candidate for increased Fgf signaling. In apc mutants, canonical Wnt signaling is hyperactivated due 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 (supplementary material Fig. S11C,G); however, this likely 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 and this was exacerbated at 72 hpf (Fig. 8B,F,J,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, pea3 and spry 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
The Polycomb group protein Ring1b is essential for pectoral fin development expression was not detectable at this stage. Expression of both \textit{fgf24} and \textit{fgf10} in 72 hpf \textit{apc/ring1b} fins resembles that of \textit{apc} mutants, but the expression domains are smaller (Fig. 8T,X). Similarly, Fgf target genes are expressed in \textit{apc/ring1b} mutants, but not in \textit{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.

to grow and gave rise to a small, albeit misshapen fin at 72 hpf (Fig. 8BB). We analyzed expression of the \textit{tbx5-fgf24-fgf10} axis in the \textit{apc/ring1b} fin buds at different stages of development. At 32 hpf, \textit{tbx5} expression in \textit{apc/ring1b} mutants is similar to that of \textit{ring1b} mutants: compaction of pectoral fin mesenchyme occurs, but \textit{tbx5} is poorly expressed and the domain is not well demarcated. Expression of \textit{fgf24} and \textit{fgf10}, while increased in \textit{apc/ring1b} embryos when compared to \textit{ring1b} mutants, did not reach wild-type levels. Interestingly, \textit{tbx5} expression is well maintained in \textit{apc/ring1b} mutants at 72 hpf. This is in striking contrast to \textit{ring1b} mutants, in which \textit{tbx5} expression was not detectable at this stage. Expression of both \textit{fgf24} and \textit{fgf10} in 72 hpf \textit{apc/ring1b} fins resembles that of \textit{apc} mutants, but the expression domains are smaller (Fig. 8T,X). Similarly, Fgf target genes are expressed in \textit{apc/ring1b} mutants, but not in \textit{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.

Fig. 8. Restoration of mesenchymal gene expression and fin bud outgrowth in \textit{apc/ring1b} mutants. (A-X) Dorsal views of embryos of the indicated genotypes stained for \textit{tbx5}, \textit{fgf24} and \textit{fgf10} at 32 hpf (A-L) and 72 hpf (M-X). \textit{tbx5} (B,N), \textit{fgf24} (E,R) and \textit{fgf10} (J,V) are overexpressed in the \textit{apc} pectoral fin mesenchyme at both 32 and 72 hpf. Expression of \textit{fgf24} (H) and \textit{fgf10} (L) is increased in \textit{apc/ring1b} mutants compared to \textit{ring1b} mutants (G,K) at 32 hpf. Expression of \textit{tbx5} (P), \textit{fgf24} (T) and \textit{fgf10} (X) is maintained in \textit{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 is formed in \textit{apc/ring1b} mutants at 72 hpf (BB).
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 signaling occurs and FGF signaling is not sufficiently activated in the pectoral fin mesenchyme, culminating to 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 correlated 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

alldh1a2 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 alldh1a2 expression is essential for tbx5 expression and initiation of the pectoral fin field, whereas alldh1a2 expression in the LPM is less critical, since chemical inhibition of RA signaling after 16-22 hpf does not abrogate pectoral fin emergence (Gibert et al., 2006). Despite the high alldh1a2 expression levels, we did not detect general upregulation of RA target genes in the LPM of ring1b mutants. However, the RA target gene dhrs3 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, but is
The Polycomb group protein Ring1b is essential for pectoral fin development

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 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 mechanisim, 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 surprisingly tissue-specific defects, such as loss of pectoral fins. These mutants include the lazarus/phox (Poppet 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 Stoop 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 proteins in the coordinated control of gene expression during development. Future work will aim at elucidating the exact mechanisms of the role of PcG protein epigenetic gene regulation on the limb developmental program.

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References


The Polycomb group protein Ring1b is essential for pectoral fin development


The Polycomb group protein Ring1b is essential for pectoral fin development

mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development 125, 2381-2395.


**Supplementary Figures**

**Fig. S1. ring1b mRNA and protein are maternally deposited.** (A,B) Whole-mount in situ hybridization with a riboprobe against *ring1b* shows expression at the eight-cell stage, no staining is detected with the sense probe. (C) Western blot analysis of Ring1b protein in wild-type embryos at 2.5 hpf shows maternal Ring1b protein. 24 hpf as control. (D) Ring1b protein is detected in lysates of *ring1b*-mutant embryos at 15 somites but is not or hardly detectable at 24 hpf (D). Histone H3 is used as a loading control.
The Polycomb group protein Ring1b is essential for pectoral fin development

**Fig. S2. Hox gene expression in ring1b mutants.** (A-X) Lateral views of in situ hybridizations for **hoxa9b** (A,B,I,J,Q,R), **hoxc6a** (C,D,K,L,S,T), **hoxc8a** (E,F,M,N,U,V) and **hoxd9a** (G,H,O,P,W,X) at 24 hpf (A-H), 48 hpf (I-P) and 72 hpf (Q-X). Axial Hox gene expression is anteriorly restricted in wild-type larvae (A,C,E,G), whereas the anterior border of axial Hox gene expression is slightly less defined in *ring1b* mutants at 24 hpf (B,D,F,H) (arrowheads). Ectopic Hox gene expression is progressively upregulated in *ring1b* mutants (B,D,F,H versus J,L,N,P and R,T,V,X). Strong ectopic expression throughout the brain is observed especially for **hoxa9b** (R), **hoxc6a** (T) and **hoxc8a** (V) in *ring1b* mutants at 72 hpf. Also note that Hox gene expression is absent or severely reduced in the pectoral fin field of *ring1b* mutants.
Fig. S3. Lateral views of live embryos at 72 hpf. *ring1b* homozygotes display heart edema (line) and jaw abnormalities (arrow).

Fig. S4. Injection of morpholinos against *ring1b* phenocopies the *ring1b* developmental phenotype. Lateral views of embryos at 72 hpf. MO1- and MO2-injected embryos display the heart edema (lines) and jaw abnormalities (arrows) similar to *ring1b* mutants (A-C). Expression of *tbx5* (E,F), *hand2* (H,I) and *fgf24* (K,L) in *ring1b* MO-injected embryos at 48 hpf, shows the same pattern as the one in *ring1b* mutants. No Ring1b protein is detected in lysates from *ring1b* MO-injected embryos (M). Histone H3 serves as loading control.
The Polycomb group protein Ring1b is essential for pectoral fin development

Fig. S5. Nuclear Tbx5 localization in the ring1b pectoral fin mesenchyme. Transverse sections of wild type (A,C) and ring1b mutants (B,D) stained with an antibody against Tbx5 at 32 (A,B) and 48 hpf (C,D). Nuclei in the pectoral fin mesenchyme of ring1b mutants stain positive for Tbx5 at 32 and 48 hpf (B,D).
Fig. S6. DEAB treatment initiated at 24 hpf leads to partial restoration of mesenchymal gene expression in *ring1b* fin buds. (A) In situ hybridizations for the RA-responsive genes *dhrs3* (A-F), *hoxc6a* (G-L) and *hoxc8a* (M-R) in wild-type and *ring1b* embryos at 48 hpf, treated with DEAB from 24 hpf. DEAB (10 µM and 100 µM) potently suppresses expression of *dhrs3* in wild-type and *ring1b* embryos (A-F). While 10 µM DEAB suppresses ectopic overexpression of *hoxc6a* and *hoxc8a* in *ring1b* embryos, 100 µM DEAB leads to more potent inhibition (I,K,O,Q).
The Polycomb group protein Ring1b is essential for pectoral fin development.

(B) In situ hybridizations for tbx5 (A-F), hand2 (G-L) and fgf24 (M-R) in wild-type and ring1b embryos at 40 hpf, treated with DEAB from 15 ss. Inhibition of RA signaling by DEAB leads to downregulation of tbx5 (C,E), hand2 (I,K) and fgf24 (O,Q) expression in wild-type embryos in a dose-dependent manner. DEAB (10 µM) leads to almost complete downregulation of tbx5 expression in ring1b fin buds (D) and this effect is more pronounced with 100 µM DEAB (F). DEAB treatment initiated at 15 ss in ring1b embryos does not have an effect on the (lack of) expression of hand2 (J,L) and fgf24 (P,R) in ring1b fin buds. (C) In situ hybridizations for tbx5 (A-F), hand2 (G-L) and fgf24 (M-R) in wild-type and ring1b embryos at 40 hpf, treated with DEAB from 24 hpf. DEAB treatment initiated at 24 hpf does not have a significant impact in expression of tbx5 (C,E), hand2 (I,K) and fgf24 (O,Q) in wild-type fin buds. DEAB treatment of ring1b embryos initiated at 24 hpf leads to partial restoration of tbx5 (D,F) and hand2 (J,L) expression in a dose-dependent manner. However, treatment does not lead to restoration of fgf24 expression (P,R).
Fig. S7. Time-course expression analysis of the Fgf receptors. (A-R) Dorsal views of in situ hybridizations for *fgfr1a* (A-F), *fgfr2* (G-L) and *fgfr3* (M-R). *fgfr1a* is not expressed in either wild-type or *ring1b* LPM at 18 ss (A,B; asterisks indicate absence of expression). At 24 hpf, *fgfr1a* is expressed in the fin buds of wild-type embryos and at weaker levels in *ring1b* fin buds (C,D). At 32 hpf, strong *fgfr1a* expression is detected in the wild-type fin buds but is greatly reduced in the *ring1b* fin buds (E,F). *fgfr2* is expressed in the LPM of wild-type and *ring1b* embryos at 18 ss (brackets, G,H). At 24 hpf, *fgfr2* expression in *ring1b* fin buds is weak (I,J) and is hardly detectable in *ring1b* fin buds at 32 hpf (K,L). *fgfr3* is expressed at higher levels in the LPM of *ring1b* mutants at 18 ss (M,N). At 24 hpf, *fgfr3* expression is upregulated and expanded in mutants (O,P). At 32 hpf, *fgfr3* remains high and the expression domain is expanded in *ring1b* embryos (Q,R). Brackets in M-R indicate the LPM.
The Polycomb group protein Ring1b is essential for pectoral fin development

Fig. S8. Impaired AP/DV patterning and loss of AER gene expression in ring1b mutants. (A) Dorsal view of in situ hybridizations for the AP markers msxc (A,B) and shh (C,D) and the DV markers eng1a (E,F) and wnt7a (G,H) at 32 hpf. msxc is not restricted to the anterior pectoral fin mesenchyme because shh is not expressed in the zone of polarizing activity (ZPA) of ring1b mutants (B,D). eng1a expression is greatly reduced, whereas wnt7a is not expressed in the ring1b pectoral fin ectoderm (F,H). (B) Dorsal (A-H) and lateral (A’-H’) views of in
situ hybridizations for the AER markers *dlx2a* (A-B), *fgf8* (C-D), *fgf24* (E-F) and *versican* (G-H) at 40 hpf. The examined AER markers are not expressed in the pectoral fin ectoderm of *ring1b* mutants at 40 hpf and fin bud outgrowth is not initiated (B-H).

**Fig. S9. Decreased cell proliferation in the *ring1b* pectoral fin field.** (A) Quantification of pH3-positive cells in pectoral fins of wild-type and *ring1b* embryos at 24, 32 and 48 hpf. Proliferation in the *ring1b* pectoral fin field is normal at 24 hpf, but decreased at 32 and 48 hpf. (B) Quantification of apoptotic cells in wild-type and *ring1b* pectoral fins. Very few pectoral fin cells stain TUNEL-positive in wild type and *ring1b* mutants. Although the difference between wild type and *ring1b* mutants at 48 hpf is statistically significant, it is unlikely that increased apoptosis contributes to the *ring1b* fin phenotype as the number of apoptotic cells is very low and it occurs at such a late stage. In all experiments, at least 15 embryos were analyzed.

**Fig. S10. FGF4-coated bead implantation induces ectopic expression of Fgf target genes.** (A-C) Whole-mount in situ hybridization for the Fgf target genes *dusp6* (A), *pea3* (B) and *spry4* (C) in embryos at 90% epiboly following FGF4-coated bead implantation at the 1000-cell stage. Arrowheads indicate the position of the bead and ectopic expression of the FGF-target genes induced in the proximity of the bead.

**Fig. S11. The Wnt-target genes *myca* and *axin2* are overexpressed in *apc* and downregulated in *ring1b* fin mesenchyme.** (A-H) Embryos of the indicated genotypes were stained at 56 hpf with *axin2* (A-D) and *myca* (E-H). *myca* and *axin2* are overexpressed in *apc* mutants, including the fin mesenchyme (B,F). In *ring1b* fin mesenchyme, *myca* and *axin2* expression is very weak (C,G). *myca* and *axin2* expression is restored in the nascent fin bud of *apc/ring1b* mutants (D,H).
The Polycomb group protein Ring1b is essential for pectoral fin development

Fig. S12. The Fgf target genes dusp6, pea3 and spry4 are overexpressed in apc fin mesenchyme. Embryos of the indicated genotypes were stained at 32 hpf (A-L) and 72 hpf (M-X) with the indicated Fgf-target genes. dusp6, pea3 and spry4 are expressed in wild-type fin buds at 32 hpf (A,E,I). Expression of all three genes is upregulated in apc fin buds (B,F,J) not detectable in ring1b mutants (C,G,K) and expression is restored, albeit weakly, in apc/ring1b mutants (D,H,L). dusp6, pea3 and spry4 are expressed in wild-type growing fins at 72 hpf (M,Q,U). Expression of all three genes is upregulated in the apc fin buds (N,R,V) and not detectable in ring1b fin buds (O,S,W). Expression of the Fgf-target genes is restored in the apc/ring1b fin buds (P,T,X).

Tabel S1. primer sequences.

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