Zebrafish prdm12b acts independently of nkx6.1 repression to promote eng1b expression in the neural tube p1 domain

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Abstract

Background: Functioning of the adult nervous system depends on the establishment of neural circuits during embryogenesis. In vertebrates, neurons that make up motor circuits form in distinct domains along the dorsoventral axis of the neural tube. Each domain is characterized by a unique combination of transcription factors (TFs) that promote a specific fate, while repressing fates of adjacent domains. The prdm12 TF is required for the expression of eng1b and the generation of V1 interneurons in the p1 domain, but the details of its function remain unclear.

Methods: We used CRISPR/Cas9 to generate the first germline mutants for prdm12 and employed this resource, together with classical luciferase reporter assays and co-immunoprecipitation experiments, to study prdm12b function in zebrafish. We also generated germline mutants for bhlhe22 and nkx6.1 to examine how these TFs act with prdm12b to control p1 formation.

Results: We find that prdm12b mutants lack eng1b expression in the p1 domain and also possess an abnormal touch-evoked escape response. Using luciferase reporter assays, we demonstrate that Prdm12b acts as a transcriptional repressor. We also show that the Bhlhe22 TF binds via the Prdm12b zinc finger domain to form a complex. However, bhlhe22 mutants display normal eng1b expression in the p1 domain. While prdm12 has been proposed to promote p1 fates by repressing expression of the nkx6.1 TF, we do not observe an expansion of the nkx6.1 domain upon loss of prdm12b function, nor is eng1b expression restored upon simultaneous loss of prdm12b and nkx6.1.

Conclusions: We conclude that prdm12b germline mutations produce a phenotype that is indistinguishable from that of morpholino-mediated loss of prdm12 function. In terms of prdm12b function, our results indicate that Prdm12b acts as transcriptional repressor and interacts with both EHMT2/G9a and Bhlhe22. However, bhlhe22 function is not required for eng1b expression in vivo, perhaps indicating that other bhlh genes can compensate during embryogenesis. Lastly, we do not find evidence for nkx6.1 and prdm12b acting as a repressive pair in formation of the p1 domain – suggesting that prdm12b is not solely required to repress non-p1 fates, but is specifically needed to promote p1 fates.

Keywords: CRISPR, Dorsoventral patterning, Hindbrain, Spinal cord, Interneuron, Locomotion, Transcription

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Background
Appropriate function of the adult nervous system requires the establishment of neural circuits during embryonic development. For such circuits to form properly, neurogenesis has to occur at the right time and place, neurons must migrate to the correct site and they must make appropriate connections. Disruptions to any step in this process result in improper neural circuit formation and such disruptions are thought to underlie many neurodevelopmental disorders – including schizophrenia and autism [1].

The embryonic vertebrate neural tube represents a well-studied system of neural circuit formation where various progenitor types form in distinct domains arrayed along the dorsoventral (DV) axis. These progenitor domains form in response to morphogen gradients – particularly dorsally derived Bone morphogenic protein (BMP) and ventrally derived Sonic hedgehog (Shh; reviewed in [2, 3]). In response to these morphogens, each progenitor domain acquires a unique gene expression profile that initially consists primarily of transcription factors (TFs). Strikingly, TFs unique to one progenitor domain frequently cross-repress the expression of TFs associated with adjacent domains, thereby establishing distinct boundaries that delineate individual progenitor domains along the DV axis. The graded morphogen signal, and the resulting distinct transcriptional programs, leads to the development of sensory neurons in the dorsal domains (pd1-pd5) and interneurons and motor neurons in the ventral domains (pd6-p0, p1, p2, pMN, p3) of the neural tube. Neurons from each of these domains then make connections to establish motor circuits that control the activity of limb and trunk musculature [4].

Many TFs that control establishment of progenitor domains along the DV axis belong to the homeodomain (HD) and basic Helix-Loop-Helix (bHLH) families. For instance, work in mouse and chick indicate that Shh activates genes such as Nkx6.1, Nkx6.2, Nkx2.2, and Olig2, while it represses Pax3, Pax6, Pax7, Dbx1, Dbx2 and Irx3 [5–13]. These TFs then repress each other’s expression to establish distinct progenitor domains. For instance, Irx3 and Olig2 are mutually repressive at the p2/pMN boundary [8, 14] such that loss of Olig2 leads to a ventral expansion of Irx3 expression, causing the pMN domain to give rise to V2 interneurons and astrocytes in place of motor neurons and oligodendrocytes [14]. More recently, members of the Prdm TF family have also been implicated in the formation of progenitor domains and the establishment of functional motor circuits (reviewed in [15]). The Prdm family consists of many members (Prdm1–16) that harbor an N-terminal PR domain, as well as a variable number of zinc fingers [16, 17], and that appear to preferentially act in complexes with bHLH TFs [15]. Hence, Prdm13 acts together with Ascl1 to promote formation of GABAergic neurons [18, 19], while Prdm8 interacts with the Bhlhe22 (a.k.a. Bhlhb5) TF to regulate axon outgrowth [20]. Of particular interest, Prdm12 is expressed in the developing CNS of mouse, frog, chick and zebrafish [21–23] – specifically in the p1 domain, which gives rise to V1 interneurons. Prdm12 deficiency in zebrafish and frog results in loss of engl expression from the p1 domain and animals lacking prdm12 function demonstrate a defective touch-evoked escape response [22, 23], suggesting that the V1 interneurons are absent. However, key aspects of Prdm12 function remain unclear.

First, Prdm12 activity has only been assessed via overexpression and transient knock-down approaches – particularly antisense morpholino oligonucleotides (MOs) – that have recently come under scrutiny as prone to non-specific off-target effects. Furthermore, Prdm12 is suggested to act as a transcriptional repressor, but this is based on overexpression in fish and frog embryos [23, 24] and has not been stringently tested. Here, we generate and characterize the first germline prdm12 mutants using CRISPR/Cas9 to inactivate zebrafish prdm12b. prdm12b mutants display embryonic lethality and, in accordance with previous prdm12b MO analyses, we find that prdm12b mutants exhibit loss of engl expression in the p1 domain together with an abnormal touch-evoked escape response. We also employ luciferase reporter assays to reveal that Prdm12b acts as a bona fide repressor. This repression requires a conserved zinc finger domain that interacts with the Bhlhe22 TF, but, when we generate a bhlhe22 germline zebrafish mutant, it displays a normal p1 territory – indicating that bhlhe22 does not need to act with prdm12b for p1 progenitor formation in vivo. Lastly, while Nkx6.1 is known to repress p1 fates in other systems, we find that prdm12b and nkx6.1 does not form a reciprocally repressive TF pair in the zebrafish. Therefore, instead of the p1 domain taking on a p2 fate, a residual domain with unknown properties persists at the p1 position in prdm12b zebrafish mutants.

Methods
Zebrafish care
Wild type and mutant zebrafish were raised in the University of Massachusetts Medical School Aquatics Facility. All embryos were staged according to previously described morphological standards [25].

Generation of CRISPR/cas9 mutant zebrafish lines
We designed single guide RNAs (sgRNA) for the zebrafish prdm12b, bhlhe22 and nkx6.1 genes (Table 1) using the CHOPCHOP web tool [26]. Each sgRNA was assembled by annealing two single stranded oligonucleotides containing the T7 promoter and the target sequence (Additional file 1) followed by PCR amplification, purification and in vitro transcription using T7 RNA polymerase (Promega) as described previously [27]. A linearized
plasmid encoding cas9 was used for in vitro transcrip-
tion using the SP6 mMessage mMACHINE Kit (Ambion) according to the manufacturer’s instructions [28]. cas9 mRNA and sgRNA was co-injected into 1-cell stage zebrafish embryos at the following concentrations: 150 ng/μL sgRNA plus 200 ng/μL cas9 mRNA for prdm12b, 100 ng/μL sgRNA plus 200 ng/μL cas9 mRNA for bhlhe22 and 150 ng/μL sgRNA plus 200 ng/μL cas9 mRNA for nkx6.1. The next day, injected embryos were assayed for sgRNA activity by DNA extraction, PCR amplification, restriction digestion and DNA sequencing (Table 1). Detection of F0 founders was done by crossing sgRNA/cas9-injected animals with wildtype zebrafish and screening their offspring for mutagenic events using the diagnostic restriction enzymes listed in Table 1. Confirmed founders were crossed to wildtype animals to raise F1 carriers for each mutant.

Antisense morpholino oligonucleotide injections
Antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools LLC. MO injections were performed into the yolk of 1-cell stage embryos using 1-2 ng of solution containing dilutions of 3 mM morpholino stock, distilled water and phenyl red. An MO with the sequence 5‘-GCAGGCAACACTGAACCCATGATGA-3’ was used to target the prdm12b translation start site. This MO was reported previously [22] and our analyses in this manuscript demonstrate that the effects of MO-mediated prdm12b knockdown are indistinguishable from the effects of prdm12b germ line mutations.

In situ RNA hybridization
Embryos were fixed in 4% paraformaldehyde (PFA) and stored in 100% methanol at −20 °C. In situ RNA hybridization was performed as described [29] followed by a color reaction using NBT/BCIP in 10% polyvinyl alcohol. RNA probes for the genes eng1b, evx1, vsx2, pax3, nkx6.1, dbx1 and prdm12b were synthesized as previously described [27]. Embryos were dissected from the yolk and flat mounted in 80% glycerol for imaging on bridged coverslips or sectioned as described [30]. Images were captured using a Nikon Eclipse E600 microscope equipped with spot RT color camera (model 2.1.1). Images were imported into Adobe Photoshop and adjustments were made to contrast, levels, color matching settings and cropping only. All adjustments were made to the entire image.

Luciferase reporter assays
0.5 × 10⁶ HEK293T cells were seeded in 6-wells plate and cultured in antibiotic free Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Hyloclone) overnight. Transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. For each transfection, 200 ng of the pGL4.31[luc2P/GAL4UAS/Hydro] reporter plasmid and 50 ng pRL-SV40 control plasmid was combined with varying concentrations of GAL4DBD expression plasmids (the fusion proteins were cloned into the pCS2 expression plasmid; exact concentrations are given in figure legends). Empty vector DNA was included to keep the total amount of DNA constant for all transfections. Luciferase activity was measured 24 h post transfection and firefly luciferase levels were normalized to renilla luciferase levels using the Dual Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions in a Perkin Elmer Envision 2104 Multiplate reader. For Trichostatin A (TSA) treatment, transfected cells were exposed to either DMSO, 50 nM or 250 nM TSA for 12 h starting 24 h after transfection and then harvested for luciferase assays.

Co-immunoprecipitation and Western blotting
3 × 10⁶ HEK293T were seeded in 10 cm dishes and transfected as above. Transfected cells were lysed in 4 mL of ice-cold co-IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5% Triton X100, 1X Complete Protease Inhibitor (Roche)) followed by incubation on ice for 30 min. Cell lysates were centrifuged at 2000 g for 10 min at 4 °C to eliminate cell debris. For immunoprecipitation, 8 μg of the mouse anti-Flag antibody (Sigma-Aldrich, F3165) was used in each sample and incubated at 4 °C overnight. 40 μL of Dynabeads was added in each sample and incubation was done for 4 h at 4 °C. Four washes of 1 mL co-IP buffer was used to eliminate non-specific binding. Lastly, immune complexes were eluted in 80 μL of 1X Laemmli buffer (Biorad) containing 2.5% beta-mercaptoethanol. Samples were agi-
tated at 95 °C for five minutes prior to Western blotting.

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**Table 1 Characteristics of CRISPRs targeting prdm12b, bhlhe22 and nkx6.1**

| Target gene | Start Coordinate | Target sequence | Enzyme | Strand | Mutagenesis Ratea | Transmission Rateb |
|-------------|------------------|-----------------|--------|--------|------------------|-------------------|
| prdm12b     | Chr5:66656496    | GCTGGGGGACACCT GTTCG | TaqI | + | 1/4 | 71/92 um318 |
|             |                  |                  |        |        |                  | 43/79 um319 |
| bhlhe22     | Chr24:25069884   | TTCACACACAAGATCCGGT | BstYI | - | 6/14 | 24/37 um320 |
| nkx6.1      | Chr21:17886500   | AGTGGAGGATGCATCTCCAG | Avall | - | 8/12 | 18/21 um321 |

aThe fraction of screened F0 animals that carried a mutagenic event
bThe fraction of screened F1 animals that were heterozygous for a mutagenic event
Western Blotting was performed using rabbit HA antibody (Abcam, ab91110) as described previously [31].

**Immunocytochemistry**

Primary antibodies: mouse 3A10 (1:100; Developmental Studies Hybridoma Bank (DSHB) [32]), mouse F310 (1:100; DSHB [33]), mouse anti-Isl (39.4D5, 1:100; DSHB [34]), mouse 81.5C10 (Hb9; 1:400; DSHB [35]). Alexa Fluro secondary antibodies: 488, 568 goat anti-mouse (both at 1:200; Molecular probes). Embryos were fixed in 4% AB fix (4% paraformaldehyde, 8% sucrose, 1x PBS) overnight at 4 °C. Whole-mount fluorescent labeling was performed as described [36]. Images were captured on either Nikon Eclipse E600 (3A10, Isl1 and Hb9 staining) or a Zeiss LSM700 confocal microscope (F310 staining). Images were imported into Adobe Photoshop and adjustments were made to contrast, levels, color matching settings and cropping only. All adjustments were made to the entire image.

**Behavioral analysis**

Escape responses were elicited by a light tap to the head or tail of an embryo with a 3.22/0.16 g of force Von Frey filament. A high-speed digital camera (Fastec Imaging, San Diego, CA) mounted to a 35 mm lens (Nikon, Melville, NY), recorded each response at 1000 frames/s. Computer software generated in the Downes laboratory [37] quantified the head-tail angle for each frame, which was then plotted in Prism. The calculated escape response began in the frame preceding the first movement until movement was no longer observed.

**Genotyping**

CRISPR-generated mutant alleles of *prdm12b*, *bhlhe22* and *nkx6.1* were genotyped by Taq1 digestion, respectively, of PCR products amplified from genomic DNA using primers listed in Additional file 2. *prdm12b<sup>asR887</sup>* mutants were genotyped by sequencing of PCR products amplified from genomic DNA using primers listed in Additional file 2.

Total RNA from zebrafish embryos was extracted with the RNeasy kit (Qiagen) following manufacturer's instructions. Total RNA was then used in cDNA kit (ThermoFisher Scientific). Wildtype and *bhlhe22* mutant transcripts were identified by sequencing of PCR products amplified from cDNA using primers listed in Additional file 2.

**Results**

**Germline disruption of *prdm12b* blocks *eng1b* expression in the p1 domain**

The *prdm12* TF is known to be expressed in the developing CNS of mouse, chick, *Xenopus* and zebrafish [21–23] – particularly in sensory ganglia and in the p1 domain of the neural tube. The p1 domain gives rise to *eng1b*-expressing V1 interneurons that regulate motor circuits in several vertebrate species [38–40]. Disruption of *prdm12* function using antisense morpholino oligonucleotides (MOs) leads to the loss of *eng1b* expression in the p1 domain, but not in other *eng1b* expressing tissues – such as the midbrain-hindbrain boundary (MHB) and the somites – in zebrafish and *Xenopus* [15, 23], but there have been no germline mutations for *prdm12* produced in any organism. Importantly, recent work has demonstrated several cases where apparently specific MO-derived phenotypes do not match the phenotypes of germ line mutants for the same gene [41]. The underlying causes of such discrepancies are varied, but include off-target effects, as well as compensatory changes in the expression of genes with similar functions to the targeted gene [42]. Hence, it is essential to confirm MO-derived phenotypes by comparisons to the phenotypes of germline mutant animals. To this end, we used the CRISPR/Cas9 genome editing system [43, 44] to generate *prdm12b* germline mutant zebrafish. We tested five sgRNAs targeting the first exon of the *prdm12b* gene and identified one that efficiently disrupts a diagnostic Taq1 site at position 129 of *prdm12b* exon 1 in 24hpf zebrafish embryos (Fig. 1A, B). Injected embryos were raised to adulthood and screened to identify founders that carry mutations in the *prdm12b* gene (Fig. 1c). In this manner, we identified one mutant F0 founder out of four tested (Table 1). Since zebrafish F0 founders are usually mosaic, this founder was crossed to wildtype fish and the resulting F1 generation raised to adulthood (Fig. 1d). Genotyping revealed that the F0 founder transmitted mutations to 77% (114/171) of its F1 offspring (Table 1). Subsequent sequencing of genomic DNA from individual F1 fish identified two different alleles (*prdm12b<sup>trans318</sup>* and *prdm12b<sup>trans319</sup>*; Fig. 1e; f; Additional file 3). In both alleles, the mutant sequence leads to a frameshift and premature termination of translation upstream of the conserved PR domain and the zinc finger domains. In addition, while we were in the process of generating *prdm12b* mutants, a mutant *prdm12b* allele became available from the zebrafish information resource center (ZIRC) as a product of the zebrafish mutation project (ZMP). This mutant allele (*prdm12b<sup>asR887</sup>*) is ENU-derived and carries a T > C change in an essential splice site at the beginning of intron 2, within the PR domain and upstream of the zinc finger domains (Additional file 4A). We obtained this line from ZIRC and confirmed the presence of the expected mutation by sequencing (Additional file 4B, C).

Since the effects of MOs wear off as development progresses (largely due to MO degradation) they are not a reliable tool to assess genetic effects on embryo viability. However, having generated *prdm12b* germ line mutants, we were able to examine the effect of *prdm12b* on viability by crossing heterozygous carriers and genotyping the resulting offspring at different stages of embryogenesis.
**prdm12b** mRNA does not appear to be maternally deposited (Fig. 2a, b) and is not detected until the end of gastrulation [15], suggesting a relatively late role in development. Accordingly, we observe the expected ~25% homozygous **prdm12b** mutants (26/139 for **um318** and 29/116 for **um319**) at 4dpf (Fig. 2c), but by 15dpf only ~13% of embryos are homozygous mutant (22/172 for **um319**) and by 21dpf we no longer detect any homozygous mutants (0/129 for **um319**). We also do not observe homozygous mutants when genotyping adult offspring.
(2 months of age; 0/92 for um318 and 0/145 for um319) from these crosses. Since prdm12b mutants start dying between 4dpf and 15dpf, we monitored developing embryos more closely during this time interval and noticed that a fraction of embryos grew at a slower rate (Fig. 2d, e). When the smaller embryos were genotyped, 82% (18/22) turned out to represent homozygous prdm12b mutants. This slower rate of growth suggests that the mutants may be unable to feed properly (perhaps due to the motility defects described below). However, when fed brine shrimp, even the mutant embryos show evidence of food in their digestive tract (orange/yellow color in Fig. 2d, e). Hence, the mutants are capable of feeding, although we cannot exclude the possibility that they do so sub-optimally.

Since loss of eng1b expression in the p1 domain is the key feature of the zebrafish prdm12b morphant phenotype, we next assayed eng1b expression in all three prdm12b mutant alleles by in situ hybridization at 24hpf. For both CRISP/Cas9-generated alleles, ~ 25% of embryos from crosses of heterozygous carriers lack eng1b expression in hindbrain and spinal cord (Fig. 2f-q). In the affected embryos, eng1b expression is lost from the p1 domain, but persists at the MHB and in the somites (whole mount in Fig. 2h, k, n, q and section in Fig. 2s). Genotyping revealed that all embryos lacking eng1b expression in the p1 domain represent homozygous prdm12b mutants (45/45 for prdm12bum318 and 13/13 for prdm12bum319). Similarly, eng1b expression is lost in both hindbrain and spinal cord in 27% of embryos from a cross of prdm12bum98477/+ heterozygous fish, while the remaining embryos show unaffected eng1b expression (Additional file 4D-G). We conclude that germ line mutants for prdm12b display the same loss of eng1b expression as previously reported for prdm12b morphants.
**Prdm12b mutant animals display an abnormal escape response**

V1 inhibitory interneurons are responsible for the modulation of motor circuits in many species, including zebrafish, *Xenopus* and mouse ([25, 32], reviewed in [45]). Accordingly, we previously demonstrated that *prdm12b* morphants display abnormal movements in response to touch [15]. The touch-evoked escape response is a classical method of assessing functionality of motor output in aquatic species [46] and it has been applied to zebrafish [47, 48]. In this test, a touch stimulus causes the fish to undergo a large amplitude body bend (C bend), which reorients the animal away from the stimulus. The initial large amplitude body bend is followed by lower amplitude counter bends, allowing the fish to propel itself away. Strikingly, the escape response of *prdm12b* morphants is exaggerated, such that morphants perform not just one, but several repetitive C-bends and, compared to a wild type response—which lasts ~100 ms—the response of *prdm12b* morphants is prolonged and may continue for several hundred milliseconds [15]. To determine if this defect is observed also in germline mutants, we assessed the escape response of 4dpf old *prdm12b* mutant fish to a head tap, followed by genotyping. We find that all *prdm12b* mutants (9/9 for um318 and 8/8 for um319), respond by carrying out repetitive C-bends (up to seven C-bends) for extended periods of time (Fig. 3a, b; Additional files 5, 6 and 7). We extended this analysis to also score the response of *prdm12b*um319 homozygous mutant animals when tapped on the tail. We observed no differences between responses to head versus tail stimulation—in all 11 cases were the responses exaggerated to both stimuli.

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**Fig. 3** *prdm12b* mutant fish display an abnormal touch evoked response. a–d. Representative kinematic traces for 10 wildtype (a) and 11 *prdm12b* mutant (b) fish stimulated with a head touch, as well as for 11 *prdm12b* mutants first assayed with a head touch (c) and subsequently with a tail touch (d). Zero degrees on the y-axis indicate a straight body while positive and negative angles represent body bends in opposite directions. All fish were at 4dpf. e. Anti-3A10 labeling of Mauthner neurons in a cross of *prdm12b*um318 heterozygotes (*n* = 117). f. Anti-F310 labeling of somites in a cross of *prdm12b*um319 heterozygotes (*n* = 16)
(Fig. 3c, d; Additional files 5, 8, 9). The touch-evoked escape response is mediated via reticulospinal neurons – most notably the Mauthner cells, but also MiD2 and MiD3 cells – and our results therefore indicate that this pathway is abnormal in prdm12b mutants. Notably, there is no known circuit connecting V1 interneurons to the reticulospinal cells, suggesting that the abnormal escape response observed in prdm12b mutants may be independent of the loss of V1 interneurons. Indeed, the behavior of the mutants is consistent with enhanced or excessive activity of this pathway, perhaps due to impaired synapse function or circuit regulation. Accordingly, we do not detect structural defects in either the morphology of Mauthner cells (Fig. 3e), or the structure of trunk/tail musculature (Fig. 3f). We conclude that prdm12b germ line mutant animals display a defective escape behavior that is qualitatively and quantitatively indistinguishable from that of prdm12b morphants.

**Prdm12b acts as a repressor in vitro**

The fact that prdm12b belongs to a family of transcription factors, together with the finding that loss of prdm12b function abolishes engl1b expression, suggests that this factor may function as a transcriptional activator. Accordingly, transfection of prdm12 into P19 cells upregulates p27 mRNA and protein levels [49]. However, recent reports instead suggest that prdm12 acts as a repressor [23], but this conclusion was based on overexpression experiments in vivo and has not been tested directly. To more directly determine whether prdm12b acts as an activator or repressor, we made use of classical reporter assays. While prdm12b possesses three putative zinc-fingers (ZnFs), it is not clear if these are sufficient for DNA binding and there is no well-defined genomic motif for Prdm12b binding. We therefore fused the well-characterized DNA binding domain (DBD) from the GAL4 transcription factor in-frame to the N-terminus of zebrafish Prdm12b (Fig. 4a; Additional file 10). Transcriptional activity was measured using the pGL4.31 reporter vector that contains multiple GAL4 binding sites (upstream activation sequence; UAS) in front of the firefly luciferase gene. Co-transfection of the reporter plasmid together with the GAL4-DBD alone led to a modest increase in Luciferase activity (Fig. 4b). Strikingly, when the GAL4DBD-Prdm12b fusion protein was instead co-transfected with the reporter plasmid, a dose-dependent reduction in Luciferase activity was observed (Fig. 4b), indicating that the Prdm12b protein functions as a repressor.

Pdrmd12b contains two types of conserved domains – the PR domain and the zinc fingers. The PR domain is related to SET domains that function as histone lysine methyl transferases (HMTs). Most PR domain proteins lack the H/RxxNHxC motif that is essential for HMT activity [50]; however, Pdrmd2, Prcmd3, Pdrmd6, Prcmd8, Pdrmd9 and Prcmd13 were recently shown to exhibit intrinsic methyltransferase activity [51–55]. Accordingly, the PR domain of Pdrmd12b has been postulated to act as a H3K9 methyltransferase – to deposit methyl groups onto lysine 9 of histone 3 – thereby repressing gene expression [24]. A recent study of Prcmd9 demonstrated that cysteine 321 (Cys321) is highly conserved among Prcmd family members that have intrinsic histone methyl transferase activity and that substituting Cys321 with a proline decreases Prcmd9 activity ~1000 fold [56]. Our sequence comparison of Prcmd1, 9, 10 and 12b revealed that Prcmd12b carries a cysteine residue (Cys164) at the analogous position to Cys321 in Prcmd9, while Prcmd1 and Prcmd10 (that lack methyltransferase activity) contain a proline at this position. To determine the functional contribution of Cys164, we tested the activity of several substitution mutants using the luciferase assay, but neither a cysteine -> proline, nor a cysteine -> alanine, substitution at position 164 affected the repressive activity of Prcmd12b (Fig. 4c). Deletion of the entire PR domain proved to be uninformative as this protein was unstable in HEK293 cells (Additional file 10). Previous work also demonstrated that some Prdm proteins act as repressors by recruiting histone deacetylases (HDACs) via the PR domain [57–59], but we find that Trichostatin A (TSA; a HDAC inhibitor) does not affect the repressive activity of Prcmd12b (Fig. 4d). Lastly, we deleted the conserved zinc fingers in Prcmd12b in order to determine if they might be required for its repressive function. Strikingly, deletion of the ZnFs completely abolished the repressive activity of Prcmd12b and instead appears to produce a protein with slight activator activity (Fig. 4e). Taken together, our results indicate that Prcmd12b functions as a repressor and that this activity requires intact zinc finger domains, at least in the context of a GAL4DBD fusion protein.

**Pdrmd12b interacts with the Bhlhe22 transcription factor and the EHMT2 methyltransferase**

As discussed, it is unclear if Pdrmd12b binds DNA directly and it may instead be recruited to genomic binding sites by forming complexes with a DNA-binding factor. Since prdm12b is expressed only in the p1 domain, we focused our search for DNA-binding Pdrmd12b-interactors to ones that are co-expressed with prdm12b in the p1 domain. Based on this criterion, the Bhlhe22 transcription factor (also known as Bhlhb5) represents a potential binding partner for Pdrmd12b. In particular, Bhlhe22 is expressed in the pdl6, p1, p2 and p3 domains and has been implicated in the specification of V1 and V2 interneurons [60]. Furthermore, Bhlhe22 has been shown to form complexes with Pdrmd8, suggesting that it may act broadly as a partner for Pdrmd proteins [20]. Using co-immunoprecipitation, we confirmed the interaction between Bhlhe22 and Pdrmd8 (Fig. 5a, lane 9) and further demonstrated robust binding
between Bhlhe22 and Prdm12b (Fig. 5a, lane 6). More detailed analyses using Prdm12b deletion constructs indicated that the ZnF domain – that we already identified as necessary for Prdm12b-mediated repression (see Fig. 4d) – is required for Bhlhe22 binding (Fig. 5a, lane 7). In contrast, the PR domain does not appear to be absolutely required for the Prdm12b-Bhlhe22 interaction (Fig. 5a, lane 8).

Moreover, since Prdm12b appears to lack intrinsic methyltransferase activity, it must function by recruiting factors to mediate its repressive effects. Accordingly, Prdm family members recruit various transcriptional repressors ([61–64] and reviewed in [16]). In particular, Prdm1, 5 and 6, as well as Prdm12, have been shown to bind EHMT2/G9a – a H3K9 methyltransferase [57, 59, 65, 66]. In the case of Prdm12, binding to EHMT2/G9a is reportedly mediated by the ZnF domains [49]. Since this is the same domain that we find to be required for binding to Bhlhe22, we examined this in further detail.

We confirmed that Prdm12b interacts with EHMT2/G9a (Fig. 5b, lane 2), but find that neither the ZnF, nor the PR domain, is required for this binding (Fig. 5b, lanes 5 and 8).

We conclude that Prdm12b binds to both Bhlhe22 and EHMT2/G9a. Additionally, the Prdm12b ZnF domain –
that is essential for Prdm12b-mediated repression – is required for binding to Bhlhe22, but not to EHMT2.

**bhlhe22 is not required for eng1b expression in the zebrafish p1 domain**

Previous work reported that siRNA-mediated knock-down of bhlhe22 in the chick spinal cord leads to a reduction in eng1 expression in the p1 domain [60], akin to the effect we observe in prdm12b mutants. The similarity of the bhlhe22 and prdm12b loss-of-function phenotypes, taken together with our finding that these two proteins form complexes, suggests that bhlhe22 and prdm12b may cooperate to control eng1b expression. To test this possibility, we generated germline mutants for zebrafish bhlhe22 using the CRISPR/cas9 system. Specifically, a sgRNA targeting the 5’ end of the bhlhe22 coding sequence (that is contained on a single exon) was used to generate six founders carrying mutations in the bhlhe22 gene (Table 1; Additional file 11A-D). One founder was characterized further and found to transmit a small deletion that introduces a frameshift, which is predicted to cause premature termination of Bhlhe22 protein synthesis upstream of the bHLH domain (Additional file 3B, Additional file 11E). We find that animals homozygous for this mutant allele (bhlhe22<sup>um320</sup>) are viable to adulthood (Fig. 6a). As expected, sequencing of bhlhe22 transcripts from such homozygous animals detected only the mutant sequence confirming presence of the mutant allele (Fig. 6b). To test if bhlhe22 might function with prdm12b in p1 formation, we examined eng1b expression...
in bhlhe22um320 animals by in situ hybridization. We find that expression of eng1b is unaffected in homozygous bhlhe22 mutants (Fig. 6c). Since siRNA-mediated knock-down of bhlhe22 reportedly disrupts gene expression in p0-p2 of chick embryos [60], we also examined expression of vsx2 in the p2 domain (Fig. 6d) and evx1 in the p0 domain (Fig. 6e), but do not observe any disruptions. We conclude that, in contrast to the situation in chick, zebrafish bhlhe22 is not required for p1 domain formation.

**prdm12b does not maintain the p1 domain by repressing nkx6.1**

Repressive interactions are common during formation of the neural tube, whereby mutually repressive pairs of TFs are involved in the establishment of individual progenitor domains (reviewed in [2, 15]). Since prdm12b appears to act as a repressor, it is plausible that it forms a repressive pair with nkx6.1 to establish the p1 domain and permit eng1b expression. Accordingly, nkx6.1 mutant mice display a ventral expansion of the p1 domain at the expense of the p2, pMN and p3 domains [67]. Furthermore, dorsal expansion of nkx6.1 has been reported in prdm12 MO-injected fish and frog embryos [22, 23] and overexpression of prdm12 inhibits nkx6.1 expression in frog embryos. To test this model further, we generated nkx6.1 mutant zebrafish by targeting a sgRNA to the 5′ end of exon 1. This produced eight founders carrying mutations in the nkx6.1 gene (Table 1; Additional file 3C; Additional file 12). Five of these were characterized further and found to transmit two different mutant alleles. The nkx6.1um321 allele contains a 23 bp deletion while the nkx6.1um322 allele carries a 1 bp insertion (as well as three single base pair substitutions). In both alleles, this leads to frameshifts that terminate at a premature stop codon upstream of the HOX domain. Accordingly, immunostaining with an anti-Nkx6.1 antibody revealed loss of Nkx6.1 protein in homozygous nkx6.1um321/um321 mutant animals (Fig. 7a). Similar to the situation with prdm12b mutations, we find that homozygous nkx6.1um321 mutant animals are observed at the expected ratio during early development, but we detect only a few homozygous nkx6.1um321 animals at adulthood (Fig. 7b). While nkx6.1 mutant mice display a profound loss of motor neurons [67], nkx6.1 MO-injected zebrafish show defective formation in only a subset of
Fig. 7 (See legend on next page.)
motor neurons and only at later stages of development [68, 69]. In general agreement with these MO-based zebrafish studies, we do not detect overt changes in expression of the hbr9 motor neuron marker in nkx6.1 mutant zebrafish (Fig. 7c), but we do observe subtle defects in the formation of branchiomotor neurons in the hindbrain (Fig. 7d).

We next used the nkx6.1 mutant fish to test if nkx6.1 and prdm12b act as a repressive pair to establish the p1 domain and enable eng1b expression. However, we do not find evidence for expansion of the eng1b expression domain in 24hpf embryos from a cross of nkx6.1+/um321 heterozygous mutants, a representative wildtype embryo (left panel) and a representative embryo from a cross of nkx6.1+/um321 heterozygotes (right panel). e Expression of eng1b in 24hpf uninjected wildtype embryos (left panel), 24hpf prdm12b MO-injected wildtype embryos (middle panel) and 24hpf prdm12b MO-injected embryos from a cross of nkx6.1+/um321 heterozygotes (right panel). g Expression of prdm12b in a representative wildtype embryo (left panel) and a representative embryo from a cross of nkx6.1+/um321 heterozygotes (middle panel) at 24hpf. Right panel shows quantification of the size of the prdm12b expression domain in 11 wildtype embryos and 20 embryos from a cross of nkx6.1+/um321 heterozygotes. Numbers in panels indicate the fraction of embryos displaying the phenotype shown.

We conclude that prdm12b does not maintain the p1 domain by repressing nkx6.1. a Anti-Nkx6.1 immunostaining of nkx6.1+/um321/um321 mutant (left) and wildtype (right) embryos at 30hpf. b Chart indicating the frequency of each genotype at various time points in broods from crosses of nkx6.1+/um321 heterozygous mutants. c Hb9 immunostaining in wildtype (left) versus a cross of nkx6.1+/um321 heterozygous embryos (right) at 33hpf. d Islet-1/2 immunostaining of 50hpf embryos from a cross of nkx6.1+/um321 heterozygotes. e Expression of eng1b in 24hpf embryos from a cross of nkx6.1+/um321 heterozygotes. f Expression of eng1b in 24hpf uninjected wildtype embryos (left panels), 24hpf prdm12b MO-injected wildtype embryos (middle panels) and 24hpf prdm12b MO-injected embryos from a cross of nkx6.1+/um321 heterozygotes (right panels). g Expression of prdm12b in a representative wildtype embryo (left panel) and a representative embryo from a cross of nkx6.1+/um321 heterozygotes (middle panel) at 24hpf. Right panel shows quantification of the size of the prdm12b expression domain in 11 wildtype embryos and 20 embryos from a cross of nkx6.1+/um321 heterozygotes. Numbers in panels indicate the fraction of embryos displaying the phenotype shown.

prdm12b germ line mutants recapitulate the phenotype observed using antisense-based approaches

Prdm12 function has been addressed previously, but only by transient loss of function approaches. In particular, antisense morpholino oligos (MOs) were first used in zebrafish [22] and subsequently in frog [23, 24] to disrupt prdm12 function. The resulting animals lack expression of eng1 in the p1 domain of the neural tube, but gene expression appears relatively normal in adjacent domains. eng1-expressing progenitors in the p1 domain are known to give rise to V1 interneurons that act in motor circuits (reviewed in [45]). Accordingly, fish and frogs lacking prdm12 function display abnormal escape responses [22, 23], but the nature of this effect (excessive C-bends) suggests a defect in a reticulospinal cell-controlled circuit that is likely independent of the loss of V1 interneurons. Importantly, recent work has highlighted significant concerns with MO-based approaches. In particular, there are many instances where
Germ line mutations do not confirm previous reported MO-based phenotypes [41]. While some of these cases may be explained by underappreciated compensatory mechanisms [70], there are striking examples of MO phenotypes that turn out to be due to non-specific or off-target effects [27]. Against this background, it is
essential to determine the phenotype of prdm12 germ-line mutants. To address this, we used CRISPR/Cas9 to generate two lines carrying frameshift mutations in the zebrafish prdm12b gene and also obtained an ENU-induced splice-site mutation from the zebrafish resource center. All three lines display a phenotype that is in good agreement with MO-derived data. In particular, germ-line mutants lack eng1b expression and display escape response defects indistinguishable from those in MO injected embryos. Hence, our findings indicate that, in this case, the various MOs act specifically. Since there is currently no available prdm12 knockout line in mouse, it remains possible that there will be species-specific differences in prdm12 function, as was recently observed when comparing MO-injected, zebrafish germ line mutants and mouse germ line mutants of the PG1 hox genes [71].

**prdm12b is a bona fide transcriptional repressor**

The Prdm12 TF has been suggested to act as a repressor based on overexpression studies in vivo and in dissected frog embryos [23, 24], but as an activator based on transfection experiments in P19 cells [49]. To address this discrepancy, we made use of classical reporter assays and find that zebrafish Prdm12b efficiently represses expression from a luciferase reporter gene. Other members of the Prdm family have been reported to act as repressors, but appear to use distinct mechanisms to do so. For instance, several Prdm TFs recruit histone deacetylases (HDACs) to repress transcription, but we find that an HDAC inhibitor does not affect the repressive properties of Prdm12b, indicating that it functions independently of HDACs. Overexpression of Prdm12b also promotes the deposition of repressive methyl marks on H3K9 [23, 24, 49]. Accordingly, the PR domain of some Prdm proteins exhibits methyltransferase activity and this domain is required for Prdm12 function in Xenopus [23]. However, we find that mutating a key conserved PR domain residue does not affect the repressive activity of prdm12b. Accordingly, in vitro analyses using core histone substrates failed to detect intrinsic methyltransferase activity for Prdm12 [49]. Notably, murine Prdm12 binds EHMT2/G9a (an H3K9 methyltransferase; [49]) and EHMT2/G9a is required for Prdm12 function in Xenopus [23], suggesting that Prdm12 may act as a repressor by recruiting EHMT2/G9a. We show that zebrafish Prdm12b also binds EHMT2/G9a, but in contrast to the situation in the mouse, the Prdm12b zinc finger domains are not required for this interaction.

In spite of the presence of several zinc finger domains, many Prdm proteins require interactions with other TFs for targeting to genomic binding sites. In particular, several Prdm proteins form complexes with bHLH TFs [15]. For instance, Bhlhe22 is known to interact with Prdm TFs [20] and is required for expression of eng1 in the chick neural tube [60], making it a candidate interaction partner for Prdm12b. Indeed, we show by co-immunoprecipitation that Prdm12b and Bhlhe22 can form a complex. Furthermore, this interaction requires the Prdm12b zinc finger domain that we find is required for Prdm12b repressor activity. To test the role for bhlhe22 in vivo, we used CRISPR/Cas9 to generate a germ-line mutant in zebrafish, but we do not find any evidence that bhlhe22 is required for formation of the p1 domain in zebrafish embryos. It is not clear why loss of bhlhe22 function produces different effects in zebrafish versus chick, but this may stem from the different approaches used – germ-line mutation in zebrafish versus transient siRNA-mediated knock-down in chick. The lack of a phenotype may also be the effect of compensatory mechanisms, either by other bHLH TFs - which are broadly expressed in the neural tube [72] – or by more general mechanisms operating to suppress the effects of genetic lesions [73]. We conclude that Prdm12b acts as a repressor of transcription – most likely by recruiting EHMT2/G9a – and that the Prdm12-mediated induction of genes such as p27 is most likely the result of indirect events.

**An undefined domain persists at the p1 position in prdm12b mutants**

The mechanism whereby prdm12 promotes formation of the p1 domain remains unclear. Mutual repression between TFs expressed in adjacent domains is the predominant mechanism for the creation of distinct domains along the dorsoventral axis of the vertebrate neural tube. Since prdm12 functions as a repressor, it is possible that it acts to repress the formation of adjacent domains. Indeed, overexpression and MO-based approaches in the frog have led to the suggestion that prdm12 and nkx6.1 (that is expressed in the p2, p3 and pMN domains) forms such a cross-repressive pair [23]. In this model, loss of prdm12 would lead to loss of eng1 expression due to nkx6.1 expression (and p2 fates) expanding into the p1 domain. However, our initial analyses of nkx6.1 mutants zebrafish do not support this model. First, if prdm12b is required for eng1 expression in the p1 domain due to its repression of nkx6.1, eng1b should be restored to the p1 domain in embryos lacking both nkx6.1 and prdm12b, but this is not what we observe. Second, if prdm12b and nkx6.1 cross-repress each other's expression, prdm12b expression should expand ventrally in nkx6.1 mutants and vice versa, but this also does not occur. Lastly, when one member of a cross-repressive pair is mutated, the corresponding progenitor fate is usually replaced by the adjacent fate, but this is not the case in prdm12b mutants – where a domain persists at the p1 position, albeit in a narrower form. Since this domain does not express any of the genes diagnostic for various fates along the DV axis, its exact state is not
clear. We note that prdm12 is reported to have anti-proliferative activity [49] and that p1 progenitor cells must exit the cell cycle prior to differentiating into V1 interneurons. It is therefore possible that prdm12 is required for this transition and that loss of prdm12 leaves cells in a proliferative progenitor state.

**Conclusion**

Our results demonstrate an essential role for prdm12b in zebrafish neurogenesis. By generating germline mutations, we show that a loss of function prdm12b allele results in lack of eng1b-expressing V1 interneurons, defective Mauthner cell-dependent locomotion—which is indistinguishable from prdm12b morphants—and ultimately embryonic lethality. Further analyses revealed that the Prdm12b zinc finger domain, which is essential for repression, is also necessary for binding to the Blhle22 TF, but not to EHMT2/G9a. We generated a blhle22 mutant zebrafish line, but find no evidence for blhle22 function in the formation of the p1 domain in zebrafish embryos. Lastly, upon examination of cross-repressive interaction between prdm12b and nkx6.1, we do not find evidence for nkx6.1 and prdm12b acting as a repressive pair in the formation of the p1/p2 boundary. Our results suggest that prdm12b does not only regulate eng1b expression in the p1 domain, but also takes part in regulating the size of this domain.

**Additional files**

- **Additional file 1:** Sequences of oligos used to generate CRISPR guide RNAs. Detailed features of the oligos used as templates for each guide RNA. (DOCX 13 kb)
- **Additional file 2:** Sequences of primers used to genotype mutant lines. Detailed features of primers used to genotype mutant lines. For blhle22, the blhle22–1 and blhle22–2 primers were used to amplify genomic DNA while the blhle22–3 and blhle22–4 primers were used to amplify cDNA. (DOCX 13 kb)
- **Additional file 3:** Sequences of mutagenic oligos used to amplify genomic DNA from fish. The predicted amino acid sequence for each mutant allele was aligned to the corresponding wildtype sequence using Clustal Omega. (PDF 236 kb)
- **Additional file 4:** Characterization of the prdm12bumb777 mutant. a. Schematic showing genomic sequence of prdm12b. Exons are indicated as boxes and black lines represent introns. The PR domain and three zinc fingers (ZnF) are highlighted in dark red and blue, respectively. The black arrow indicates a single base pair change in the second intron of prdm12bumb777. b. Sequence traces confirming the expected single nucleotide change in wildtype (b) versus prdm12bumb777 (c) animals. d-g. eng1b expression in 24hpf embryos from a cross of prdm12bumb777 (c) animals. Embryos are shown in dorsal (d), e, or lateral (f, g) view with anterior to the left. eng1b expression is lost in 27% of embryos compared to 73% of embryos showing wildtype eng1b staining. (PDF 1962 kb)
- **Additional file 5:** Detailed analysis of the touch-evoked escape response in prdm12b mutant and wild type animals. a, b. Representative kinematic traces of individual wild type (a) and prdm12b mutant (b) animals stimulated with a head tap (from Fig. 3a, b). c. Quantification of number of body bends with an amplitude similar to the C-bend (defined as exceeding 100°) from data collected in Fig. 3a, b. d. Quantification of C bend duration (from data collected in Fig. 3a, b). e, f. Representative kinematic traces of individual prdm12b mutant animals stimulated with a head (left panels) or a tail (right panels) tap (from Fig. 3c, d) (212 MB)
- **Additional file 6:** Movie of wild type touch-evoked response. Movie of representative wild type animal tapped on the head (from Fig. 3a; recorded at 1000 frames/second). (MP4 794 kb)
- **Additional file 7:** Movie of prdm12b mutant touch-evoked escape response. Movie of representative prdm12b mutant animal tapped on the head (from Fig. 3b; recorded at 1000 frames/second). (MP4 1842 kb)
- **Additional file 8:** Movie of prdm12b mutant touch-evoked escape response. Movie of representative prdm12b mutant animal tapped on the head (from Fig. 3c; recorded at 1000 frames/second). (MP4 771 kb)
- **Additional file 9:** Movie of prdm12b mutant touch-evoked escape response. The same prdm12b mutant animal as in Additional file 8; was instead tapped on its tail (from Fig. 3d; recorded at 1000 frames/second). (MP4 1040 kb)
- **Additional file 10:** Expression of GAL4DBD–Prdm12b constructs used in transfection experiments. a. Immunoblot showing expression of GAL4DBD–Prdm12b constructs in transfected HEK293T cells. All constructs are stable except Myc–GAL4–Prdm12b. b. Immunoblot showing expression of Myc–Flag–G9a and Myc–Flag–Blhle22 constructs in transfected HEK 293 T cells. (PDF 619 kb)
- **Additional file 11:** Generation of blhle22 germline mutant. a. Schematic showing genomic sequence of blhle22 with the bHLH domain indicated in blue. Note that blhle22 is contained on a single exon. The CRISPR target sequence is shown in red with the BstY1 restriction site bracketed and the black arrow indicating the BstY1 cut site. b. Identification of functional guide RNAs. sgRNA and cas9 mRNA was injected into 1-cell stage embryos. Injected embryos were raised to 24hpf and BstY1 digestion of PCR amplicons from pools of embryos was used to identify CRISPR-induced mutations (black arrow). c. Identification of individual F0 founders. sgRNA/cas9 injected embryos were raised to adulthood and crossed to wildtype fish. BstY1 digests of PCR amplicons from pools of embryos was used to identify F0 mosaic founders (black arrow). d. Identification of F1 animals. Adult F0 mosaic founders were out-crossed to wildtype fish and the F1 offspring raised to adulthood. BstY1 digests of PCR amplicons from pools of embryos was used to identify F0 mosaic founders (black arrow). e. Sequencing of lncRNA genomic DNA revealed the transmission of one mutant allele (um329) carrying a 5 base pair deletion (black dashes). The CRISPR target sequence is shown in red. f. Predicted amino acid sequence of mutant allele. The um320 peptide shares its first 67 amino acids with the wildtype protein before going out of frame and terminating at a premature stop codon N-terminal to the bHLH domain. (PDF 485 kb)
- **Additional file 12:** Generation of germ line nkx6.1 mutants. a. Schematic showing genomic sequence of nkx6.1 with the homeodomain indicated in green. The CRISPR target sequence is shown in red with the AvaI restriction site bracketed and the black arrow indicating the AvaI cut site. b. Identification of functional guide RNAs. sgRNA and cas9 mRNA was injected into 1-cell stage embryos. Injected embryos were raised to 24hpf and AvaI digestion PCR amplicons from pooled embryos was used to identify CRISPR-induced mutations (black arrow). c. Identification of individual F0 founders. sgRNA/cas9 injected embryos were raised to adulthood and crossed to wildtype fish. Avail digests of PCR amplicons from pools of embryos was used to identify F0 mosaic founders (black arrow). d. Identification of F1 animals. Adult F0 mosaic founders were out-crossed to wildtype fish and the F1 offspring raised to adulthood. Avail digests of PCR amplicons from fin clip genomic DNA was used to identify heterozygous F1 animals. e. Sequencing of F1 genomic DNA revealed the transmission of two mutant alleles (um338) carrying a 23 base pair deletion (black dashes). The CRISPR target sequence is shown in red. f. Predicted amino acid sequence of mutant allele. The um330 peptide shares its N-terminal 67 amino acids with the wildtype protein before going out of frame and terminating at a premature stop codon N-terminal to the conserved homeodomain. g. Quantification of the size (along the dorsoventral axis) of the nkx6.1 expression domain in prdm12b MO-injected embryos (data from Fig. 8). (PDF 651 kb)
Abbreviations
bHLH: Basic Helix-Loop-Helix; CNS: Central nervous system; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; DBD: DNA binding domain; dpf: Days post fertilization; DV: Dorsal-ventral; ENU: N-ethyl-N-nitrosourea; HD: Homeodomain; HDAC: Histone deacetylase; HMT: Histone methyl transferase; hpf: Hours post fertilization; MHV: Mid-Hindbrain boundary; MO: Morpholino; sgRNA: Single guide RNA; Shh: Sonic hedgehog; TF: Transcription factor; TSA: Trichostatin A; WT: Wildtype; ZIRC: The Zebrafish International Resource Center; ZNF: Zinc Finger

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Availability of data and materials
Mutant lines generated in this study are available upon request.

Authors’ contributions
OY participated in the design of the study, generated the CRISPR-based mutant lines, performed all in situ and immunohistochemistry analysis of the mutant lines, carried out all reporter assays and co-immunoprecipitation experiments, performed the computational analyses of escape responses and drafted the manuscript. GBD carried out and recorded the escape response, assisted in data interpretation, reviewed, edited and approved the manuscript. CGS conceived the study, secured funding, participated in study design, and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval
This study was submitted to and approved by the University of Massachusetts Medical School Institutional Review Board.

Consent for publication
Not applicable.

Competing interests
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