The Chromatin Regulator BRPF3 Preferentially Activates the HBO1 Acetyltransferase but Is Dispensable for Mouse Development and Survival*

Received for publication, November 9, 2015, and in revised form, December 2, 2015 Published, JBC Papers in Press, December 16, 2015, DOI 10.1074/jbc.M115.703041

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To interpret epigenetic information, chromatin readers utilize various protein domains for recognition of DNA and histone modifications. Some readers possess multdomains for modification recognition and are thus multivalent. Bromodomain- and plant homeodomain-linked finger-containing protein 3 (BRPF3) is such a chromatin reader, containing two plant homeodomain-linked fingers, one bromodomain and a PWWP domain. However, its molecular and biological functions remain to be investigated. Here, we report that endogenous BRPF3 preferentially forms a tetrameric complex with HBO1 (also known as KAT7) and two other subunits but not with related acetyltransferases such as MOZ, MORF, TIP60, and MOF (also known as KAT7) but is not required for mouse development and survival, indicating that BRPF3 forms a functional tetrameric complex with HBO1 (24). By contrast, it is still unclear how BRPF3 may recognize such modifications through different structural modules, including the methyl CpG-binding domain, bromodomain, chromodomain, plant homeodomain-linked (PHD)4 finger and PWWP (Pro-Trp-Trp-Pro containing) domain (5–7). Such readers are crucial for transducing signals from various cellular and environmental cues to regulate the structure and function of chromatin in the nucleus. Some chromatin readers are multivalent because they contain multiple domains for recognition of different modifications. Human BRPF3 (bromodomain- and PHD finger-containing protein 3) is one such multivalent chromatin reader, comprising two PHD fingers joined by a C2HC zinc knuckle and followed by a bromodomain and a PWWP domain (8, 9). BRPF3 is paralogous to BRPF1 and BRPF2 (10, 11). These three human proteins form a subgroup within the bromodomain superfamily, within which there are 39 other members (12, 13).

Several recent studies have provided insights into the molecular and biological functions of BRPF1 and BRPF2 (11, 14). The bromodomain and PWWP domain of BRPF1 bind specifically to acetylated and methylated histone H3, respectively (15–18). Along with a connecting zinc knuckle, two PHD fingers of BRPF1 or BRPF2 form a unique bivalent domain for interaction with DNA and the N terminus of histone H3 (19–21). Flanking the PHD fingers are two conserved sequence motifs with similarity to EPC (enhancer of polycomb) proteins (8, 22). These motifs are important for formation of tetrameric complexes containing ING5 (inhibitor of growth 5), MEA6 (MYST/Esa1-associated factor 6), the histone acetyltransferase MOZ (monocytic leukemia zinc finger protein), MORF (MOZ-related factor), or HBO1 (histone acetyltransferase bound to ORC1). Although BRPF1 forms distinct tetrameric complexes with these three acetyltransferases (8, 9, 23), BRPF2 interacts only with HBO1 (24). By contrast, it is still unclear how BRPF3 may interact with these acetyltransferases.

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In the past 2 decades or so, chromatin has been gradually recognized as an important signaling platform for regulation of diverse nuclear processes, thereby forming the foundation of epigenetic phenomena (1–4). Although chromatin modifiers confer or remove covalent modifications, chromatin readers recognize such modifications through different structural modules, including the methyl CpG-binding domain, bromodomain, chromodomain, plant homeodomain-linked (PHD)4 finger and PWWP (Pro-Trp-Trp-Pro containing) domain (5–7). Such readers are crucial for transducing signals from various cellular and environmental cues to regulate the structure and function of chromatin in the nucleus. Some chromatin readers are multivalent because they contain multiple domains for recognition of different modifications. Human BRPF3 (bromodomain- and PHD finger-containing protein 3) is one such multivalent chromatin reader, comprising two PHD fingers joined by a C2HC zinc knuckle and followed by a bromodomain and a PWWP domain (8, 9). BRPF3 is paralogous to BRPF1 and BRPF2 (10, 11). These three human proteins form a subgroup within the bromodomain superfamily, within which there are 39 other members (12, 13).

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*This work was supported by grants from Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada (to X.-J. Y.), Canadian Institutes of Health Research-National Science Foundation of China, and Fonds de Recherche Santé-Quebec-National Science Foundation of China joint research initiatives (to D. M. and X.-J. Y.). The authors declare that they have no conflicts of interest with the contents of this article.

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4 The abbreviations used are: PHD, plant homeodomain-linked; qPCR, quantitative PCR; MEF, mouse embryonic fibroblast; hMOF, human MOF.
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mouse Brpf1 led to embryonic lethality at E9.5, with severe defects in the vasculature and neural tube (25, 26), forebrain-specific deletion caused abnormal cerebral and hippocampal development (27, 28). Global inactivation of mouse Brpf2 resulted in embryonic lethality at E15.5, with growth retardation, abnormal eye development, and faulty erythropoiesis (24). Specific deletion of the same gene in hematopoietic and endothelial cells revealed an important role in early thymocyte development (29). These studies indicate that mouse Brpf1 and Brpf2 have distinct functions in vivo. It remains elusive what roles BRPF3 plays in vivo.

To determine the molecular and biological functions of BRPF3, we compared its interaction with different members of the MYST family of histone acetyltransferases. The results indicate that BRPF3 preferentially interacts with and activates HBO1. We then utilized a mouse strain containing a knock-in reporter and determined the spatiotemporal expression atlas from embryos to adults. We found that mouse Brpf3 is widely expressed during embryonic development and highly expressed in the adult brain and tests. Unexpectedly, the homozygous mutant mice displayed no overt phenotypes. These results support that BRPF3 activates HBO1 but is dispensable for mouse development and survival.

Materials and Methods

Mice—Mouse strains were maintained in a newly established animal facility at McGill University, and all procedures for use of mice were carried out according to guidelines and protocols approved by the McGill University Animal Use Committee. Brpf3+/− mice were obtained from European Conditional Mouse Mutagenesis Program. Inserted at the Brpf3 locus is a promoterless lacZ-Neo cassette flanked with two FRT sites. In addition, two loxP sites are inserted before and after exons 5–7 of the Brpf3 gene. The line was maintained on the C57BL/6J background. The original mutant allele was designed based on the knock-out-first strategy (30–32). To ensure complete disruption, Brpf3−/− mice were crossed with Ella-Cre mice (The Jackson Laboratory). Intercross between the resulting male and female heterozygotes was employed to assess the mortality of homozygous animals. Mice were genotyped by PCR amplification of specific fragments from genomic DNA extracted from yolk sac or tail samples as described (25). Primers Brpf3-F (ATCCTTAGCCGGGTGTTGTG) and Brpf3-R1 (ACCCCT-TGCTCGGGTGCTTT) were used to amplify a 505-bp fragment from the wild-type allele, whereas primers Brpf3-F and CAS-R1 (TCGTGGTATCGTTATGCGCC) yielded a 358-bp fragment for the mutant Brpf3A allele and Brpf3−/− allele. Primers Brpf3-ex02 (AAAGTATAGGAACTTCGTCGAGA) and Brpf3-ex03 (CCTGGGGCTGATGCAAGGGCAGGCA) were used to detect a 269-bp fragment from the Brpf3A allele after Cre-mediated recombination (to delete exons 5–7 of the Brpf3 gene). Primers Brpf3-F and Brpf3-ex03 were used to detect a 702-bp fragment for the Brpf3−/− allele after FLPO-mediated recombination (to remove the lacZ-Neo cassette) and Cre-mediated recombination. Primers Cre01 (GCATTACCGGTGATGC-AACGGACTG) and Cre02 (GAACGCTAGAGCCTTTTTTG-CACGTTC) were used for detection of a 380-bp fragment of the Cre transgene. RT-PCR, histological analysis, and X-Gal staining were carried out as described (25, 33).

Plasmids—Expression constructs for MOZ, HBO1, TIP60, and hMOF were engineered on pcDNA3.1-FLAG, a derivative of the mammalian expression vector pcDNA3.1 (Invitrogen). Expression plasmids for BRPF1, BRPF2, BRPF3, four BRPF3 deletion mutants, ING5, and MEAF6 were prepared on pcDNA3.1-HA, another derivative of pcDNA3.1. The BRPF3 mutants were generated by PCR with the Pfu DNA polymerase (Roche Applied Science). Green fluorescent protein (GFP) constructs were derived from pEGFP-C2 (BD Biosciences). The vector for mCherry-BRPF3 was derived from pEGFP-C2 by replacing the GFP coding sequence with that for mCherry. All mutants were verified by DNA sequencing.

Antibodies—Anti-FLAG (Sigma, F3165), anti-HA (BABCO/Covance), anti-mouse HRP IgG (Amersham Biosciences, NA931V), anti-rabbit HRP IgG (Fisher, AP307FMI), anti-histone H3 (Abcam, ab1791), anti-H3K9ac (Abcam, ab10812), anti-H3K14ac (Millipore, 07-353), anti-H3K18ac (Millipore, 07-354), anti-H3K27ac (Millipore, 07-360), anti-histone H4 (Millipore, 05-858), and anti-H4K16ac (Abcam, ab109463) antibodies were purchased from the indicated commercial sources. The anti-BRPF3 polyclonal antibody was raised in two rabbits against the N-terminal 194 residues of human BRPF3 and affinity-purified with the antigen as the affinity tag (9).

Brpf3 Knockdown—Four shRNA lentiviral vectors against mouse Brpf3 were purchased from Sigma (MISSION®), SHCLND- NN_001081315): TRCN0000239203, TRCN0000239205, TRCN0000239202, and TRCN0000239204, which are herein referred to as shRNA1–4, respectively. The corresponding vector pLKO.1 (Sigma, SHC001) was used as a negative control. Lentiviral particles were prepared in 293FT cells (34). Wild-type mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos as described (33) and used for lentiviral transduction. After 48 h, cells were treated with puromycin (5 μg/ml) for 24 h to select transduced cells. Knockdown efficiency was assessed by RT-qPCR and immunoblotting.

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. MEFs were cultured in a MEF culture medium (DMEM, 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM β-mercaptoethanol) (Sigma) in a 5% O2 and 5% CO2 incubator at 37 °C. For cell growth analysis with an IncuCyte live cell imaging system (Essen BioScience), MEFs were seeded at a density of 5 × 104 cells/well in a 12-well plate and monitored for up to 10 days. For fluorescence microscopy, cells were seeded at a density of 5 × 104 cells/well in a 12-well plate. Transfection was performed using 1.5–2.5 μl of Lipofectamine 2000 (Invitrogen) per 1 μg of DNA according to the manufacturer’s instructions. For immunoprecipitation, cells were seeded at 7 × 105 cells per 6-cm dish. Transfection was carried out with 4–6 μl of Lipofectamine 2000 and 3 μg of DNA.

Fluorescence Microscopy—For analysis of co-localization, the expression plasmid for GFP-HBO1 was transiently transfected into HEK293 cells with or without the vector for mCherry-tagged BRPF3. Green and red fluorescence signals were exam-
ined under an inverted Axio Observer.Z1 microscope (Zeiss), linked to an Axiocam HRm camera, and controlled by a ZEN software package (Zeiss). Fluorescence images were captured with the software package and exported for further processing with CS5 Adobe Photoshop and Illustrator.

**Immunoprecipitation and Immunoblotting—**Plasmids for expression of FLAG-tagged MOZ, HBO1, TIP60, and hMOF were transfected into HEK293 cells along with expression plasmids for HA-tagged BRPF3, ING5, and MEAF6 as specified under “Results” and in the figure legends. In some experiments, the plasmid for wild-type BRPF3 was replaced with those for its deletion mutants. About 48 h post-transfection, cells were washed twice with PBS, and soluble protein extracts were prepared for affinity purification on anti-FLAG M2-agarose (Sigma) as described (9). The FLAG peptide was used to elute bound proteins for immunoblotting with anti-FLAG and -HA antibodies or for acetyltransferase activity determination.

To determine knock-out efficiency by immunoblotting, wild-type, heterozygous, and homozygous Brpf3 mutant embryos were collected at E12.5, and protein extracts were prepared in the RIPA buffer (150 mM sodium chloride, 1.0% Nonidet P-40 (or Triton X-100), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, and protease inhibitor mixture) for immunoblotting with the anti-BRPF3 antibody (9). For immunoblotting to detect acetylated histones, the nonspecific histone deacetylase inhibitor sodium butyrate (5 mM) was included in the RIPA buffer.

**Co-immunoprecipitation of Endogenous Proteins—**One 15-cm dish of confluent HeLa cells was lysed with 1 ml of Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1% Nonidet P-40, 50 mM NaF, and protease inhibitors). Whole cell lysis was then sonicated on ice for 4–6 times (10 s each) with a Virsonic 100 sonicator (at setting 5, VirTis). After being rotated for 30 min at 4 °C, soluble extracts were collected by centrifugation at 13,000 rpm for 10 min at 4 °C. A mixture of 5 mg of soluble extract, 4 µg of goat anti-HBO1 antibody (Santa Cruz Biotechnology, sc-13284), and 25 µl of protein G Dynabeads (Life Technologies, Inc., 10030D) were incubated with end-to-end rotation overnight at 4 °C. After washing four times with 500 µl of PBS containing protease inhibitors, the beads were resuspended in an SDS sample buffer. After boiling for 5 min, the supernatant was used for SDS-PAGE and immunoblotting with anti-HBO1 and -BRPF3 antibodies.

**Histone Acetyltransferase Activity Determination—**Acetylation of HeLa histone octamers and oligonucleosomes was performed as described (9, 35). Notably, high concentration acrylamide gels (e.g. 15%) were essential for efficient separation of histones H3, H4, H2A, and H2B by SDS-PAGE.

**Whole-mount β-Gal Staining of Tissues and Embryos—**The staining was performed as described (25, 33). Mice were anesthetized and perfused with PBS and then 2% paraformaldehyde/PBS. After perfusion, tissues were collected and fixed at 4 °C for 2–3 h (or 1 h for embryos) in the fixative solution (PBS containing 2% paraformaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40, and 0.2 mM MgCl₂). The solution was then changed to the detergent rinse (0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40). After 5 min, the detergent rinse was replaced with a fresh one. This was repeated once, and whole-mount tissues (or embryos) were then incubated in the X-Gal staining solution (5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], and 1 mg/ml X-Gal) at 37 °C until a desired color intensity was developed (33).

**β-Gal Staining for Frozen Sections of Brpf3⁻/⁻ Embryos—**Frozen sections of Brpf3⁻/⁻ embryos were prepared at E9.5, 12.5, 14.5, 16.5, and E18.5 for β-gal staining as described (33).

**RT-qPCR—**The TRizol reagent (Life Technologies, Inc., 15596-018) was used to isolate total RNA from MEFs derived from embryos, lentivirus–transduced MEFs, or mouse tissues as described (25, 33). Reverse transcription was performed with the QuantiTect Reverse Transcription kit (Qiagen, 205311). Real time PCR was carried out on a Realplex2 Mastercycler (Eppendorf) with the Green-2-Go qPCR Mastermix (Bio Basic, QPCR004-5). Primers were designed on a web-based qPCR primer designer (IDT Biotechnology) as follows: Brpf1 (CAGTAAGATCCACCAACCGGC and GAGGAAAGGGGTACGC-TGCA), Brpf2 (AACACTGACTACCCACAGG and GCCCTCTCAGTTTCTCTTTATT); and Brpf3 (GACTTTTACCAGATTCTCTCGG and CACCGAGTCAGTTTCTCCCA). For regular RT-PCR, primers BR77 (AGGGTAAAGTAGGCTTGAAG) and BR78 (CCAGTATTTGAGCTCCTCCTCC) were used to detect a 310-bp fragment from exons 2 to 4 of Brpf3, whereas BR79 (CATCAGTCGACTCCATGG), BR80 (GACCTTTACCCAGTCTCTCGG), and BR81 (CACCAGATCGATTCTCCCA) were used for analysis of exons 5–7.

**Complete Blood Counts—**Bloods were drawn from tails of 5-month-old wild-type and mutant littersmates for complete blood counts and glucose level determination performed at McGill Animal Comprehensive Center.

**Behavioral Tests—**Nest-building tests were performed as described (27, 36). Hind limb clasping tests were performed as reported (38).

**Statistical Analysis—**Multiple t tests by GraphPad Prism (version 6.0) were used to determine statistical significance.

**Results**

**Human BRPF3 Interacts with the MYST Domain of HBO1—**BRPF1 forms tetrameric complexes with the histone acetyltransferases MOZ, MORF, and HBO1, stimulating their enzymatic activity and restricting their substrate specificity toward nucleosomal histone H3 (9, 23, 24). Different from BRPF1, BRPF2 preferentially interacts with and activates HBO1 but not MOZ and MORF (23). Thus, an interesting question is how BRPF3 targets these acetyltransferases. Our previous pulldown assays showed BRPF interacts with the MYST domains of MOZ and MORF in vitro (9). To investigate whether this is true in cells, we transiently expressed BRPF3 with MOZ and three other acetyltransferases, HBO1, TIP60, and hMOF. These four proteins are members of the same family and share the MYST acetyltransferase domain (39, 40). Immunoprecipitation was performed, and bound proteins were eluted for immunoblotting. As shown in Fig. 1A, BRPF3 co-precipitated with all four acetyltransferases, albeit most efficiently with HBO1 (lanes 1–8 and 13). Interestingly, when ING5 and MEAF6 were co-expressed, BRPF3 was expressed to the highest level when HBO1 was co-expressed (Fig. 1A, compare lanes 9–12, top panel), suggesting that HBO1 stabilizes BRPF3. Moreover, in
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FIGURE 1. Differential interaction of BRPF3 with four MYST acetyltransferases. A, expression plasmids for HA-tagged BRPF3, ING5, and MEAF6 were co-transfected into HEK293 cells along with plasmids for FLAG-tagged MOZ, HBO1, TIP60, and hMOF as specified. After 48 h, extracts were prepared for immunoprecipitation (IP) on anti-FLAG M2-agarose. After extensive washing, bound proteins were eluted with the FLAG peptide for immunoblotting (IB) with anti-HA and anti-FLAG antibodies as indicated. In the absence of BRPF3, ING5 and MEAF6 did not express well (compare lanes 9–12 with lane 14). Asterisks denote degraded MOZ proteins.

B, FLAG-tagged MOZ, HBO1, TIP60, and hMOF were transiently expressed and affinity-purified without ectopic expression of BRPF3 for immunoprecipitation as in A and immunoblotting with a rabbit anti-BRPF3 antibody (9) to detect endogenous BRPF3.

C, HeLa cell extracts were used for immunoprecipitation with a goat anti-HBO1 polyclonal antibody and immunoblotting with the anti-BRPF3 and anti-HBO1 antibodies as indicated. IgH and IgL, IgG heavy and light chains, respectively. Asterisks denote degraded BRPF3 proteins.

D and E, FLAG-HBO1 and its deletion mutants were transiently expressed with HA-tagged BRPF3, ING5, and MEAF6 as specified. Transfection, immunoprecipitation, and immunoblotting were carried out as in A. The immunoprecipitation and immunoblotting results are shown in D, and the domain organizations of HBO1 and its mutants are illustrated in E with the mapping results summarized at right. ZF, zinc finger; MYST, MYST domain. Mutant ΔC14 contains the MYST domain but not its C-terminal 14 residues, which correspond to a small region of MOZ or MORF essential for BRPF1 interaction (9).
the presence of ING5 and MEAF6, BRPF3 formed a tetrameric complex predominantly with HBO1, albeit also weakly with TIP60 (Fig. 1A, lanes 9–12 and 14). ING5 and MEAF6 promoted the binding of BRPF3 to HBO1 (Fig. 1A, compare lanes 6 and 10). These results suggest that BRPF3 prefers to bind HBO1 in cells.

To substantiate this, FLAG-tagged MOZ, HBO1, TIP60 and hMOF were transiently expressed in HEK293 cells for immunoprecipitation on anti-FLAG M2-agarose. Eluted proteins were then analyzed by immunoblotting with anti-FLAG and -BRPF3 antibodies. The latter antibody was to detect endogenous BRPF3 (9). As shown in Fig. 1B, among the four MYST proteins, only HBO1 co-precipitated endogenous BRPF3 protein. Together, these results indicate that BRPF3 preferentially interacts with HBO1.

To substantiate the interaction further, we immunoprecipitated endogenous HBO1 from HeLa cell extracts for immunoblotting with anti-HBO1 and -BRPF3 antibodies. As shown in Fig. 1C, the anti-HBO1 antibody efficiently co-immunoprecipitated endogenous BRPF3, supporting the interaction between endogenous HBO1 and BRPF3 proteins.

We next mapped the HBO1 domain required for BRPF3 interaction. For this, we analyzed four HBO1 deletion mutants. As shown in Fig. 1, D and E, removing the N-terminal 166 or 245 residues had no impact on BRPF3 binding, but deletion of the MYST domain in the N-terminal mutant NT abolished the binding, indicating that the MYST domain is both necessary and sufficient for BRPF3 interaction. This is very similar to the report that the MYST domains of MOZ and MORF mediate interaction with BRPF1 (9). A small region at the C-terminal end of the MYST domain of MOZ or MORF is essential for interaction with BRPF1, so we generated mutant ΔC14 (Fig. 1E) to investigate whether this is true for the interaction of HBO1 with BRPF3. As shown in Fig. 1D, this mutant interacted with BRPF3, albeit at a slightly lower efficiency than full-length HBO1 or the MYST domain (compare lane 4 with lanes 1 and 3). Thus, the MYST domain of HBO1 is both necessary and sufficient for BRPF3 interaction. This interacting mode is similar to but also slightly distinct from that for MOZ and MORF with BRPF1 (9).

A Small N-terminal Domain of BRPF3 Binds to HBO1—We next investigated how differently HBO1 binds to BRPF1, BRPF2, and BRPF3. For this, FLAG-HBO1 was expressed in HEK293 cells with HA-tagged BRPF proteins, ING5 and MEAF6, for co-immunoprecipitation. Although BRPF3 was expressed at a slightly higher level than BRPF1 and BRPF2 (Fig. 2A, top panel), the binding of HBO1 to BRPF3 was the strongest (bottom three panels). Interestingly, all three BRPF proteins promoted the expression of HBO1, but BRPF2 and BRPF3 were more efficient than BRPF1 in doing so (Fig. 2A, bottom two panels). Moreover, expression of BRPF proteins induced a small mobility shift (Fig. 2A, compare lane 1 with lanes 2–4 in the bottom two panels), which is perhaps due to post-translational modifications such as phosphorylation. These results support that like BRPF2, BRPF3 forms a tetrameric complex with HBO1.

We then mapped the BRPF3 domain required for HBO1 interaction. For this, three BRPF3 deletion mutants were expressed as GFP fusion proteins for co-immunoprecipitation with FLAG-HBO1. As shown in Fig. 2, Band C, all three mutants (N602, N193 and N127) co-immunoprecipitated with HBO1, indicating that the N-terminal 127 residues of BRPF3 are sufficient for HBO1 binding. This is consistent with published reports about BRPF1 and BRPF2 (9, 23, 24). Moreover, as shown for full-length BRPF3 (Fig. 2A), all three mutants promoted mobility shift of HBO1 (Fig. 2B, compare lanes 2–4 with lane 1).

We also performed fluorescence microscopy to examine subcellular co-localization of HBO1 and BRPF3. For this, GFP-HBO1 and mCherry-BRPF3 were transiently expressed in HEK293 cells. HBO1 and BRPF3 were localized to the nucleus (Fig. 2, D and E). In a small population of cells, GFP-HBO1 accumulated in nucleoli (Fig. 2D). When GFP-HBO1 and mCherry-BRPF3 were co-expressed along with ING5 and MEAF6, HBO1 and BRPF3 formed uniform nuclear distribution (Fig. 2F), supporting that BRPF3 promotes uniform nuclear distribution of HBO1 but exclusive from nucleoli. In addition, mutant N127 co-localized with HBO1 in the nucleus (data not shown). These results support that the N-terminal domain of BRPF3 mediates HBO1 interaction at the subcellular level (Fig. 2C).

BRPF3 Stimulates Acetyltransferase Activity of HBO1—Having established that BRPF3 preferentially interacts with HBO1, we next investigated how BRPF3 may functionally regulate the acetyltransferase activity and substrate specificity of HBO1. For this, histone acetyltransferase assays were performed to detect acetylation of HeLa histone octamers and oligonucleosomes. To detect acetylation, [14C]acyetyl-CoA was included as the coenzyme, and acetylated histones were detected by autoradiography. When histone octamers were used as substrates, BRPF3 stimulated the acetyltransferase activity of HBO1 toward histones H3, H2B, and H4 (Fig. 3A). To a lesser extent, BRPF3 stimulated the acetyltransferase activity of TIP60 toward these three histones (Fig. 3A). By comparison, no effects were detected on MOZ and hMOF (Fig. 3A). When oligonucleosomes were used as substrates, BRPF3 stimulated the acetyltransferase activity of HBO1 toward histones H3 and H4 (Fig. 3B), but no effects were detected on TIP60, MOZ, and hMOF (Fig. 3B). These results indicate that BRPF3 preferentially stimulates the acetyltransferase activity of HBO1 toward nucleosomal histone H3 and H4.

Detection by autoradiography did not provide information about specific lysine residues that were acetylated, so we carried out immunoblotting with antibodies specific to acetylated forms of histones H3 and H4 to determine the site specificity of HBO1. HBO1 itself and its BRPF3 complex, as well as equivalent BRPF1 and BRPF2 complexes, were incubated with oligonucleosomes and cold acetyl-CoA. Reaction mixtures were resolved by SDS-PAGE for immunoblotting with antibodies specific to acetylated forms of histones H3 and H4. Compared with HBO1 itself, the BRPF3 complex increased the levels of H3K9ac, H3K14ac, and H4K16ac, but not that of H3K18ac (Fig. 3C). A similar trend was also observed with the BRPF1 complex, but the BRPF2 complex stimulated only the H3K14ac level (Fig. 3C). Thus, consistent with their
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physical interaction (Figs. 1 and 2), BRPF3 functionally stimulates the acetylation ability of HBO1 toward H3K9, H3K14, and H4K16 in vitro (Fig. 3C).

Dynamic Brpf3 Expression during Mouse Development—Having determined the molecular functions of BRPF3 in vitro, we next investigated its biological functions in vivo. For this, we...
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first analyzed a knock-out-first mouse strain containing a lacZ knock-in cassette and two LoxP sites flanking exons 5–7 of the Brpf3 gene (Fig. 4A, top). This strain was generated by the international knock-out consortium but has not been characterized (30–32). The knock-in lacZ cassette serves as an efficient reporter for transcriptional activity (31), so we carried out whole-mount β-galactosidase staining to assess Brpf3 expression in embryos and adult tissues. Brpf3 was widely expressed in embryos at E12.5 (Fig. 4B) and highly expressed in the adult testis and brain (Fig. 4C). By contrast, expression was low or not detectable in the liver and kidney (Fig. 4C) or other major organs (data not shown).

To gain insights into the expression at the cellular levels, we performed systematic β-galactosidase staining in serial frozen sections. Shown in Fig. 4D are two representative images. The β-galactosidase activity was widely detectable in the embryo proper at E9.5 (Fig. 4D). The activity was also high in the yolk sac and ectoplacental cone (data not shown). For later days of development, we systematically analyzed serial sagittal and transverse frozen sections at E12.5, 14.5, 16.5, and 18.5. Shown in Fig. 4E are four representative images at E16.5. At this stage, β-galactosidase activity was high in the placenta, yolk sac, retina, nasal epithelium, lung, stomach, kidney, adrenal gland, testis, intestine, and bladder (Fig. 4E). Expression in some of these tissues such as placenta, yolk sac, retina, and lung started at as early as E12.5 and was maintained at E14.5 (data not shown). However, except for the kidney and testis, the expression was generally low at E18.5 (data not shown). Thus, Brpf3 expression appeared to be dynamic during mouse embryogenesis.

Phenotypes of Brpf3-deficient Mice—The dynamic expression pattern suggests that BRPF3 may play a role in regulating mouse embryogenesis. To investigate this directly, we generated Brpf3(-/-) mice (Fig. 4A). Because we previously noticed that the knock-out-first strategy (30–32) was not 100% faithful in leading to gene inactivation, Brpf3(-/-) mice were mated with the Ella-Cre strain, in which expression of the Cre recombinase starts at the pre-implantation stage (41), to generate global excision at the LoxP sites. The resulting Brpf3(-/-) mice were intercrossed to generate homozygous Brpf3(-/-) mice. Unexpectedly, mutant Brpf3(-/-) embryos and mice were grossly normal (Fig. 5, A and B), which was in stark contrast to embryonic lethality observed with Brpf1 or Brpf2 knockouts (24–26). Analysis of 131 mice from intercrosses between male and female Brpf3(+/-) mice yielded expected Mendelian inheritance and revealed no obvious defects in survival and fertility. In addition, comparison of four pairs of wild-type and Brpf3(+/-) mice showed no significant difference in their body weight. To analyze the mutant mice further, we performed histological analysis of the brain and testis. No obvious defects were detected. In addition, we prepared MEFs and monitored proliferation in

FIGURE 3. BRPF3 preferentially stimulates the acetyltransferase activity of HBO1. A and 8, expression plasmids for FLAG-tagged MOZ, HBO1, TIP60, and hMOF were transfected into HEK293 cells with or without the expression plasmid for HA-tagged BRPF3 as specified. For cells used for lanes 2–9, HA-tagged INGS and MEAF6 were also co-expressed. Extracts were prepared for immunoprecipitation as in Fig. 1A, and the precipitated proteins were eluted for acetylation of HeLa histone octamers (A) or oligonucleosomes (B) in the presence of [14C]acetyl-CoA. Reaction mixtures were resolved by SDS-PAGE and fluorography. C, FLAG-HBO1 was expressed with or without HA-tagged BRPF proteins (along with HA-tagged INGS and MEAF6) for affinity purification as in A. Purified proteins were used to acetylate oligonucleosomes in the presence of cold acetyl-CoA. Reaction mixtures were resolved by SDS-PAGE for immunoblotting with antibodies recognizing histones H3 and H4 or their acetylated forms as indicated. The H3K27ac level was not detectable.

FIGURE 2. Interaction of HBO1 with different BRPF proteins. A, FLAG-HBO1 was transiently expressed with HA-tagged BRPF1, BRPF2, BRPF3, INGS, and MEAF6 as specified. Transfection, immunoprecipitation (IP), and immunoblotting (IB) were carried out as in Fig. 1A. B and C, mapping the HBO1-binding site on BRPF3. FLAG-HBO1 was transiently expressed with GFP-tagged BRPF3 mutants. Transfection and immunoprecipitation were carried out as in Fig. 1A. Anti-GFP and anti-HA antibodies were used for immunoblotting as indicated in B. The domain organizations of BRPF3 and its mutants are illustrated in C, with the mapping results summarized at right. Mutant N579 corresponds to a truncated mutant that is expressed in BRPF3 mutant mice (see Fig. 5F). Different from mutant N579, mutant N602 contains an intact EPC-N2 domain, the corresponding region of which is important for interaction of BRPF1 with INGS and MEAF6 (9). Domain abbreviations are as follows: BN, BRPF N-terminal domain; EPC-N1 and EPC-N2, separate regions similar to the N-terminal domain of Drosophila enhancer of polycomb (Epc); PZF, PHD-zinc knuckle-PH domain; bromo, bromodomain; PWWP, PWWP domain. D and E, expression plasmid for GFP-HBO1 (D) or mCherry-BRPF3 (E) was transfected into HEK293 cells. After fixation and GFP staining, cells were analyzed by fluorescence microscopy. Scale bar, 40 µm.
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**FIGURE 4. Brpf3 knock-out strategy and determination of the expression pattern.**

**A**, schematic representation of two Brpf3 mutant alleles. On the Brpf3<sup>Δ</sup> allele, there are two FRT sites flanking a promoterless lacZ cassette, which itself contains an upstream E2 splicing acceptor (E2 SA) and two copies of the coding sequence for the porcine teschovirus-1 peptide 2A (T2A) ribosome-skipping signal along with a downstream polyadenylation signal (pA). The two T2A signals separate the β-galactosidase coding sequence from exon 4 of the Brpf3 gene in the upstream and from the neomycin resistance (Neo) coding sequence in the downstream, so the fusion transcript transcribed from the Brpf3<sup>Δ</sup> allele produces three proteins as follows: a truncated BRPF3 mutant (corresponding to mutant N579, Fig. 2C), encoded by exons 1–4; β-galactosidase from the lacZ cassette; and aminoglycoside 3-phosphotransferase from the Neo sequence. Breeding with EIIa-Cre mice removes exons 5–7 (which encode a small portion of the EPC-N2 domain, the entire bromodomain, and a region C-terminal to the bromodomain, Fig. 2C) of the Brpf3 gene to generate the Brpf3<sup>-</sup>-<sup>Δ</sup> allele. Primers used for genotyping are indicated with small arrowheads: F, Brpf3-F; R1, Brpf3-R1; ex02, Brpf3-ex02; ex03, Brpf3-ex03. **B**, whole-mount β-galactosidase staining for examining lacZ expression in wild-type (WT) and Brpf3<sup>-</sup>-<sup>Δ</sup> embryos at E12.5. The expression was wide in the embryo proper. Scale bar, 2 mm. **C**, whole-mount β-galactosidase staining of adult Brpf3<sup>-</sup>-<sup>Δ</sup> tissues. Brpf3 was highly expressed in the brain and testis but displayed low or no detectable expression in the liver and kidney, or other major organs (data not shown). Scale bar, 5 mm. **D**, β-galactosidase staining for analyzing lacZ expression in frozen transverse sections of wild-type (WT) and Brpf3<sup>-</sup>-<sup>Δ</sup> embryos at E9.5. NT, neural tube; NR, nucleated erythrocytes; LB, lung bud. Scale bars, 100 μm. **E**, β-galactosidase staining for detecting lacZ expression in four transverse sections of E16.5 Brpf3<sup>-</sup>-<sup>Δ</sup> embryos. Asterisks denote areas that were damaged during section staining. Sections in D and E were annotated according to a published atlas on mouse embryo development (37). NE, nasal epithelium. Scale bars, 1 mm.
vitro with an IncuCyte live cell imaging system. As shown in Fig. 5C, the proliferation rate of Brpf3Δ/Δ MEFs was similar to that of wild-type and heterozygous MEFs. Therefore, Brpf3Δ/Δ mice and fibroblasts show no obvious defects.

Brpf3Δ/Δ Mice Produce a Truncated Mutant—According to the knock-out strategy (Fig. 4A), exons 5–7 are removed in the Brpf3Δ/Δ allele, but exons 1–4 remain intact. RT-PCR revealed that a transcript containing these four exons was expressed at a normal level in Brpf3Δ/Δ mice (Fig. 5E), raising the question whether a truncated BRPF3 protein is produced from the mutant allele. Because of the presence of the T2A ribosome-skipping signal after exon 4 in the transcript from the
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allele (Fig. 4A), these four exons are expected to produce N579, a truncated protein containing the N-terminal 579 residues of BRPF3 (Fig. 2C). To investigate this possibility, protein extracts were prepared from wild-type, heterozygous, and homozygous embryos at E12.5 for immunoblotting with the anti-BRPF3 antibody raised against the N-terminal 194 residues of BRPF3 (9). As shown in Fig. 5F, a band corresponding to mutant N579 was detected in heterozygous and homozygous but not wild-type embryos, indicating that the Brpf3Δ4 allele produces a truncated mutant. This raises an interesting issue about whether this mutant interacts with and activates HBO1.

To address this issue experimentally, we performed immunoprecipitation. Mutant N579 does not contain an intact EPC-N2 domain (Fig. 2C). For BRPF1, this domain is important for interaction with ING5 and MEAF6 (9), so we included mutant N602 (which contains an intact EPC-N2 domain, Fig. 2C) for comparison. FLAG-HBO1 was expressed with HA-tagged BRPF3, N579, and N602, in the presence or absence of co-expression of HA-tagged ING5 and MEAF6 for immunoprecipitation. As shown in Fig. 5G, both mutants co-precipitated with HBO1 (lanes 1–4). Moreover, ING5 and MEAF6 co-precipitated with HBO1 only when BRPF3 or mutant N602 was co-expressed (Fig. 5G, lanes 5 and 7), indicating that BRPF3 and N602 are able to bridge the association of ING5 and MEAF6 with HBO1. However, mutant N579 failed to do so (Fig. 5G, lane 6). Interestingly, mutant N602 was not as efficient as full-length BRPF3 to promote expression of ING5 and MEAF6 (Fig. 5G, top two panels, lanes 11, 13, and 14), indicating that the C-terminal part of BRPF3 (i.e. the bromodomain and PWWP domain, Fig. 2C) contributes to complex formation with ING5 and MEAF6. Moreover, mutant N579 was unable to stimulate expression of ING5 and MEAF6 (Fig. 5G, lanes 12 and 14), suggesting that the EPC-N2 domain of BRPF3 is essential for interaction with ING5 and MEAF6 (Fig. 2C).

We next analyzed how BRPF3 and its truncated mutants may stimulate the acetyltransferase activity of HBO1. For this, HBO1 itself and its complexes were incubated with oligonucleosomes and cold acetyl-CoA. Reaction mixtures were resolved by SDS-PAGE for immunoblotting with antibodies specific to acetylated forms of histones H3 and H4. BRPF3 but not its mutants N579 and N602 increased levels of H3K9ac and H3K14ac (Fig. 5H). Interestingly, compared with full-length BRPF3, mutants N579 and N602 inhibited H3K14ac acetyltransferase activity of HBO1, supporting that the C-terminal region of BRPF3 is important for stimulating the acetyltransferase activity of HBO1. Thus, although mutant N579 is expressed in Brpf3Δ4/Δ4 mice (Fig. 5F), it is not functional and may even lead to dominant-negative inhibition.

We also examined global histone acetylation in wild-type and mutant embryos. For this, protein extracts were prepared from wild-type, heterozygous, and homozygous embryos at E12.5 in the RIPA buffer, supplemented with 5 mM sodium butyrate, for immunoblotting with antibodies specific to H3K9ac, H3K14ac, H3K18ac, and H4K16ac. As shown in Fig. 5I, no difference was detected in extracts from wild-type and mutant embryos.

Generation and Characterization of Brpf3Δ/Δ Mice—Brpf3Δ/Δ mutant mice still possess the lacZ knock-in reporter and two T2A signals (Fig. 4A), which may facilitate production of mutant N579. After removal of these elements flanked by the two FRT sites, excision of exons 5–7 of the Brpf3 gene is expected to trigger nonsense mRNA decay (due to a reading frameshift) and to decrease production of mutant N579. We thus crossed Brpf3Δ/Δ mice with the PGK1-FLPo strain (42), to induce excision at the two FRT sites (Fig. 6A) and to generate the floxed Brpf3Δ allele (Fig. 6A). Mating Brpf3Δ/Δ mice with the Ella-Cre strain yielded Brpf3Δ/Δ mice. Intercross of Brpf3Δ/Δ mice resulted in Brpf3Δ/Δ homozygotes. As observed with Brpf3Δ/Δ mutant mice, Brpf3Δ/Δ homozygotes appeared normal. RT-PCR revealed that a transcript lacking exons 5–7 was still produced in E13.5 MEFs (Fig. 6, B and C), suggesting that nonsense mRNA decay is ineffective at this stage in the mutant strain.

After deletion of exons 5–7, the resulting transcript produces a fusion protein containing the N-terminal 579 residues of BRPF3 from exons 1–4 and 31 residues from read-through of exon 8 (Fig. 6B). Immunoblotting indicated that this fusion protein was not detectable in Brpf3Δ/Δ MEFs (Fig. 6D, lanes 1–3). It was still detectable in Brpf3Δ/Δ embryos at E12.5, but the level was much lower (Fig. 6D, lanes 4–6) than that of mutant N579 in Brpf3Δ/Δ embryos (Fig. 5F). As in Brpf3Δ/Δ MEFs, the fusion protein was not detectable in Brpf3Δ/Δ testis (Fig. 6E).

These results suggest that perhaps due to the extra 31 residues, the fusion protein expressed in Brpf3Δ/Δ mice is less stable than mutant N579 produced in Brpf3Δ/Δ animals. As a result, Brpf3Δ/Δ mice represent complete knockouts.

Wild-type and Brpf3Δ/Δ mice grew at similar rates (Fig. 6F). In addition, complete blood counts did not reveal obvious defects, although the monocyte level was slightly increased.

FIGURE 5. Comparison of wild-type and Brpf3Δ/Δ mice. A, morphology of wild-type and Brpf3Δ/Δ embryos at E9.5 and E12.5. The embryos were subjected to whole-mount β-galactosidase staining to determine Brpf3 expression. Scale bars, 0.5 mm (E9.5) and 2 mm (E12.5). B, 8-week old mutant mice showed no obvious phenotypes. C, MEF proliferation was monitored by an IncuCyte live cell imaging system for 7 days. The analyses were performed in triplicates with MEFs from one set of embryos. MEFs from another set of embryos showed similar results. D, schematic illustration of the transcript produced from the Brpf3Δ allele. In the transcript, the coding sequence from the lacZ cassette (Fig. 4A) is fused to exon 4 of the Brpf3 gene. Primers BR77 and BR78 are to assess exons 1–4, whereas BR77 and BR79 are to determine the presence of exon 5. E, RT-PCR analysis of Brpf3 transcripts in wild-type and Brpf3Δ/Δ tissues. The Gapdh transcript levels were used as the internal control. F, protein extracts were prepared from wild-type, heterozygous, and homozygous embryos at E12.5 for immunoblotting with anti-BRPF3 and -β-actin antibodies. N579 denotes the Brpf3 mutant containing the N-terminal 579 residues (Fig. 2C). Single asterisks denote two potential Brpf3 isoforms, and the double asterisk marks a nonspecific band at ~100 kDa. G, expression plasmid for FLAG-HBO1 was transfected into HEK293 cells with those for HA-tagged BRPF3, N579 and N602 with or without expression vectors for HA-tagged ING5 and MEAF6 as specified. Extracts were prepared for immunoprecipitation (IP) on anti-FLAG M2-agarose. After extensive washing, binding proteins were eluted with the FLAG peptide for immunoblotting (IB) with anti-FLAG and -HA antibodies. H, along with HA-tagged ING5 and MEAF6, FLAG-HBO1 was co-expressed with HA-tagged BRPF3 and deletion mutants as indicated. Affinity-purified complexes were used to testify HeLa oligonucleosomes in the presence of acetyl-CoA. Reaction mixtures were resolved by SDS-PAGE for immunoblotting with anti-acetylated histone antibodies. Immunoblotting with anti-histone H3 and H4 antibodies was used as the internal control. I, protein extracts were prepared from wild-type, heterozygous, and homozygous embryos at E12.5 with the RIPA lysis buffer, supplemented with 5 mM sodium butyrate, for immunoblotting as in H.
The blood glucose level was normal in the mutant mouse (Fig. 6G). Thus, Brpf3 loss did not affect development and survival of either Brpf3+/+ (Fig. 5) or Brpf3−/− (Fig. 6) mice.

We next asked whether BRPF3 is important for behavioral regulation due to its high expression in the brain (Fig. 4C). For this, we assessed nest-building skills and hind limb clamping. Pups learn nest-building skills by watching their mothers and other cage mates (36). Three pairs of wild-type and Brpf3−/− mice were evaluated. This test revealed that Brpf3−/− mice possess the same learning ability as wild-type littermates (data not shown). Hind limb clamping tests are often used for evaluating motor and coordination skills (38). As shown in Fig. 7A, no difference was observed between wild-type and Brpf3−/− mice.

### Complete blood counts in control and Brpf3−/− mice

|                  | Control          | Brpf3−/−          |
|------------------|------------------|-------------------|
| Erythroid        |                  |                   |
| Hemoglobin (g/l) | 0.46 ± 0.01      | 0.45 ± 0.05       |
| Hematocrit (l/l) | 149 ± 0.71       | 147 ± 0.13       |
| RBCs x1012/l    | 9.83 ± 0.01      | 9.52 ± 0.91       |
| MCH (pg)         | 46.50 ± 0.71     | 47.33 ± 0.58      |
| MCHC (g/l)       | 327.50 ± 4.95    | 327.67 ± 8.14     |
| Leukocytes       |                  |                   |
| WBCs x109/l     | 2.30 ± 0.67      | 4.13 ± 2.44       |
| Neutrophils (%)  | 8.50 ± 3.54      | 10.67 ± 12.22     |
| Lymphocytes (%)  | 91.00 ± 4.24     | 87.67 ± 11.68     |
| Monocytes (%)    | 0.00 ± 0.00      | 1.33 ± 0.58*      |
| Eosinophils (%)  | 0.50 ± 0.71      | 0.33 ± 0.58       |
| Platelets        | Platelets x109/l |                   |
|                  | 536.50 ± 375.47  | 446.00 ± 335.43   |
| Glucose (mmol/l)| 12.5 ± 0.28      | 13.3 ± 1.76       |
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(Fig. 7A). Thus, these tests indicate that Brpf3 deletion does not affect related behaviors.

Brpf3 was highly expressed in the testis (Fig. 4C), so we examined fertility of male Brpf3<sup>−/−</sup> mice. For this, we bred male Brpf3<sup>−/−</sup> with female Brpf3<sup>−/−</sup> mice to compare the fertility with wild-type male mice. Analysis of the resulting litter size did not reveal any difference.

Wild-type and Brpf3<sup>−/−</sup> MEFs were monitored with the IncuCyte system for 9 days. As shown in Fig. 7B, no significant difference in the proliferation rates was observed. We next investigated whether there is redundancy with, or compensation from, Brpf1 and Brpf2. We thus performed RT-qPCR to determine Brpf1 and Brpf2 transcript levels in Brpf3<sup>−/−</sup> MEFs. As shown in Fig. 7, C and D, levels of Brpf1 and Brpf2 transcripts were not altered in Brpf3<sup>−/−</sup> MEFs. In addition, similar results were obtained in the mutant testis and brain (Fig. 7, E and F). Moreover, levels of Moz, Morf, Hbo1, and Tip60 transcripts remained unaltered in Brpf3<sup>−/−</sup> MEFs (Fig. 7G). Thus, there is no compensation from BRPF1 and BRPF2 or their acetyltransferase partners.

We also determined global histone acetylation in wild-type and Brpf3<sup>−/−</sup> MEFs, E12.5 embryos, and adult tissues by immunoblotting with antibodies specific to H3K9ac, H3K14ac, H3K18ac, H3K27ac, and H4K16ac. As shown in Fig. 7H, except for H3K27ac, no significant changes were observed in mutant MEFs (lanes 1 and 2), embryos (lanes 3 and 4), and adult tissues (lanes 5–8). Thus, results from different analyses of Brpf3<sup>−/−</sup> mice support further that BRPF3 is not essential for mouse development and survival.

Analysis of Brpf3 Knockdown in MEFs—Genetic knock-out and transient knockdown may yield distinct impact (43). To complement the genetic knock-out strategies (Figs. 4 and 6), we performed Brpf3 knockdown by lentiviral expression of shRNAs in MEFs. As shown in Fig. 8A, shRNA-2 was the most efficient among four vectors tested, resulting in almost complete disappearance of BRPF3. We chose shRNA-1 and shRNA-2 for subsequent analysis. At the RNA level, shRNA-1 and shRNA-2 led to Brpf3 knockdown to 50 and 20%, respectively (Fig. 5B). Interestingly, this also affected levels of Brpf1 and Brpf2 mRNA levels (Fig. 5B). However, knockdown by neither shRNA affected histone H3 and H4 acetylation, except for H3K27ac (Fig. 8C). Interestingly, Brpf3 knockdown inhibited MEF proliferation (Fig. 8D). Thus, Brpf3 knockdown in cultured MEFs in vitro yielded slightly different results from genetic knockouts in mice in vivo. This is reminiscent of what was recently reported for another gene (43). Similar to this, apparently contradictory results have been reported for genetic inactivation of the mouse Hbo1 gene in vivo and knockdown of human HBO1 in cultured cells (44).

Discussion

Chromatin regulators are important for various epigenetic phenomena. The human genome encodes hundreds of chromatin regulators. Although extensive molecular studies have been carried out for some of these regulators, much less is known about their functions at the organismal level. This study is the first report on genetic analysis of BRPF3, a novel multivalent chromatin regulator. We demonstrate herein that it preferentially interacts with and activates HBO1 but is dispensable for mouse development and survival.

BRPF3 Preferentially Interacts with and Activates HBO1 in Vitro and in Cultured Cells—Molecular and cell-based assays show that BRPF3 interacts specifically with HBO1 and forms a tetrameric complex with ING5 and MEAF6, to activate the enzymatic activity and restrict substrate specificity of HBO1 (Figs. 1–3). These results are consistent with recent mass spectrometric identification of BRPF3-interacting proteins (45). Thus, BRPF3 is more similar to BRPF2 than to BRPF1 (Fig. 9). Together with published studies about the interaction of BRPF1 and BRPF2 with HBO1, our results support that the BRPF family of proteins is important for regulating the substrate specificity and enzymatic activity of HBO1 (Fig. 9). Notably, this acetyltransferase forms similar tetrameric complexes with the JADE family of proteins, which shares the EPC structural modules with BRPF proteins (8, 46–48). Compared with JADE1 and JADE2, a BRPF protein possesses a bromodomain and a PWWP domain (Fig. 2C), known to bind acetylated and methylated histone H3, respectively (15–18). ING4 and ING5 are similar, and both can be incorporated into the complexes (8, 23). Thus, HBO1 forms up to 12 tetrameric complexes with BRPF1/2/3, JADE1/2, ING4/5, and MEAF6, thereby diversifying the functions under different biological contexts in vivo.

Because of its high sequence similarity to BRPF1 and BRPF2, BRPF3 was considered to target MOZ and MORF (8, 9). Unexpectedly, the current findings indicate that at the molecular level BRPF3 is different from BRPF1 but similar to BRPF2 in preferential interaction with HBO1 but not with MOZ and MORF (Fig. 1). As reported for BRPF1 and BRPF2 (9, 23, 24), the N-terminal part of BRPF3 mediates its interaction with the MYST domain of HBO1 (Fig. 2C). In agreement with this, the N-terminal region is essential for HBO1 interaction (45). Moreover, the MYST domain is sufficient and necessary for BRPF3 binding (Fig. 1E). Further
analysis of specific residues of BRPF3 responsible for the differential interaction with HBO1 should yield insights into the underlying molecular and structural basis.

Pathologically, two recurrent missense mutations associated with bile duct cancer patients have been identified in the human BRPF3 gene (49). Two other mutations are present in pediatric leukemia (50) and adult medulloblastoma (51). Our results provide some insights into how the resulting mutants, R378C (49), E419K (51), S846F (49), and T1031M (50), interact differently with HBO1 in related cancer.

The finding that endogenous BRPF3 does not interact with MOZ and MORF (Fig. 1B) highlights BRPF1 as a unique target.
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of MOZ and MORF (Fig. 9). The MOZ gene was identified as a fusion partner in a chromosomal translocation causing monocytic leukemia (52). Subsequent studies established that the MOZ and MORF genes are fused to different partners in leukemia-associated chromosomal rearrangements (40, 53). In addition, both genes are altered in non-hematologic malignancies (54–60). In addition to cancer, the MOZ and MORF genes are mutated in multiple developmental disorders with a common characteristic of intellectual disability (61–68). Our results lend support for the notion that BRPF1, but not BRPF2 and BRPF3, serves as the authentic target of MOZ and MORF under physiological and pathological conditions (Fig. 9). This thus provides some explanation about why global and conditional Brpf1 knock-out mice display severe phenotypes, including embryonic lethality at E9.5 (25, 26) and abnormal forebrain development (27, 28).

BRPF3 Is Non-essential for Mouse Development and Survival—About the biological function of BRPF3, mouse genetic analysis indicates that it is non-essential (Figs. 4–7). Thus, BRPF3 is distinct from BRPF1 and BRPF2, whose loss leads to embryonic lethality at E9.5 and E12.5, respectively (24, 25). These results suggest that the three proteins have distinct functions in vivo (Fig. 9). As a major partner of BRPF2 and BRPF3, HBO1 is highly relevant. Loss of mouse HBO1 leads to lethality prior to E9.5 and global reduction of H3K14ac (44). The lethality is more severe than Brpf2 inactivation but less so than Brpf1 disruption (24–26). Results presented herein indicate that BRPF3 does not play a major role during mouse embryogenesis. In this context, it will be interesting to investigate physiological roles of JADE1 and JADE2 in vitro (8, 14, 23, 48). It is possible that different BRPF and JADE proteins work together to mediate the action of HBO1 to maintain the H3K14ac level in vivo.
BRPF proteins are conserved from *Caenorhabditis elegans* to humans, with one member in the worm or fly but three in vertebrates such as zebrafish and mammals. *Drosophila* Brpf is highly similar to BRPF1, whereas the *C. elegans* member Lin-49 is relatively distant and does not possess the PWWP domain (40). Although little is known about the function of the fly protein, Lin-49 regulates Hox gene expression, neuron asymmetry, hindgut development, and fecundity (69–71). Hbo1 but not Moz is conserved in *C. elegans*, suggesting that Lin-49 may interact with the Hbo1 ortholog. Inactivation of zebrafish *Brpf1* affects anterior Hox gene expression and alters pharyngeal segmental identity (72), whereas disruption of medaka *Brpf1* alters craniofacial and caudal skeletons (73). Thus, fish *Brpf1* regulates skeletal development. Deletion of fish Moz phenocopies *Brpf1* inactivation to some extent (74), indicating a potential genetic interaction. It remains unclear what roles fish Brpf2 and Brpf3 play *in vivo*.

This study shows that Brpf3 knock-out mice display no obvious phenotypes (Figs. 4–7), so an important question is what roles BRPF3 plays *in vivo*. There are different possibilities. First, all three BRPF proteins interact with HBO1 (Fig. 9), so they may be functionally redundant under certain biological contexts. Second, more careful analysis will be needed to characterize potential functions of BRPF3 in tissues with high expression such as the adult testis and brain (Fig. 4). It remains possible that BRPF3 regulates advanced brain functions such as learning, memory, and intellectual reasoning. Third, BRPF3 may play a role in the hematopoietic system, for which phenotypes may only appear under adverse or challenged conditions. Related to this, monocyte levels appeared to be elevated in *Brpf3*–/– mice (Fig. 6G). Fourth, BRPF3 was recently found to be crucial for DNA replication origin firing and damage response in immortalized cell lines (45), so an interesting question is whether this is also the case in primary cells and *in vivo*. Fifth, a potential role in metabolic control is an important issue worth further investigation. For example, specific deletion of the mouse Klotho gene does not lead to obvious gross phenotypes, but the mutant mice display defects in mineral metabolism (75). Sixth, BRPF1, BRPF2, and BRPF3 may have overlapping functions, so it will be important to generate double and triple knockouts to understand the biological functions of BRPF3 *in vivo*. Finally, there are potential isoforms (Figs. 5F and 6D) that need to be characterized. How they contribute to BRPF3 functions remains to be investigated. Knock-out mice and primary cells described herein (Figs. 4–7) should be valuable for examining all of these interesting possibilities about biological functions of BRPF3 *in vivo*.

In summary, we have demonstrated that BRPF3 preferentially interacts with HBO1 (Figs. 1–3) and that inactivation of the mouse *Brpf3* gene yields no overt phenotypes (Figs. 4–7). Mouse BRPF1 is critical for embryogenesis (25, 26) and forebrain development (26, 28), whereas BRPF2 is required for erythropoiesis and thymocyte development (24, 29). Our results about BRPF3 support that despite their high sequence homology (9), the three BRPF proteins have distinct functions *in vivo* (Fig. 9).

**Author Contributions**—K.Y. carried out the majority of experiments and drafted the manuscript; L.Y. and C.D. started the project; M.G. prepared lentiviruses for Brpf3 knockdown; L.C., X.L., and I.L. provided technical help; D.M. participated in data analysis; X.-J.Y. supervised the project and wrote the manuscript.

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