The multidomain protein Brpf1 binds histones and is required for Hox gene expression and segmental identity

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INTRODUCTION
Anterior-posterior patterning of the vertebrate head, including segmental identity determination of the seven pharyngeal arches, is governed by differential Hox gene expression (reviewed by Santagati and Rijli, 2003). Chondrocytes of the arches derive from cranial neural crest (CNC) cells of the midbrain and hindbrain region (Le Douarin, 1982), CNC forming the first pharyngeal arch (mandibular) is devoid of Hox gene expression, whereas cells contributing to the second (hyoid) and posterior (branchial, gill) arches display a nested pattern of Hox gene expression (the ‘Hox code’) (Hunt et al., 1991) (reviewed by Santagati and Rijli, 2003). The most anteriorly expressed Hox genes, Hoxa2 and hoxa2b and hoxb2a in zebrafish, determine second arch identity. Ectopic expression of hox2 in the first arch causes it to acquire second arch identity, resulting in two hyoids (Grammatopoulos et al., 2000; Hunter and Prince, 2002; Pasqualetti et al., 2000). Conversely, loss of hox2 results in an anterior homeotic transformation of the second arch to first arch identity and a bimandibular phenotype (Gendron-Maguire et al., 1993; Hunter and Prince, 2002; Rijli et al., 1993). Interestingly, tissue-specific deletion of mouse Hoxa2 in post-migratory neural crest cells reproduces the conventional knockout phenotype, demonstrating the requirement for maintained Hox expression in CNC (Santagati et al., 2005).

Maintenance of Hox gene expression is regulated by the antagonistic function of Polycomb group (PcG) and Trithorax group (TrxG) proteins. Many PcG and TrxG factors were identified in Drosophila by mutations that produce or suppress specific homeotic phenotypes in segment identity. They have been fairly well conserved throughout evolution. Most of them act in large complexes and modify the local properties of chromatin to maintain transcriptional repression (PcG) or activation (TrxG) of their target genes through the cell cycle, thereby accounting for epigenetic transcriptional memory (reviewed by Ringrose and Paro, 2004; Ringrose and Paro, 2007; Simon and Tamkun, 2002). Biochemically, the roles of the different TrxG proteins are diverse. Some members bind to particular cis-regulatory DNA sequences in their target genes [e.g. Polycomb/Trithorax response elements (PRE/TREs) in Drosophila], whereas others are involved in histone binding or enzymatic histone modification. Prominent examples are Trithorax itself and its mammalian orthologs, the Mll (Mixed-lineage leukemia) proteins, which are histone H3 lysine 4 (K4H3) methyltransferases (reviewed by Popovic and Zeleznik-Le, 2005). In Mll-deficient mouse embryos, Hox gene expression is not properly maintained, leading to anterior homeotic transformations of segmental identities and defects during hematopoiesis (Yagi et al., 1998; Yu et al., 1998; Yu et al., 1995). More recently, based on genetic analysis in zebrafish, a TrxG-like function required to maintain cranial Hox gene expression was assigned to Moz (Monocytic leukemia zinc-finger protein; Myst3 – ZFIN) (Miller et al., 2004), a histone acetyltransferase (HAT) of the MYST family, which in mouse is also required for maintenance of hematopoietic stem cells (Katsumoto et al., 2006; Thomas et al., 2006).

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The Trithorax group (TrxG) is composed of diverse, evolutionary conserved proteins that form chromatin-associated complexes accounting for epigenetic transcriptional memory. However, the molecular mechanisms by which particular loci are marked for reactivation after mitosis are only partially understood. Here, based on genetic analyses in zebrafish, we identify the multidomain protein Brpf1 as a novel TrxG member with a central role during development. brpf1 mutants display anterior transformations of pharyngeal arches due to progressive loss of anterior Hox gene expression. Brpf1 functions in association with the histone acetyltransferase Moz (Myst3), an interaction mediated by the N-terminal domain of Brpf1, and promotes histone acetylation in vivo. Brpf1 recruits Moz to distinct sites of active chromatin and remains at chromosomes during mitosis, mediated by direct histone binding of its bromodomain, which has a preference for acetylated histones, and its PWWP domain, which binds histones independently of their acetylation status. This is the first demonstration of histone binding for PWWP domains. Mutant analyses further show that the PWWP domain is absolutely essential for Brpf1 function in vivo. We conclude that Brpf1, coordinated by its particular set of domains, acts by multiple mechanisms to mediate Moz-dependent histone acetylation and to mark Hox genes for maintained expression throughout vertebrate development.

KEY WORDS: Brpf1, Bromodomain, PWWP domain, Moz, Hox gene expression, Craniofacial development, Cranial neural crest, Pharyngeal arch, Anterior-posterior patterning, Homeotic transformation, Zebrafish

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Here, based on the positional cloning of bimandibular zebrafish mutants, we identify the multidomain protein Brpf1 (Bromodomain and PHD finger containing 1; also known as Br140 and Peregrin) as a TrxG member and close partner of Moz. Brpf1 contains a unique combination of domains typically found in chromatin-associated factors, including PHD fingers, a bromodomain and a PWWP domain. Bromodomains interact with acetylated lysines on N-terminal tails of histones and other proteins (reviewed by Yang, 2004), and PHD fingers were recently shown to bind to methylated K4H3 (Shi et al., 2006; Wysocka et al., 2006), whereas the histone-binding properties of PWWP domains remain to be shown. Based on its domains, Brpf1 has been proposed to be involved in chromatin remodeling (Thompson et al., 1994). However, its exact function in vertebrates is currently unknown.

In this study, we show that during zebrafish development, Brpf1 is required for histone acetylation, maintenance of cranial Hox gene expression and proper determination of pharyngeal segmental identities. We demonstrate genetic and physical interaction of Brpf1 with the HAT Moz. This interaction can explain how Brpf1 promotes histone acetylation. Furthermore, in contrast to Moz, Brpf1 remains associated with the chromatin even during metaphase, contributing to transcriptional memory throughout mitosis. We further show that the previously largely unappreciated PWWP domain is essential for histone binding and chromatin association of Brpf1 in interphase and mitosis, as well as for Brpf1 function in vivo. Together, these data identify Brpf1 as a novel TrxG protein with essential roles in epigenetic memory during vertebrate development.

**MATERIALS AND METHODS**

**Zebrafish lines and genotyping**

brpf1[t20002] and brpf1[t25114] alleles were obtained during the Tübingen 2000 screen at the Max-Planck Institute of Developmental Biology, brpf1[t9043] during a screen at the University of Oregon (Eugene, OR) (Miller et al., 2004). Unless stated otherwise, the t20002 allele was used for phenotypic analyses. brpf1[t20002] and brpf1[t9043] larvae were genotyped taking advantage of restriction fragment length polymorphisms. The brpf1[t20002] mutation creates a Ddel restriction site, whereas brpf1[t9043] creates a Tsp45I site.

**Genetic mapping and cloning of brpf1**

For genetic mapping, carriers of the brpf1[t20002] mutation were crossed to the Tübingen 2000 screen at the Max-Planck Institute of Developmental Biology, brpf1[t9043] during a screen at the University of Oregon (Eugene, OR) (Miller et al., 2004). Unless stated otherwise, the t20002 allele was used for phenotypic analyses. brpf1[t20002] and brpf1[t9043] larvae were genotyped taking advantage of restriction fragment length polymorphisms. The brpf1[t20002] mutation creates a Ddel restriction site, whereas brpf1[t9043] creates a Tsp45I site.

**RESULTS**

**Zebrafish brpf1 mutants display anterior shifts in segmental identities of pharyngeal arches 2-6**

Zebrafish forward genetic screens after ENU mutagenesis and cartilage staining at 120 hours post-fertilization (hpf) yielded three non-complementing mutants (t20002, t25114, b943) with shifts in...
segmental identities of craniofacial arches, but otherwise normal morphology (Fig. 1A,B). In wild-type larvae at 120 hpf (Fig. 1C), the ventral part of the first pharyngeal arch (Meckel’s cartilage of mandibular) is characterized by the absence of a basal element, whereas such basal elements are present in arch 2 (basihyal of hyoid) and pharyngeal arches 3–7 (basibranchial of gill arches). In mutants, the basihyal was absent (Fig. 1D). In addition, both ventral and dorsal elements of arch 2 (ceratohyal and hyosymplectic) had shapes more similar to the corresponding elements of arch 1 (Meckel’s cartilage and palatoquadrate) (Fig. 1C,D,J,L). An anterior transformation was also apparent with the molecular marker bapx1 (nkr3.2–ZFHN), which in wild-type animals is exclusively expressed in joint cells between the ventral and dorsal element of arch 1 (Fig. 1M) (Miller et al., 2003), whereas mutants displayed ectopic bapx1 expression in arch 2 (Fig. 1N). Corresponding anterior shifts also occurred for arch 2-associated muscles (Fig. 1O,P) and dermal bones, which were absent or strongly reduced in mutants (Fig. 1J,K,Q,R). Similarly, mutants lacked the specific ossification in both the dorsal and the ventral element of arch 2 (Fig. 1Q–T), leaving their central regions unossified as in arch 1. Furthermore, mutants displayed anterior transformations of pharyngeal arches 3–6 (gill arches), which acquired shapes and ossification patterns similar to arch 2 of wild-type animals (Fig. 1F–I,S,T).

Using meiotic segregation analysis, we mapped the t20002 mutation within a 0.1 cm interval of chromosome 8 (Fig. 1U). Subsequent chromosomal walking led to the identification of a genomic fragment that contained sequences with high similarity to the mammalian bromodomain and PHD finger-containing 1 (brpf1) gene (see Materials and methods). The predicted protein of the full-length cDNA (3777 bp; GenBank accession number EU486162) is 71.4 and 71.1% identical with human and mouse Brpf1, respectively. Similarity is even higher within the conserved domains (C2H2 zinc finger, PHD, bromo and PWWP; see Fig. 1V), indicating that it is a true zebrafish Brpf1 ortholog. We identified molecular lesions in the brpf1 gene of all three mutant alleles (Fig. 1V). brpf1t20002 contains a C135A transversion (see Fig. S1A in the supplementary material), introducing a TAA stop codon that leads to premature termination of the protein within the N-terminal C2H2 zinc finger. In the brpf1t2043 allele, a G1044A transition introduces a TGA stop codon, resulting in a truncation of the protein within the PHD-finger domain. Finally, in brpf1t25114, a T→A transversion creates a new intronic splice acceptor site 10 bp from the regular site at nucleotide position 2463 (see Fig. S1B in the supplementary material). This new splice site is preferentially used. Thus, 50/50 independent cDNA clones contained the corresponding 10 bp insertion that results in a frame shift and premature termination of the protein directly upstream of the C-terminal PWWP domain.

Strikingly, comparative analyses revealed that the phenotype of t25114 mutants with the loss of the PWWP domain only, was at least as strong as that of the severely truncated and putative brpf1-null allele t20002 (Table 1; see Fig. S1F–I in the supplementary material). Whole-mount in situ hybridizations (see Fig. S1C in the supplementary material) and semi-quantitative RT-PCR (see Fig. S1D in the supplementary material) further showed that the t25114 mutation did not affect brpf1 transcript stability, and t25114-truncated GFP-Brpf1 fusion protein was as stable as the full-length version (see Fig. S1E in the supplementary material). Together, this points to a pivotal role of the PWWP domain for proper Brpf1 function in vivo.

To provide further evidence for the causative nature of the identified brpf1 mutations, we knocked down zygotic Brpf1 in wild-type embryos by injection of antisense morpholino oligonucleotides (MOs) targeting the splice donor site between exon 1 and intron 1 (see Fig. S1J in the supplementary material). Morphant embryos displayed a complete loss of wild-type Brpf1 transcripts, as determined by semi-quantitative RT-PCR (see Fig. S1K in the supplementary material) and northern blotting (see Fig. S1L in the supplementary material), and displayed craniofacial defects indistinguishable from those of the mutants (Fig. 1D,E). Finally, zebrafish brpf1 mutants injected with mouse Brpf1 mRNA displayed a partial, but significant rescue of anterior Hox gene expression at 33 hpf (Fig. 2, compare I-L with E-H).

Table 1. Quantification of skeletal alterations of the three brpf1 alleles and of brpf1 morphants

<table>
<thead>
<tr>
<th>Skeletal alteration</th>
<th>Percentage of animals with each phenotypic trait</th>
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<tbody>
<tr>
<td></td>
<td>t20002</td>
</tr>
<tr>
<td><strong>Arch 2 (hyoid), ventral elements</strong></td>
<td></td>
</tr>
<tr>
<td>Basihyal absent</td>
<td>91</td>
</tr>
<tr>
<td>Basihyal reduced</td>
<td>9</td>
</tr>
<tr>
<td>Ceratohyal inversion</td>
<td>3</td>
</tr>
<tr>
<td>Ceratohyal shape change</td>
<td>44</td>
</tr>
<tr>
<td>Shorter</td>
<td>44</td>
</tr>
<tr>
<td>Broader</td>
<td>44</td>
</tr>
<tr>
<td>Distal ends bifurcated</td>
<td>35</td>
</tr>
<tr>
<td>Fusion ceratohyal–Meckel’s cartilage</td>
<td>0</td>
</tr>
<tr>
<td><strong>Arch 2 (hyoid), dorsal elements</strong></td>
<td></td>
</tr>
<tr>
<td>Hyosymplectic shape change</td>
<td>32</td>
</tr>
<tr>
<td>Sympatheic extension shortened</td>
<td>32</td>
</tr>
<tr>
<td>Hyomandibular reduction (dorsal of foramen)</td>
<td>26</td>
</tr>
<tr>
<td>Fusion hyosymplectic-palatoquadrate</td>
<td>6</td>
</tr>
<tr>
<td><strong>Arches 3–6</strong></td>
<td></td>
</tr>
<tr>
<td>Hyobranchials absent</td>
<td>100</td>
</tr>
<tr>
<td>Ceratobranchials distally broadened</td>
<td>55</td>
</tr>
<tr>
<td><strong>Total number of animals examined</strong></td>
<td>34 (100%)</td>
</tr>
</tbody>
</table>

Skeletal alterations were assessed by Alcian Blue staining at 120 hpf (compare with Fig. S1F–H in the supplementary material). If anything, the t25114 and b943 alleles, which encode less severely truncated Brpf1 proteins, display slightly stronger, rather than weaker, phenotypes than the potential null allele t20002. For details, see text and Fig. 1. These subtle differences in phenotypic strengths are most likely due to differences in the genetic backgrounds.
Altogether, the data indicate that Brpf1 is absolutely essential for segmental pharyngeal identity, and that the functionality of Brpf1 has been conserved between zebrafish and mouse.

**brpf1 is required for maintenance of cranial Hox gene expression**
Anterior-posterior patterning of the vertebrate head, including the determination of pharyngeal arch identity, is governed by differential Hox gene expression (Santagati and Rijli, 2003). Therefore, we studied whether Brpf1 might act upstream of Hox genes. CNC cells contributing to arch 1 cartilage lack Hox gene expression, whereas cells contributing to arch 2 display expression of hoxa2b and hoxb2a, and cells contributing to arches 3-7 display expression of hoxa2b and hoxb3a (Fig. 2B-D) (Hunter and Prince, 2002). In brpf1 mutants, expression of all Hox genes in hindbrain and CNC was initiated normally during segmentation stages (see Fig. S2 in the supplementary material). However, at 26 hpf (data not shown) and 35 hpf, CNC expression of hoxa2b and hoxb2a was completely absent in mutant embryos (Fig. 2F,G). hoxb3a transcripts were only lost in the anterior, being present at reduced levels in the posterior CNC (Fig.

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Fig. 1. See next page for legend.
mutant Brpf1 proteins, with the C2H2, PHD finger, bromo and PWWP (brpf1 and physical map of the causative of the later pharyngeal segmental defects of To address whether the reduction of Hox gene expression is by forced expression of brpf1 mutant phenotype is partially rescued is largely normal (see Fig. S4A-D in the supplementary material). This indicates that partial loss of Brpf1 activity synergistically enhances the effects caused by partial loss of Moz activity and vice versa. By contrast, brpf1 mutants injected with the highest amounts

brpf1 is expressed in different craniofacial cell types and promotes Hox gene expression in a cell-autonomous fashion

Anterior Hox genes are expressed in hindbrain, CNC and pharyngeal endoderm and ectoderm (Crump et al., 2006). brpf1 displayed transient expression in all of these cell types. During blastula, gastrula and early segmentation stages, it was uniformly expressed throughout the entire embryo (see Fig. S3A-C in the supplementary material; 0-11 hpf), whereas during mid-segmentation stages, brpf1 expression was largely confined to the anterior half of the embryo (see Fig. S3D in the supplementary material; 17 hpf). At 26 hpf, strong expression was observed in brain, eyes, post-migratory CNC and pharyngeal endoderm (Fig. 3A-D). However, at 55 hpf, brpf1 expression in specifying chondrocytes and endodermal pouches of the pharyngeal arches had largely ceased, while expression was maintained in pharyngeal and oral ectoderm (Fig. 3E-I). Strong and persistent brpf1 expression could also be detected in brain and retina, and in the gastrointestinal tract, including liver and pancreas (see Fig. S3E-K in the supplementary material).

Studies with chimeric embryos showed that Brpf1 regulates Hox expression both in CNC (hoxa2b; Fig. 3J-O) and hindbrain (hoxb1a; see Fig. S4E-H in the supplementary material) in a cell-autonomous manner, indicating that the effect is direct and is not mediated via secreted posteriorizing signals. Chimeric studies with transplanted cells exclusively in the endoderm further revealed that Brpf1 expression in the pharyngeal endoderm is neither necessary nor sufficient for segmental identity of pharyngeal arches (see Fig. S5 in the supplementary material), pointing to a direct effect in the CNC.

Brpf1 genetically interacts with the HAT Moz, and the brpf1 mutant phenotype is rescued by HDAC inactivation

A pharyngeal segmental identity phenotype very similar to that of brpf1 mutants has been reported for zebrafish mutants in Moz, a transcriptional coactivator and HAT of the MYST family (Crump et al., 2006; Miller et al., 2004). Upon co-injection of sub-optimal amounts of brpf1 and moz MOs, which upon single injections did not produce any apparent phenotype, we obtained reduced hox2 gene expression (Fig. 4A-D, n=25/25; see Fig. S6A-D in the supplementary material, n=15/16) and pharyngeal identity defects as severe as in the strongest brpf1 or moz morphants (Fig. 4E-H, n=15/15; see Fig. S6E-H in the supplementary material, n=12/12). This indicates that partial loss of Brpf1 activity synergistically enhances the effects caused by partial loss of Moz activity and vice versa. By contrast, brpf1 mutants injected with the highest amounts

The brpf1 mutant phenotype is partially rescued by forced expression of hox2 genes

To address whether the reduction of Hox gene expression is causative of the later pharyngeal segmental defects of brpf1 mutants, we tried to rescue the bimandibular phenotype by reintroducing hox2 transcripts. Ectopic hox2 gene expression during early segmentation stages can be obtained by injecting hoxb1a mRNA at the 1-cell stage (Hunter and Prince, 2002). hoxb1a RNA-injected wild-type embryos displayed a transformation of first to second arch identity, characterized by the loss of bapx1 expression (Fig. 2O). The same effect was observed upon injection into brpf1 mutants (Fig. 2, compare left side of P with N), indicating a conversion of the bimandibular to a bihidyloid phenotype. Alternatively, injected mutants showed a particular reduction of ectopic bapx1 expression in the second arch, resembling the wild-type situation (Fig. 2, compare right side of P with M). We conclude that Brpf1 regulates segmental identity of pharyngeal arches via its positive effect on Hox gene expression.

2H), whereas expression of the more-posterior Hox genes (hoxb6b, b7a, b8a, b9a) was unaffected (data not shown). However, CNC cells displayed normal migration patterns, as revealed by dlx2a in situ hybridizations (Akimenko et al., 1994) (data not shown). We conclude that Brpf1 is specifically required for the maintenance, but not for the initiation, of anterior Hox gene expression. Interestingly, in the hindbrain, effects of brpf1 mutations on hox2 and hox3 expression maintenance (Fig. 2G,H and see Fig. S2F in the supplementary material) were less severe than in the CNC. This suggests that Brpf1 function in the hindbrain is less crucial than that in the CNC, consistent with our findings that hindbrain patterning in brpf1 mutants is largely normal (see Fig. S4A-D in the supplementary material).
Moz showed a phenotype no more severe than that of moz single morphants (see Fig. S6I,J in the supplementary material, n=23/23). Moz is a HAT, suggesting that the segmental defects of brpf1 mutants might be due to histone hypoacetylation. To test this, we treated brpf1 mutants with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). Treatment of mutants from 20-33 hpf rescued hoxa2b expression in CNC (Fig. 4I-L, n=9/9) and hoxb1a expression in the hindbrain (see Fig. S6K-N in the supplementary material, n=13/13) to wild-type levels, and led to a significant alleviation of skeletal defects at 120 hpf (see Fig. S6O-R in the supplementary material). Together, this suggests that Brpf1 and Moz tightly cooperate to determine pharyngeal segmental identities by promoting histone acetylation and maintenance of anterior Hox gene expression.

**Fig. 2. Brpf1 regulates segmental identity by maintaining anterior Hox gene expression.** Whole-mount in situ hybridizations with the probes indicated bottom left at the stages indicated bottom right; genotypes and treatment of zebrafish embryos as indicated in upper right corners. (A-L) Lateral views; (M-P) ventral views. (A-D) Hox gene expression in wild type (WT). Hox-expressing hindbrain rhombomeres (r) and arch-forming cranial neural crest (CNC) (2-7) are indicated. (E-H) Absent or reduced Hox gene expression in brpf1 mutants (−/−). Arrow in H indicates the remaining hoxb3a expression in the posterior CNC. (I-L) Partially rescued Hox gene expression in the hindbrain (l, arrow) and the CNC (J-L, arrows) of brpf1 mutants injected with mouse Brpf1 mRNA. (M-P) The bimandibular phenotype of the brpf1 mutant (N) can be overcome by injection of hoxa1a mRNA. (O) hoxa1a-injected wild-type embryo lacking bapx1 expression, indicative for bihyoid phenotype [compare with Hunter and Prince (Hunter and Prince, 2002)]. (P) hoxa1a-injected brpf1 mutant with bihyoid pattern on left side and wild-type pattern on right side. Arch numbers are indicated.

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**Fig. 3. Expression pattern and cell-autonomous function of brpf1 in zebrafish CNC.** Staining with reagents indicated at lower right at the stages indicated upper right. Numbers of pharyngeal arches are indicated (1-7). (A-J) Wild-type embryos; (K) brpf1b943 mutant (−/−); (L-O) mutant transplanted with wild-type cells (WT → −/−). (A) Dorsal view; (B-J, O) lateral views; (F-H) horizontal section; (I) longitudinal section. (A-D) At 26 hpf, brpf1 is co-expressed with the CNC marker dlx2a (A) and with fli1a (D), stained by anti-GFP immunostaining of tg(fli1a:EGFP) embryo (Isogai et al., 2003). brpf1-positive cells between CNC include pharyngeal endoderm [D; compare with Fig. 1A in Crump et al. (Crump et al., 2004)]. (E-I) At 55 hpf, sox9a-positive chondrocytes of cartilage condensates (cc; H) (Yan et al., 2002) and pax9a-positive pharyngeal endodermal cells (pe; G) (Nornes et al., 1996; Okabe and Graham, 2004) lack brpf1 expression, which, however, is strongly expressed in p63 (tp63 – ZFIN)-positive cells (Carney et al., 2007) of the pharyngeal ectoderm (pec; F-H), in the oral ectoderm (pe; I) and in facial ectoderm ventral to arches 1 and 2 (vfe; I) (Crump et al., 2006). (J-O) Analysis of chimeric embryos with rhodamine-dextran (RD)-labeled (N) and tg(fli1a:EGFP)-positive (M) wild-type cells integrated in the CNC of a brpf1 mutant host [for procedure, see Crump et al. (Crump et al., 2006)]. Only wild-type, not adjacent mutant CNC, cells display hoxa2b expression (arrows in L and M; n=3/3). g, gut; op, opercle.
**Brpf1 co-localizes and physically interacts with Moz**

To examine and compare the subcellular localization of Brpf1 and Moz proteins, we transfected HEK 293 cells with expression constructs for GFP- or HA-tagged full-length Brpf1 and FLAG-tagged Moz. Immunofluorescence analyses revealed that Brpf1 and Moz co-localized in a specific punctate pattern in interphase nuclei (Fig. 5A). These domains most likely represent active chromatin, as indicated by co-localization with active histone marks (H2AK5Ac, H3K4me1, H3K4me3) and exclusion from regions with inactive marks (H3K9me3), both in interphase (data not shown) and during mitosis (Fig. 6I-K).

Reciprocal co-immunoprecipitations (Co-IP) with protein extracts of transfected HEK 293 cells further revealed that Brpf1 and Moz undergo (direct or indirect) physical association (Fig. 5G, upper panels). Importantly, the Brpf1-associated HAT activity towards histones H3 and H2A was similar to the specificity of immunoprecipitated Moz (Fig. 5G, bottom panels, lanes 3 and 7). A Moz-G657E mutant lacking HAT activity (Collins et al., 2006) was still able to associate with Brpf1, whereas only very little Brpf1-associated HAT activity could be detected (Fig. 5G, bottom panels, lanes 4 and 8). This indicates that Moz is the major HAT associated with Brpf1 and that its HAT activity is not required for the interaction with Brpf1. Confirming this, neither the co-localization with Brpf1 nor chromatin association required the HAT activity of Moz (Fig. 5B).

To map the Moz-interaction site of Brpf1, we carried out Co-IPs and co-localization experiments with a series of deletion or mutant constructs (Fig. 5F). These revealed that the N-terminal 245 aa containing the C2H2 zinc finger are necessary and sufficient for co-localization (Fig. 5C,D) and physical interaction with Moz (Fig. 5H, lanes 3, 6). The C2H2 zinc finger does not mediate this interaction, as mutant versions of it still interacted with Moz (Fig. 5I, lanes 3, 6), whereas an N-terminal 149 aa fragment with the intact zinc finger did not (Fig. 5I, lane 4), narrowing the interaction domain to a region between aa 150 and 245. Interestingly, the N-terminal 245 aa fragment of Brpf1, although sufficient for Moz co-localization, lost the typical punctate distribution in interphase nuclei (Fig. 5D). This suggests that the more C-terminal domains of Brpf1 are essential for the characteristic association of the complex with chromatin.

The PWPP domain is necessary for chromatin association of Brpf1

Bromodomains are known to mediate binding to acetylated histones (Yang, 2004), and PHD fingers to methylated histone residues (Pena et al., 2006; Wysocka et al., 2006). Brpf1 contains a PHD finger, a bromodomain, and a C-terminal PWPP domain, for which histone-binding capacity had not yet been reported. To dissect which domains of Brpf1 mediate chromatin association, we determined the localization of different truncated versions of Brpf1 in cells (Fig. 6I). Full-length Brpf1 localized to distinct sites of condensed chromosomes (Fig. 6A,B). Strikingly, deletion of the C-terminal PWPP domain led to a total exclusion of Brpf1 from condensed chromosomes (Fig. 6, compare C with B). The same exclusion was obtained for a Brpf1 fragment consisting only of the PHD finger and bromodomain (Fig. 6D). By contrast, a fusion of the PWPP domain and bromodomain restored the typical association with mitotic chromosomes (Fig. 6E) and the co-localization with active histone marks (Fig. 6, compare L with I), whereas neither the PWPP domain nor bromodomain was alone sufficient for proper localization (data not shown). PWPP domain-dependent chromatin localization during both interphase and metaphase was also observed for GFP-Brpf1 in zebrafish embryos (see Fig. S7 in the supplementary material). Together, these data indicate that the PWPP domain is absolutely essential and, together with the bromodomain, sufficient for chromatin targeting of Brpf1, whereas the PHD and zinc-finger domains are dispensable. Interestingly, in contrast to interphase (Fig. 5A), Brpf1 and Moz did not co-localize during mitosis, when Moz was largely excluded from chromosomes (Fig. 6F;G; see Discussion).

The PWPP of Brpf1 directly binds histones

To study whether the PWPP- and bromodomain-dependent chromatin association of Brpf1 is mediated by direct binding to histones, we generated recombinant GST fusions of the PHD finger, the bromodomain and the PWPP domain (Fig. 7A), and performed affinity purifications with human core histones. In these assays, the bromodomain bound the four core histones equally, the PWPP domain displayed stronger and preferential binding to H2B and H2A, and the PHD domain no binding at all (Fig. 7B). Furthermore, the PWPP domain bound efficiently to purified calf thymus H2A or H2B, whereas no, or less, binding was observed for the bromodomain (Fig. 7C). This suggests that the PWPP domain can directly bind H2A and H2B, whereas the bromodomain might require H3 and H4 or histone octamers. Furthermore, affinity purifications and subsequent western blot analyses with normal or hyperacetylated histones revealed that the bromodomain binds preferentially to acetylated H2A, whereas no such preference was detected for the PWPP domain (Fig. 7D; compare lanes 3, 4 with 5, 6 and 7, 8), suggesting that the PWPP domain can bind H2 histones independently of their acetylation status.
DISCUSSION
Zebrafish Brpf1 is required for maintenance of Hox gene expression and pharyngeal segmental identity

Despite its identification almost 15 years ago, there had been little information about the role of Brpf1 in vertebrate systems. Here, we have studied both its biological and molecular function, applying a combination of loss-of-function studies in zebrafish with protein localization and biochemical analyses.

Zebrafish mutants lacking zygotic Brpf1 display progressively reduced expression of anterior Hox genes in CNC cells, which in turn causes anterior shifts in segmental identity of pharyngeal arches 2-6. The complexity of the craniofacial phenotype, which is very similar to that of moz mutants (Miller et al., 2004), is most likely due to misregulation of multiple Hox genes. hox2 acts as a selector gene for second arch segmental identity, and mutation of Hoxa2 in mouse or reduction of hoxa2b and hoxb2a function in zebrafish results in a homeotic transformation of second to first arch identity (Gendron-Maguire et al., 1993; Hunter and Prince, 2002; Rijli et al., 1993). In this light, the bimandibular phenotype of brpf1 mutants can be explained by the requirement of Brpf1 for maintained hoxa2b and hoxb2a expression. Similarly, the acquirement of second arch characteristics in arches 3-6 of brpf1 mutants might result from its role in hoxb3a expression, consistent

Fig. 5. Co-localization and physical interaction of Brpf1 and Moz. (A-D) Brpf1 co-localizes with Moz. Immunofluorescent staining of interphase HEK 293 cells after co-transfection with the indicated versions of GFP-Brpf1 (left panels; green) and FLAG-Moz (middle panels; red), counterstained with DAPI (for DNA; blue); merged images are shown in right-hand panels. Full-length Brpf1 co-localizes with wild-type Moz (A) and with HAT-negative Moz-G675E (B) in a punctate pattern on interphase nuclei. Co-localization is abolished when Brpf1 is N-terminally truncated (C). N-terminal fragment of Brpf1 co-localizes with Moz, but displays a more diffuse distribution (D). (E,F) Schematic structures and co-localization/immunoprecipitation properties of full-length Brpf1, full-length Moz (E), and the various truncations used (F). (G-I) Brpf1 physically associates with Moz. (G) Co-IP of full-length Brpf1 and wild-type or HAT-negative Moz-G675E from co-transfected cells with anti-FLAG (Moz) antibody (left) or anti-HA (Brpf1) antibody (right), analyzed in western blots (upper panels) with the specified antibodies, or assayed for HAT activity on core histones (lower panels). (H) Co-IP of full-length FLAG-Moz and various GFP-Brpf1 deletion constructs with anti-FLAG or anti-GFP antibodies, followed by analysis of complex formation (upper panel) and control for Brpf1 expression levels (lower panel) via anti-GFP western blotting. (I) Co-IP of full-length Moz or C-terminally truncated MozN and various HA-tagged versions of the N-terminal fragments of Brpf1 using anti-FLAG antibody, analyzed in anti-HA western blots. Lower panel shows input control. Brpf1 aa 1-245 fragments that have histidine or cysteine mutations in the zinc-finger domain can still co-precipitate with Moz (lanes 5, 6), whereas the aa 1-149 fragment with an intact zinc finger cannot (lane 4). Scale bars: 5 μm in B; 2.5 μm in D.
with data obtained in mouse reporting that loss of Hoxa3 function
leads to disruptions in the formation of third pouch derivatives
(Manley and Capecchi, 1995).

During normal development, **brpf1** is not only expressed in CNC
that forms the pharyngeal arch cartilage, but also in pharyngeal
ectoderm and endoderm. Previous studies have indicated that Hox
gene expression in premigratory CNC cells does not irreversibly
determine their segmental identity. Rather, migrating CNC cells
receive additional instructive information from surrounding tissues
(see Crump et al., 2004a; Piotrowski and Nüsslein-Volhard, 2000;
Trainor and Krumlauf, 2000). However, our grafting experiments
show that Brpf1 function in the endoderm is dispensable for
pharyngeal segmental identity, whereas its effect on Hox gene
expression in the CNC is cell-autonomous. This suggests that for the
determination of arch identity, Brpf1 is exclusively required in the
CNC, as has also recently been shown for its interaction partner Moz
(Crump et al., 2006). Future experiments will need to show whether
the same is true for arch-associated dermal bones, which are most
likely also CNC derivatives that show corresponding
transformations in **brpf1** mutants. By contrast, the anterior shifts in
facial muscular organization seem to be secondary consequences of
the cartilage transformations, as the earlier pattern of muscle
progenitor cells appears normal (K.L. and M.H., unpublished).

**Brpf1 behaves like a TrxG member**

TrxG and PcG proteins are key regulators of chromatin structure
(Ringrose and Paro, 2004; Ringrose and Paro, 2007). Several lines
of evidence suggest that Brpf1 is a novel TrxG member. First, it
is required for maintenance, but not initiation, of Hox gene
expression, a hallmark of TrxG mutants in flies (Breen and Harte,
1993) and mouse (Yu et al., 1998). Second, it genetically interacts
and physically associates with a HAT, and defects of
**brpf1** mutants can be rescued by inhibition of HDAC activity, consistent
with the HAT association and HDAC sensitivity of many TrxG
factors and mutations (Milne et al., 2002; Petruk et al., 2001).
Third, Brpf1 contains a combination of domains found in other
TrxG proteins (bromo, PWWP, PHD finger, zinc finger, AT
hooks) (Ringrose and Paro, 2004). Fourth, it directly binds
histones, as do several TrxG proteins containing bromodomains
and/or chromo/PHD-finger domains (Ringrose and Paro, 2004;
Yang, 2004). Fifth, Brpf1 co-localizes with the active chromatin markers H2AK5Ac (L; red) and H3K4me1 (J; red), but not with the inactive
chromatin marker H3K9me3 (K; red). Left panels are counterstained with DAPI (blue); merged images are shown in right-hand panels; regions with
strong co-localization (yellow) are indicated by arrows. Scale bars: 2.5 μm in A; 5 μm in B,J-L.

**Fig. 6. The PWWP domain is required for association of Brpf1 with metaphase chromosomes.** (A-G) Immunofluorescent staining of mitotic HEK 293 cells transfected with the indicated GFP-Brpf1 constructs (A-E; green) and FLAG-Moz (F,G; red). (A,F) Spreads of metaphase chromosomes. Right panels of A-E and F,G show merged images with DAPI staining of DNA (blue). Full-length Brpf1 displays punctate distribution along metaphase chromosomes (A), whereas in intact nuclei, localization is concentrated in fewer, but still distinct domains of the DNA (B). Truncated Brpf1 lacking the PWWP domain (C) and a Brpf1 fragment containing the PHD domain and the bromodomain (D) are excluded from mitotic chromosomes, whereas a Brpf1 fragment containing the bromodomain and the PWWP domain co-localizes with DNA (E) in a similar manner to full-length Brpf1 (B). (F,G) In contrast to Brpf1 (A,B), no chromatin association is apparent for Moz in metaphase chromosome spreads (F) and in intact mitotic nuclei (G). (H) Schematic structures and chromosome-targeting properties of the full-length and truncated versions of Brpf1. (H-L) Immunofluorescent staining of mitotic HEK 293 cells, revealing that full-length Brpf1 (I-K; green) and the fragment containing the bromodomain and PWWP domain (L; green) co-localize with the active chromatin markers H2AK5Ac (I,J; red) and H3K4me1 (J; red), but not with the inactive chromatin marker H3K9me3 (K; red). Left panels are counterstained with DAPI (blue); merged images are shown in right-hand panels; regions with strong co-localization (yellow) are indicated by arrows. Scale bars: 2.5 μm in A; 5 μm in B,J-L.
transcriptional reinitiation occurs at PRE/TREs, and corresponding sites, although not yet identified at the molecular level, have been suggested to exist in vertebrates (Ringrose and Paro, 2007). Interestingly, we found Brpf1 to be associated with chromatin in discrete spots in both interphase and mitotic chromosomes of HEK 293 cells and zebrafish embryos. Thus, it is tempting to speculate that Brpf1 might account for this TrxG-mediated transcriptional memory through mitosis and cell division. Interestingly, Moz, although co-localized with Brpf1 during interphase, is not retained on mitotic chromosomes, suggesting that only a specific subcomplex is involved in the physical marking of certain genes during mitosis.

Molecular mechanisms of Brpf1 function and the roles of its multiple domains

To understand the molecular mechanisms of Brpf1 function, we carried out a series of studies comparing the properties of full-length Brpf1 protein with those of various truncated or mutant versions. We found that Brpf1 physically associates with the HAT Moz, which extends previous findings (Doyon et al., 2006) and is consistent with our immunofluorescence and genetic data, which indicate that Brpf1 is required for proper chromatin localization and in vivo function of Moz. We mapped the Moz-interaction domain in Brpf1 to thus far uncharacterized motifs between aa 150 and 245. However, it remains unclear whether the binding is direct or indirect, being mediated by other proteins such as Ing5 (Doyon et al., 2006). In addition, we showed that both the bromodomain (aa 679-766) and the PWWP domain (aa 1126-1226) are involved in chromatin-binding of Brpf1. A combination of both domains localized to active chromatin, whereas the individual domains and truncated Brpf1 lacking the PWWP domain did not, pointing to an essential cooperative function of the two domains, as has recently been described for bromo-PHD modules in other proteins (Pena et al., 2006; Wysocka et al., 2006). Consistent with this, the bromodomain and the PWWP domain were found to bind histones with different specificities. The bromodomain showed a significant preference for acetylated histones, as does this domain in other proteins (Yang, 2004), whereas the PWWP domain did not. Conversely, PWWP showed a preference for histones H2A and H2B, whereas the bromodomain bound all core histones equally. Our findings are the first demonstration of direct histone binding for a PWWP domain. This puts PWWP domains on a par with the structurally related and well-known histone-binding chromo and MBT domains (Maurer-Stroh et al., 2003). The importance of this binding is highlighted by the fact that zebrafish mutants lacking only the PWWP domain displayed the brpf1-null phenotype, demonstrating that it is absolutely essential for Brpf1 function in vivo. Future experiments will need to show whether the Brpf1 PWWP domain has any preference for other histone modifications. Interestingly, histone binding of Brpf1 also seems to be required for proper localization of Moz on interphase chromatin. Although Moz has previously been reported to be able to bind nucleosomes (Deguchi et al., 2003), we found its nuclear distribution upon co-transfection with the N-terminal fragment of Brpf1 to be rather diffuse, in contrast to its localization at discrete sites upon co-transfection with full-length Brpf1.

In summary, our data propose a model in which Brpf1, as conferred by its unique set of domains, acts in multiple steps to keep Hox and possibly other genes active during vertebrate development. Mediated by its PWWP domain, it can bind to H2A/H2B histones independently of their acetylation status, and remains at discrete genomic loci even during mitosis, marking them for reinitiation of activation. After mitosis, and mediated by its N-terminal domain, it recruits Moz to chromatin, triggering acetylation of histones H3 and H2A (the latter of which had not previously been identified as a Moz substrate). Finally, mediated by binding of its bromodomain to acetylated histones, Brpf1 protects histones from deacetylation by HDACs.
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Tübingen 2000 Screen Consortium


Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/1935/DC1

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