Two proteins, one model organism: On the functional characterization of Lkb1 and Ring1b in zebrafish
van der Velden, Y.U.

Citation for published version (APA):
van der Velden, Y. U. (2013). Two proteins, one model organism: On the functional characterization of Lkb1 and Ring1b in zebrafish
Chapter 3

The Polycomb group protein Ring1b is critically involved in cranial skeleton development
The Polycomb group protein Ring1b is critically involved in cranial skeleton development

Yme U. van der Velden, Liqin Wang, Maarten van Lohuizen and Anna-Pavlina G. Haramis

Division of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands

Polycomb group (PcG) proteins form multimeric complexes that are dynamically involved in gene repression through chromatin modification. PcG proteins target literally hundreds of genes, many of which are critical regulators of embryonic development, stem cell plasticity, cell fate maintenance, cellular differentiation and cancer. Although great advances have been made in the understanding of PcG function, the role of PcG proteins in vertebrate embryogenesis remains poorly understood due to early lethality of mice deficient for several core PcG proteins. Here, we show that zebrafish Ring1b, the single E3 ubiquitin ligase in the Polycomb Repressive Complex 1 (PRC1) critically regulates the developmental program of craniofacial cell lineages. Loss of Ring1b causes a severe craniofacial phenotype, which includes an almost complete absence of all cranial cartilage, bone and musculature. This study focused on the defect in cartilage development. We show that cartilage precursors, which are derived from the neural crest, migrate into the pharyngeal arches, but differentiation into chondrocytes is abrogated. This phenotype is rather specific for cartilage precursors, since other neural crest-derived cell lineages, including glia, neurons and chromatophores are formed in ring1b mutants. Thus, our results reveal a critical and specific role for Ring1b in promoting the differentiation of cranial neural crest cells into chondrocytes.

Introduction

Polycomb group (PcG) proteins act as transcriptional repressors that were first identified in Drosophila melanogaster through their ability to silence homeotic genes. Nowadays, hundreds of PcG target genes have been identified in a vast number of organisms through genome-wide mapping studies. PcG proteins are highly enriched at promoters of transcription factors, but are also found at genes that encode receptors, signaling proteins and morphogens (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006; van der Stoop et al., 2008). Indeed, it is now appreciated that PcG proteins are key regulators of processes such as embryonic development, stem cell plasticity, cell fate maintenance, cellular differentiation and cancer. The mechanism underlying PcG-mediated gene silencing is still not fully understood, but includes 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). Historically, PcG proteins are divided into two distinct multimeric complexes, termed Polycomb repressive complex 1 and 2 (PRC1 and PRC2). In a simplified view, PRC2 can be seen as the initiator complex that acts through trimethylation of histone H3 at lysine H3 at lysine 27 (H3K27) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002). This epigenetic mark can be recognized by PRC1 through the chromodomain of Polycomb. E3-ligase activity of RING-
domain-containing proteins then leads to the mono-ubiquitination of histone H2A at lysine 119, a histone modification that is associated with gene repression (de Napoles et al., 2004; Wang et al., 2004). Recent data indicates however that the function of PcG proteins in gene silencing not this straightforward. For example, binding of PcG proteins to target genes does not per se lead to gene silencing (Beisel et al., 2007; Papp and Muller, 2006; Schwartz et al., 2006) and PRC1 can be recruited to chromatin independently of PRC2 (Dietrich et al., 2012; Schoeftner et al., 2006; Tavares et al., 2012; Yu et al., 2012). Moreover, the composition of PcG complexes is highly versatile, allowing dynamic and tissue-specific regulation of PcG function depending on the cellular context (Yu et al., 2012).

The study of PcG proteins in vertebrate embryonic development is hampered due to the early lethality of mice defective for the core proteins of PRC2 (Eed, Ezh2, and Suz12) and PRC1 (Ring1b). To gain more insight in the developmental functions of PcG proteins, we previously generated Ring1b-deficient zebrafish. Only a single ortholog of the Drosophila E3 ubiquitin ligase dRing is present in zebrafish whereas a genomic duplication event gave rise to two Ring proteins in mammals: Ring1a and Ring1b. The zebrafish ortholog is most homologous to Ring1b, and in contrast to mice, Ring1b deficiency does not cause a gastrulation arrest in zebrafish. Rather, ring1b mutants are phenotypically indistinguishable from wild-type siblings at 24 hpf. Pleiotropic developmental defects arise during organogenesis, including loss of pectoral fins, craniofacial malformations, pericardial edema and diminished blood circulation, leading to the death of ring1b mutants at 4-5 dpf (van der Velden et al., 2012).

The skeleton of vertebrates is predominantly composed of two different structural tissues: cartilage and bone (Eames et al., 2003). Both cell populations are mainly derived from the neural crest (NC), which is an ectoderm-derived, multipotent cell population that is induced at the beginning of neurulation at the border of non-neural ectoderm and the neural plate (Gans and Northcutt, 1983; LaBonne and Bronner-Fraser, 1999). As neurulation progresses, NC cells undergo epithelial-to-mesenchymal transition, allowing delamination from the dorsal neuroepithelium and subsequent migration throughout the embryo. The NC gives rise not only to cartilage and bone, but also to neurons, glia, smooth muscle cells and chromatophores. Distinct cell types are formed according to their position along the anteroposterior axis. For example, NC cells that give rise to chromatophores originate from trunk NC cells whereas NC cells that will form craniofacial cartilage are located at the level of the mid/hindbrain (Raible and Eisen, 1994; Schilling and Kimmel, 1994). During cartilage development, the most anteriorly-located cranial neural crest (CNC)-derived cartilage precursors will mainly form skeletal elements that encase the brain (neurocranium) whereas the ventral viscerocranium is derived from more posterior located NC cells. Viscerocranial precursors migrate ventrally as three streams to populate the pharyngeal arches, an embryonic structure that will form several tissues of the head-neck region in higher vertebrates. Stream 1 migrates into the most anterior located mandibular arch (arch I), stream 2 into the hyoid arch (arch II) and stream 3 into the five posterior branchial arches (arch III-VII). The mandibular arch and hyoid arch form the jaw and its supportive elements, respectively, whereas the five branchial arches give rise to the cartilaginous elements of the gill. Cranial bones are also derived from CNC cells and ossification occurs through two
distinct processes: endochondral and dermal ossification. In endochondral ossification, a skeletal template composed of replacement cartilage is formed first, which is then replaced by bone. Alternatively, bones can also be formed directly from osteogenic condensations (dermal ossification). Both processes are essential for cranial bone development.

In this study, we show that zebrafish that are deficient for the E3 ubiquitin ligase Ring1b have a severe craniofacial phenotype, including an almost complete absence of all cranial cartilage, bone and musculature. We focused on the defect in cartilage development and show that specification and initial migration of CNC-derived cartilage precursors into the pharyngeal arches is largely unaffected, however, cartilage differentiation is abrogated. Moreover, the loss of Ring1b rather specifically affects CNC-derived cartilage precursors since other NC-derived lineages, including neurons, glia and chromatophores are present in ring1b mutants. Finally, we reveal that both endochondral and dermal ossification are abrogated due to loss of Ring1b.

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:AGGAGTG TCAAACATGCAGAAAG and ring1b_R: GAGGATTGTAAACAAAGCCGC, followed by sequence analysis for the ring1b^{+4} allele or digestion of the PCR product with restriction enzyme TaqI to identify the ring1b^{Δ14} allele.

Whole-mount in situ hybridization
Whole-mount in situ hybridizations were carried out according to the standard protocol (Westerfield, 2000). BM purple (Roche) was used as alkaline phosphatase substrate. Probes for dlx2a, col2a1, col10a, hand2, runx2a, runx2b, sox9a and sox9b were described previously. Antisense riboprobes amplified from cDNA were cyp26a1, foxD3, hoxa3a, neuroD, and sox10. Primer sequences are available upon request.

Whole-mount antibody staining
Embryos were overnight fixed in 20% DMSO, 80% Methanol at 4 °C, followed by dehydration and overnight storage in methanol at -20 °C. Endogenous peroxidase was blocked by 10 minute incubation in methanol containing 0.03% H_2O_2 and after rehydration; embryos were digested in PBS containing 10 µg/ml proteinase K, 0.1% Tween 20 and refixed in PBS containing 4% paraformaldehyde for 20 minutes. After extensive washing in PBS containing 1% DMSO and 0.3% TritonX-100, embryos were blocked for one hour in PBS containing 2% normal goat serum, 2 mg/ml BSA, 1% DMSO and 0.3% Triton X-100 (PTBN). Primary antibody was mouse anti-MF20 (1:20, Developmental Studies Hybridoma Bank). Secondary antibodies was biotinylated goat anti-mouse IgG (1:200, DakoCytomation). Signal was amplified using the ABC method (Vectastain Elite).

Whole-mount cartilage stainings
Embryos were fixed in 40% ethanol, 5% acetic acid, and 10% formalin containing 0.02% alcian blue for 6 hours at room temperature, followed by dehydration and overnight storage in 100% ethanol at -20 °C. Embryos were rehydrated and washed in MQ containing 0.2% Triton-X100. Pigment
was bleached by 30 minute incubation in MQ containing 1% KOH, 3% H₂O₂ and 0.2% Triton-X100. After two washes in Mili-Q (MQ) water containing 0.2% Triton-X100, the bleaching was neutralized by 10 minute incubation in a saturated sodium tetraborate solution. Next, the embryos were digested in a 60% saturated tetraborate solution containing 0.01% trypsin (Sigma) for one hour. Embryos were cleared in a MQ containing 20% glycerol, 1% KOH and 0.2% Triton-X100 for 20 minutes and stored in 70% glycerol.

**Xanthophore staining**
Embryos were incubated from 24 hpf onwards in embryo medium (+chorion) containing 0.000005% Methylene blue.

**Live imaging**
For live imaging of melanophores, iridophores and xanthophores, embryos were anaesthetized in MS222 prior to mounting in methylcellulose and imaging on either an Olympus BX50 or Leica MZ FLIII stereo microscope.

**Results**

**ring1b mutants lack the jaw elements**
Visible craniofacial defects in homozygous ring1b mutants were first observed around 40 hpf by a reduction in the amount of tissue at the level of the anterior pharyngeal arches. At 72 hpf, ring1b mutants showed a reduction of tissue under the eye at the location of the jaw (Fig 1B). This dramatic phenotype led us to examine at which developmental stage and to which extent cartilage development is affected by loss of Ring1b. To visualize cartilage, we stained wild-type and ring1b mutants at different developmental time points with Alcian Blue, a dye that binds to carbohydrate moieties of the chondrogenic extracellular matrix.

In wild-type embryos at 56 hpf, the trabeculae cranii, which is a cartilage

![Image](image.jpg)

**Fig. 1.** ring1b mutants lack almost all head cartilage elements. Lateral view of wild-type (A) and ring1b (B) live embryos at 72 hpf. Alcian-Blue-stained head cartilages of wild-type (C,E,G,I) and ring1b (D,F,H,J) mutants at the indicated developmental stages, ventral views. Cartilage development initiates at 48 hpf with the formation of paired trabeculae in wild-type embryos (E). The elements elongate and fuse posteriorly at 56 hpf and by 72 hpf the elaborate cartilagenous skeleton of the head has been established. Note that the cartilaginous structures from the posterior gill-bearing arches have formed by 65 hpf, but are hardly visible due to the plane of focus. In contrast, two rudimentary cartilage deposits are present at both sides of the anterior notochord only at 72 hpf ring1b mutants (J; arrowheads). ch: ceratohyal; ep: ethmoid plate; hys: hyosymplectic; m: Meckel's cartilage; pc: parachordal, pq: palatoquadrate; tc: trabeculae cranii.
element of the prospective neurocranium, have fused posteriorly to the parachordals and the ethmoid plate starts to emerge anteriorly (Fig. 1E). At 65 hpf, the ethmoid plate has elongated in wild-type embryos and the first viscerocranial cartilage structures were also formed (Fig. 1G). Meckel’s cartilage and the palatoquadrate are formed respectively from ventral and dorsal groups of cartilage precursors resided in the mandibular arch. Cartilage elements derived from the hyoid arch include the ceratohyal and hyosymplectic (Knight and Schilling, 2006). At 72 hpf, craniofacial cartilage structures matured further, as illustrated by the more anterior location of the ethmoid plate and Meckel’s cartilage (Fig. 1I).

In contrast to wild-type embryos, craniofacial cartilage was absent in ring1b mutants at both 56 and 65 hpf (Fig. 1F,H). At 72 hpf, two cartilage deposits were detected at both sides of the anterior notochord (Fig. 1J). A total absence of the cartilaginous pectoral fin girdle was also observed. Together, these results show that loss of Ring1b almost completely abrogates the formation of cartilaginous elements.

**Cranial muscle development is abrogated in ring1b mutants**

Because both the viscerocranium and neurocranium are essential for the patterning of associated craniofacial musculature (Noden, 1983b; Schilling and Kimmel, 1997), we next examined to which extent loss of Ring1b affects cranial muscle development. To detect the developing muscles, we performed whole-mount stainings with the MF20 antibody that recognizes the myosin heavy chain of vertebrate striated muscles (Bader et al., 1982). The vertebrate craniofacial musculature is of paraxial mesoderm origin (Noden, 1983a; Noden, 1983b; Schilling and Kimmel, 1994). Muscle precursors residing in the first two pharyngeal arches give rise to muscles that are associated with the jaw and its support elements whereas precursors residing in arches III-VII form muscles associated with gill cartilage. The anterior mandibularis, which originates from the mandibular arch, had formed by 56 hpf in wild-type embryos (Fig. 2C,I). At 65 hpf, cranial musculature associated with the jaw started to emerge (Fig. 2E,K). The hyohyoideus and interhyoideus, derived from the hyoid arch, matured further at 72 hpf and various other muscles, including the mandibular arch-derived intermandibularis anterioris, intermandibularis posterioris, levator arcus palatine and dilator operculi as well as the hyoid arch-derived adductor hyomandibulae and adductor operculae, are also formed by this stage (Fig. 2G,M). In contrast, irregular MF20-positive patches of craniofacial musculature were only detected at 72 hpf in ring1b mutants (Fig. 2H,N).

In addition to the analysis of the pharyngeal arch-derived craniofacial musculature, we extended the analysis to somite-derived muscles. Interestingly, the posterior hypaxial muscle (phm), which eventually will give rise to the most anterior two segments of the medial obliquus inferioris (Windner et al., 2011), had delaminated from the somites in both wild-type and ring1b mutants at 48 hpf (Fig. 2A,B). At 56 hpf, the sternohyoideus (sh) had also formed in both wild-type and ring1b mutants and the pectoral fin muscle (pfm) was prominently visible in wild-type embryos (Fig. 2C,D). Although ring1b mutants lack pectoral fins, a population of MF20-positive cells, presumably pfm progenitors, was detected in between the sh and phm (Fig. 2D). The sh and phm elongated during later development and attached to the cleithrum in wild-type embryos, a bone of the fin girdle (Fig. 2E,G). Elongation of these muscles was completely abrogated in ring1b mutants (Fig. 2F,H).
The Polycomb group protein Ring1b is critically involved in cranial skeleton development

Our findings thus indicate that loss of Ring1b severely impairs the formation of craniofacial musculature, whereas initial formation and migration of somite-derived muscles is relatively unaffected.

**Ring1b is not required for early CNC specification and migration**

Because craniofacial cartilage development was abrogated in ring1b mutants, we next addressed whether CNC cells that will form the prospective viscerocranium migrate correctly into the pharyngeal arches.

We analyzed the expression pattern of two transcription factors that are required for pharyngeal arch development, the homeobox transcription factor *dlx2a*, which is already expressed in pre-migratory CNC cells, and the bHLH transcription factor *hand2*, whose expression is confined to a ventral subset of *dlx2a*-positive cells in postmigratory CNC cells (Akimenko et al., 1994; Angelo et al., 2000; Miller et al., 2000). At 32 hpf, *dlx2a*-expressing CNC cells had migrated into the pharyngeal arches in both wild-type and ring1b embryos (Fig. 3A-B’). In wild-type embryos, CNC cells from stream 3 were at this stage starting to split into five cell groups that populate the branchial arches: three distinct cell groups were readily visible. Although the three branchial *dlx2a*-expressing cell groups were also detected in ring1b mutants,
the separation process appeared slightly affected. In contrast, hand2 expression in the pharyngeal, hyoid and the two anterior branchial arches of ring1b mutants was indistinguishable from wild-type embryos (Fig. 3C-D').

We also included expression analysis of hoxa3a, a reported direct target of PcG proteins in mammals (Bracken et al., 2006) that is expressed in the branchial arches (Fig. 3E-F') (Hogan et al., 2004). hoxa3a was expressed at reduced levels in ring1b mutants, but the overall size of the expression domain was relatively unaffected.

Because the separation of stream 3 CNC cells seemed slightly impaired in ring1b mutants, we extended the analysis to the branchial pouches, which are outpockets from the foregut. During migration, CNC cells encounter the pharyngeal pouches and populate the space in between each pouch. Importantly, impaired pharyngeal pouch development often results in
The Polycomb group protein Ring1b is critically involved in cranial skeleton development impaired separation of migrating CNC cells, suggesting that pharyngeal pouches play a role in directing CNC cell migration (David et al., 2002; Knight and Schilling, 2006; Piotrowski and Nusslein-Volhard, 2000). In situ hybridization against cyp26a1, a pharyngeal pouch marker, indeed showed a severe reduction in expression, indicating that pharyngeal pouch development was indeed impaired in ring1b mutants (Fig. 3G-H’). Together, the data indicates that Ring1b is largely dispensable for the migration or specification of CNC cells into the pharyngeal arches. In addition, pharyngeal pouch development is affected by loss of Ring1b, as assessed by reduced cyp26a1 expression, which might contribute to the defect in cartilage development.

Figure 4. Loss of ring1b impairs the formation of prechondrogenic condensations and abrogates CNC cartilage differentiation. Ventral (A-I) and lateral (A’-J’) views of whole-mount in situ hybridizations with riboprobes against the indicated genes in wild-type and ring1b mutants at 50 hpf. Prechondrogenic condensation markers sox9a and sox9b are expressed in ring1b CNCs residing in pharyngeal arches 1 and 2, albeit the expression domain is smaller (A-D’). The expression domain of CNC markers sox10 and dlx2a is similarly reduced in pharyngeal arches 1 and 2 of ring1b mutants (E-H’). Expression of sox9a, sox9b and sox10 is not detected at the presumptive location of the trabeculae cranii in ring1b mutants (B,B’,D,D’,F,F’). col2a1 is expressed in the trabeculae cranii and pharyngeal arches I-II in wild-type embryos, but expression is abrogated in ring1b mutants (I-J’). Note that expression of col2a1, a marker for differentiating chondrocytes, is normal in the otic vesicles and parachordals of ring1b mutants. tc: trabeculae cranii; pc: parachordal; ov: otic vesicle. Numbers indicate the respective pharyngeal arches.
Ring1b is required for chondrocyte differentiation
We next investigated whether differentiation of post-migratory CNC cells into chondrocytes was impaired. A master regulator of vertebrate chondrocyte differentiation is the transcription factor Sox9 (Akiyama et al., 2002; Kist et al., 2002). In zebrafish, two orthologues of Sox9 exist; Sox9a and Sox9b (Yan et al., 2005). Although each ortholog has, apart from shared, unique expression domains and functions, both are critically involved in chondrocyte differentiation (Yan et al., 2005).

In 50 hpf wild-type embryos, sox9a and sox9b were expressed in prechondrogenic condensations of the mandibular arch (1), hyoid arch (2) and the posterior branchial arches (3-7) (Fig. 4A,A',C,C'). In addition, both genes were expressed in the developing trabeculae cranii. In ring1b mutants, sox9a was also expressed in the pharyngeal arches (Fig. 4B,B'). sox9a expression domains in the mandibular and hyoid arches were well demarcated, albeit the domain was clearly smaller. Faint staining was detected at the location of the pharyngeal arches, but individual branchial arches could not be identified. In addition, ring1b mutants lacked trabecular staining. sox9b showed a similar expression pattern as sox9a in the pharyngeal arches of wild-type embryos, whereas the trabecular expression domain was more extended (Fig. 4C). sox9b was not expressed in the branchial arches and at the presumptive trabeculae cranii of ring1b mutants, whereas expression in the mandibular and hyoid arch was detected and similar to sox9a expression (Fig. 4D, D'). Notably, sox9a and sox9b expression in the hindbrain domains was unaffected in the mutants suggesting a specific defect in cartilage differentiation. Similar results were obtained for the pan-neural crest markers sox10 and dlx2a, which both continue to be expressed in post-migratory CNC cells (Fig. 4E-H').

To further assess craniofacial cartilage formation in ring1b mutants, we examined the expression of col2a1, which encodes the alpha I chain of type II collagen, the main extracellular matrix protein in cartilage (Vandenberg et al., 1991; Yan et al., 1995; Yan et al., 2005). Notably, Sox9 binds to an intron of Col2a1 in mammals, thereby enhancing gene transcription (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997). In zebrafish, col2a1 is expressed in differentiating chondrocytes and Sox9a/b function is required for its expression (Yan et al., 2005). In 50 hpf wild-type embryos, col2a1 was strongly expressed in the trabeculae cranii, otic vesicle and to a lesser extent in the mandibular and hyoid arch (Fig 4I,I'). As expected, since Sox9a/b function is essential for col2a1 expression, col2a1 was not expressed at the location of the presumptive trabeculae cranii in ring1b mutants (Fig. 4J). Importantly, col2a1 expression was neither detected in the ring1b mandibular and hyoid arches, despite the observed sox9a/b expression in these structures.

Together, these results demonstrate that Ring1b is required for proper execution of the cartilage differentiation program.

Development of non-ectomesenchymal NC derivatives is modestly affected in ring1b mutants
NC cells give rise to a wide array of cell lineages including cartilage, bone, neurons, glia, smooth muscle cells and chromatophores. Since the differentiation of CNC cells into cartilage is abrogated in ring1b mutants, we addressed whether NC differentiation in general is perturbed by loss of Ring1b.

First, we analyzed whether migration of trunk NC cells was affected by loss of Ring1b. We stained embryos for sox10, a
The Polycomb group protein Ring1b is critically involved in cranial skeleton development

The Polycomb group protein Ring1b is critically involved in cranial skeleton development

marker of NC lineages that is involved in the specification of non-ectomesenchymal NC derivatives, such as chromatophores, neurons and glia (Dutton et al., 2001). sox10-positive NC cells are migrating ventrally from their dorsal premigratory position in a rostrocaudal fashion. At 24 hpf, ring1b trunk NC cells had migrated as far as in wild-type siblings and localization of sox10 expressing NC cells remained indistinguishable from wild-type siblings at 32 hpf (Fig. 5A-D). Thus, loss of Ring1b does not affect the migration of trunk NC cells while strong sox10 expression suggests normal specification of these cells.

Figure 5. Migration and initial differentiation of trunk neural crest cells is modestly affected in ring1b mutants. sox10 staining of wild-type and ring1b mutants at 24 and 32 hpf shows that sox10-expressing NC cells have migrated timely and correctly to their ventral positions (A-D). Expression of foxD3 in cranial ganglia-associated glia is comparable to wild-type siblings in ring1b mutants at 32 hpf (E,F), but reduced in the postotic ganglia of ring1b mutants at 56 hpf (G,H). neuroD expression in cranial ganglia precursors is reduced in ring1b mutants at 32 hpf (L,J), but remains detectable in the hindbrain region of ring1b mutants at 56 hpf (L). Lateral view of 32 hpf embryos shows normal distribution of melanocytes in ring1b mutants at 32 hpf (M,N). At 60 hpf, ring1b melanophores remain more stellate than wild-type melanophores, which have started to round up (S,T). Iridophores are present in near normal numbers in ring1b mutants at 60 hpf (O,P), whereas ring1b xanthophores are smaller and less stellate (Q,R,U,V). gX: vagal ganglia; mll: medial lateral line ganglia; o/gVIII: octaval/statoacoustic ganglia; pll/gP: posteriolateral line ganglia; pog: preotic ganglia; ptg: postotic ganglia; tg: trigeminal ganglia.

To address whether glial differentiation was impaired, we analyzed the expression of forkhead transcription factor foxD3 at two developmental time-points (Kelsh et al., 2000). In ring1b mutants at 32 hpf, foxD3 was expressed at near-normal levels in the cranial ganglia-associated glia of the developing trigeminal ganglion as well as in the preotic and postotic ganglia, indicating that migration and initial differentiation of cranial glial precursors was largely unaffected by Ring1b loss (Fig. 5E,F). However, at 56 hpf, glial foxD3 expression was reduced in ring1b mutants, particularly in glia associated with the...
postotic ganglia (Fig. 5H). Together, this suggests that the glial developmental program is fairly normally initiated, but not well maintained.

Next, we analyzed the expression pattern of neuroD, a marker for all neurogenic placodes (Andermann et al., 2002). At 24 hpf, four distinct neuroD expression domains were detected in wild-type embryos (Fig. 5I). These include, from anterior to posterior, the trigeminal ganglia, octaval/statoacoustic ganglia, medial lateral line ganglia and the posteriolateral line ganglia. In ring1b mutants, neuroD expression was clearly reduced and expression in the presumptive medial lateral line ganglia was too weak to be detected (Fig. 5J). However, prolonged staining revealed neuroD expression in this domain, showing that the medial lateral line ganglia were formed in ring1b mutants, but that neuroD was very weakly expressed (data not shown). At 56 hpf, neuroD was expressed in several tissues, which obscured the identification of ganglia in the depicted figures (Fig. 5K,L). Nevertheless, ring1b mutants expressed neuroD in the octaval/statoacoustic ganglia (gVIII) as well as in a single domain in which the vagal nerve (gX) and the posteriolateral line ganglia (gP) are located, which together showed that cranial ganglia development is affected, but not abrogated.

Finally, we morphologically inspected chromatophore development. Three NC-derived chromatophores are produced in zebrafish: melanophores (black), iridophores (iridescent) and xanthophores (yellow). While melanophores are found in wide variety of vertebrates, iridophores and xanthophores are not (Curran et al., 2009).

At 32 hpf, melanophore pigmentation in ring1b mutants was indistinguishable from wild-type siblings. Melanophores had migrated ventrally over the yolk and yolk extension (Fig. 5M,N) and size and shape

---

**Figure 6. Loss of endochondral and dermal ossification in ring1b mutants.**

Ventral (A-D), dorsal (E,F) and lateral (A’-F’) views of whole-mount in situ hybridizations with riboprobes against the indicated genes in wild-type and ring1b mutants. In wild-type embryos, runx2a and runx2b are expressed in both hypertrophic pharyngeal arch-derived chondrocytes as well as in the dermal ossification centers of the operculum, parasphenoid and cleithrum. Weak expression is detected in pharyngeal arches and the parasphenoid of ring1b mutants (B,B’,D,D’). At 72 hpf, col10a1 is expressed in developing dermal bones in wild-type embryos, but not in ring1b mutants (E-F’). cl: cleithrum; de: dentary; h: hyoid; m: mandibular; mx: maxilla; pa: pharyngeal arches; pq: palatoquadrate; ps: parasphenoid, op: operculum cl: the cleithrum. Numbers indicate the respective pharyngeal arches.
The Polycomb group protein Ring1b is critically involved in cranial skeleton development appeared normal (data not shown). During later stages of development, melanophores in wild-type embryos matured further and changed their morphology accordingly; from spindly to a more compacted shape (Fig. 5S). In contrast, ring1b melanophores remained spindly (Fig. 5T). The significance of this phenotypical difference remains arguable, since melanophores in 3 dpf wild-type embryos from a different genetic background have been shown to display a very similar morphology to those in 60 hpf ring1b mutants (Kelsh et al., 1996).

Iridophores terminally differentiate at around 42 hpf and initially populate the dorsal and ventral stripe (Curran et al., 2010). At 60 hpf, iridophores were present in both wild-type and ring1b mutants, albeit the number of iridophores was slightly reduced in the mutants (Fig 5Q,R,U,V). Yellow xanthophores also emerge at around 42 hpf (Odenthal et al., 1996). Individual xanthophores are difficult to distinguish. However, qualitative observations can be made by staining for methylene blue, which is specifically taken up by xanthophores (Le Guyader and Jesuthasan, 2002). Methylene blue staining showed that xanthophores were present in the tail of both wild-type and ring1b mutants (Fig. 5V).

Runx2 was shown to be a key regulator of osteoblast differentiation (Komori et al., 1997; Nakashima et al., 2002; Otto et al., 1997). In addition, Runx2 is also critically involved in the maturation step from immature chondrocytes to hypertrophic chondrocytes during the process of endochondral ossification. Of note, runx2 expression is never detected in permanent cartilage (Inada et al., 1999; Kim et al., 1999).

We analyzed the expression pattern of the two runx2 genes that are present in zebrafish. In wild-type embryos, runx2a and runx2b were expressed in both hypertrophic chondrocytes and dermal ossification centers. Runx2a was expressed in the cleithrum, dentary, maxilla, operculum, pharyngeal arches and parasphenoid whereas runx2b expression was detected in differentiating osteoblasts of the branchiostegal ray, cleithrum, operculum, palatoquadrate, parasphenoid and pharyngeal arches (Fig. 6A,A’,C,C’). Because a cartilaginous template is needed for endochondral ossification, we expected that this process was impaired in ring1b mutants. Expression of runx2a and runx2b was severely reduced or absent in presumptive cartilaginous elements of the viscerocranium in 68 hpf ring1b mutants (Fig. 6B,B’,D,D’). Thus, endochondral ossification is indeed abrogated.

Collectively, these results show that migration and differentiation of non-ectomesenchymal NC derivatives is modestly affected by loss of Ring1b. Glia, neurons and the three chromatophore lineages are formed in ring1b mutants, although further morphogenesis and maintenance of gene expression is somewhat impaired at later stages of development. Thus, the arrest in cartilage formation appears to be a rather specific developmental defect in ring1b CNC cells.

**Ring1b is required for ossification**

Because of the severe defects in cartilage development, we next addressed to which extent ossification is affected by Ring1b loss. *Col10a1* expression is specific to developing dermal bones at 72 hpf. Indeed, strong staining was observed in the cleithrum, operculum and parasphenoid of
wild-type embryos (Fig. 6E,E’). Strikingly, col10a1 expression was completely absent in ring1b mutants (Fig. 6F,F’). Thus, these results show that also dermal ossification is severely impaired in ring1b mutants.

Discussion

Transcriptional regulation of cartilage differentiation

Chondrogenesis involves a cascade of events that requires the concerted action of an intricate gene regulatory network. Factors controlling this process include signaling molecules (including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), hedgehog, transforming growth factor beta (TGF-β) and WNT), transcription factors (including basic helix-loop-helix (bHLH), forhead, HOX, NKF, PAX and SOX) and cell adhesion molecules (N-cadherin and NCAM). Despite the obvious complexity of this network, a single transcription factor, Sox9, is regarded as the master regulator of early cartilage development (Akiyama, 2008). Sox9 is expressed before the onset of pre-chondrogenic condensations and is essential for both the initiation of pre-chondrogenic condensations as well as for the early stages of chondrocyte differentiation (Mori-Akiyama et al., 2003; Yan et al., 2002). Mechanistically, Sox9 directly promotes expression of various chondrocyte-specific genes including genes encoding the extracellular matrix proteins Col2a1, Col11a2 and Aggrecan (Bell et al., 1997; Bridgewater et al., 1998; Lefebvre et al., 1997; Ng et al., 1997; Oh et al., 2010). Among these targets, the binding of Sox9 to the promoter of Col2a1 is most intensively studied. At the Col2a1 promoter, Sox9 is found in a transcriptional complex that can include CBP/p300, PGC-1α, Smad2/3, Sox5 and Sox6 (Furumatsu et al., 2005; Kawakami et al., 2005). Moreover, Sox9 also interacts with the thyroid hormone receptor-associated protein complex (Trp230/Med12), which is a co-activator that acts as a bridge to RNA polymerase II (Rau et al., 2006; Zhou et al., 2002).

At later stages of cartilage development, chondrocytes either differentiate into permanent cartilage or, alternatively, differentiate into hypertrophic chondrocytes, which are ultimately replaced by osteoblasts in the process of endochondral ossification. The core binding transcription factors (CBFs) play a central role in both processes by promoting chondrocyte hypertrophy and inhibiting differentiation into permanent cartilage. CBFs are heterodimeric transcription factors composed of distinct CBFα subunits and a common CBFβ subunit. The CBFα subunit, which is also referred to as Runt-related transcription factor (Runx), contains a DNA-binding motif, whereas the CBFβ subunit enhances the binding of the CBFα subunit to DNA (Ogawa et al., 1993). Three CBFα subunits have been described, Runx1-3. In mice, the absence of both Runx2 and Runx3 abrogates chondrocyte hypertrophy and thus endochondral ossification does not initiate (Yoshida et al., 2004). Knockdown experiments in zebrafish also indicate that runx2b and runx3 are critically involved in chondrogenesis: runx2b morphants lack the entire pharyngeal skeleton whereas only rudimentary trabeculae are formed in runx3 morphants (Flores et al., 2006). To date, a comprehensive understanding of which Runx target genes are critically involved in cartilage development remains elusive, although individual target genes have been identified, including Col10a1, Indian hedgehog and Tcf7 (Li et al., 2011; Mikasa et al., 2011; Yoshida et al., 2004). Thus, although recent studies have provided insight in the molecular mechanisms underlying chondrogenesis, much remains unknown. For instance, the role of (epigenetic) chromatin modifications, such as chromatin
remodeling, DNA (de)methylation and histone modifications has largely been overlooked. Our results illustrate the critical role of these proteins in cartilage development.

**Zebrafish as model for the study of chondrogenesis**

The zebrafish has provided a great platform for the identification of genes involved in cartilage development mainly through the numerous zebrafish mutants with affected cartilage development that were retrieved from genetic screens (Nissen et al., 2006; Piotrowski et al., 1996; Rau et al., 2006; Schilling et al., 1996a). However, surprisingly few mutants were identified with such a severe phenotype as ring1b mutants. Among the mutants in which cartilage development is totally abrogated is, not surprisingly, the sox9a/sox9b double mutant (Yan et al., 2005). In addition, three zebrafish mutants have been identified in which a transcriptional co-activator for Sox9, Trap230/Med12, is disrupted (Hong et al., 2005; Rau et al., 2006). These mutants also show a complete absence of cartilage.

Another note-worthy mutant is the lim-absent (lia) mutant, in which fgf3 is mutated (Kiefer et al., 1996). Although only posterior arch formation is abrogated in fgf3 mutants (Herzog et al., 2004), more potent inhibition of Fgf signaling, either chemically or by morholino-mediated knockdown of both Fgf3 and Fgf8, causes an almost complete loss of cartilage (David et al., 2002; Walshe and Mason, 2003). Together, these studies revealed that the endoderm is involved in patterning CNC cells through the action of both Fgf3 and Fgf8.

In addition to those, to our knowledge, only one other mutant with a near complete absence of cartilage has been described: the chinless (chn) mutant (Schilling et al., 1996b). The causative mutation has not been identified, but the phenotypical similarity with ring1b mutants is striking.
leads to a marked reduction in \textit{sox9a/b} expression, the underlying mechanism remains elusive.

One mechanism by which Ring1b possibly controls cartilage development can be deduced from recent data obtained by the group of Alan Cantor (Yu et al., 2012). In this paper, it was shown that PRC1 binds directly to the Runx1/CTFβ dimer in lymphocytes. Loss of Runx1 caused a depletion of Ring1b at sites that are occupied by both Ring1b and Runx1/CTFβ in wild-type mice, which led the authors to conclude that the Runx1/CTFβ dimer is involved in the recruitment of PRC1 to commonly bound sites. Perhaps surprisingly, knockdown of either Ring1b or CTFβ caused a co-directional change in gene expression in 88% of the genes that were commonly bound by CTFβ, Runx1 and Ring1b. Thus, Ring1b in this setting functions not merely as gene repressor, but is rather required for a specific cellular program through both gene repression and activation.

A very similar mechanism can be envisaged for the recruitment and function of Ring1b during chondrogenesis given the critical roles of Runx2a/b in this developmental process.

Indeed, various recent studies showed that vertebrate transcription factors are capable to recruit PcG complexes, suggesting that transcription factors might be more generally involved in PcG protein recruitment than previously appreciated. Although our study did not address the mechanism underlying the cartilage defect in \textit{ring1b} mutants, we do favor such a mechanism. Therefore, future studies will aim for the identification of transcription factors that interact and recruit Ring1b to critical regulators of cartilage development.

\textbf{References}


The Polycomb group protein Ring1b is critically involved in cranial skeleton development


The Polycomb group protein Ring1b is critically involved in cranial skeleton development.


Book II: Functional characterization of Ring1b in zebrafish