The Polycomb group protein Ring1b is essential for pectoral fin development

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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 chromodomains 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...
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 (AGGAGTGT CAACATGCAGAAAG) and ring1b_R (AGGAGTTGTTACAAAGGCGC), followed by sequence analysis for the ring1b+/- allele or digestion of the PCR product with restriction enzyme Taq1 to identify the ring1b+/- 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 1 mM DTT. To detect endogenous Ring1b, 30 embryos per tube were lysed in 100 mM MPES (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 was 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-beta-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 (Munich, Germany) and linearized with HI restriction sites. To generate the ring1b expression construct, the open reading frame of ring1b, excluding the 5’ and 3’ untranslated regions, was cloned into pcDNA3-Myc using BamHI restriction sites.

mRNA and morpholino injections
Vectors were linearized with Ncol. 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) (ACACACGTCTTTTATCTCAAATGTT) and 20 ng of splice-blocking MO2 (TTAAACTCTCAAACAACCTGATC) 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, hoxea6, hoxd9a, meis3, mxsc, 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. Dechondrinated 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 antibody was biotinylated goat anti-rabbit IgG (1:300; DakoCytomation). Whole-mount immunohistochemistry, embryos were fixed overnight at 4°C in Dent’s fixative, digested in PBS containing 10 μg/ml protease K, 0.1% Tween20 and blocked in PBS containing 0.5% normal goat serum, 0.5% DMSO and 0.3% Triton X-100. Primary antibody was rabbit anti-H3 (1:750; sc8565R, Santa Cruz) and secondary antibody goat anti-rabbit IgG (1:300; DakoCytomation).
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 (Δ14 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; Pirrota, 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).

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. 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.
Known marker for fin mesenchyme, we examined the expression important for fin development. In addition to genes that are expressed in the fin field mesenchyme and are expressed in the pectoral fin field (Fig. 4M,N,Q,R,U,V). At 32 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 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

**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.

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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 μM and 100 μM DEAB efficiently inhibited RA signaling, as demonstrated by inhibition of the RA-responsive genes dhrs3, 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 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 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 (Sleptsov-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...
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 (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 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...
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-accumulates in the nucleus and, together with TCF, activates Wnt-signaling cascade, although fgf24 expression remains confined to the mesenchyme and is not expressed in the ectoderm.

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

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,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, 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 missshapen, 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.

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.

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.
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 aldha2 expression is essential for tbx5 expression and initiation of the pectoral fin field, whereas aldha2 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 aldha2 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 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.
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/phox4 (Popperl et al., 2000), colgate/histone deacetylase 1 (HDAC1) (Nambari 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 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

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