Published in final edited form as:

Biochim Biophys Acta. 2015 August ; 1849(8): 1040–1050. doi:10.1016/j.bbagr.2015.06.006.

The chromatin remodeler *chd5* is necessary for proper head development during embryogenesis of *Danio rerio*

Brett Bishop^a, Kwok Ki Ho^a, Kim Tyler^a, Amanda Smith^a, Sylvia Bonilla^b, Yuk Fai Leung^b, and Joe Ogas^{a,*}

^aDepartment of Biochemistry and Purdue Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907

^bDepartment of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Abstract

The chromatin remodeler CHD5 plays a critical role in tumor suppression and neurogenesis in mammals. CHD5 contributes to gene expression during neurogenesis, but there is still much to learn regarding how this class of remodelers contributes to differentiation and development. CHD5 remodelers are vertebrate-specific, raising the prospect that CHD5 plays one or more conserved roles in this phylum. Expression of *chd5* in adult fish closely mirrors expression of *CHD5* in adult mammals. Knockdown of *Chd5* during embryogenesis suggests new roles for CHD5 remodelers based on resulting defects in craniofacial development including reduced head and eye size as well as reduced cartilage formation in the head. In addition, knockdown of *Chd5* results in altered expression of neural markers in the developing brain and eye as well as a profound defect in differentiation of dopaminergic amacrine cells. Recombinant zebrafish *Chd5* protein exhibits nucleosome remodeling activity in vitro, suggesting that it is the loss of this activity that contributes to the observed phenotypes. Our studies indicate that zebrafish is an appropriate model for functional characterization of CHD5 remodelers in vertebrates and highlight the potential of this model for generating novel insights into the role of this vital class of remodelers.

Keywords

CHD remodeler; embryogenesis; neural development; ATP-dependent chromatin remodeling

1. Introduction

ATP-dependent chromatin remodelers play a key role in both activation and repression of genes that exhibit developmental regulation [1,2]. Loss of these remodelers is often

*Correspondence to Joe Ogas, Purdue University, 175 S. University St., West Lafayette IN, 47907. Telephone: (765) 496-3969, ogas@purdue.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors do not identify any competing financial interests with this manuscript.

associated with pronounced developmental abnormalities and death in animal systems. Characterizing the diverse contributions of chromatin remodelers to transcriptional circuits is integral to understanding developmental regulation in eukaryotes.

Members of a subset of the CHD family of ATP-dependent remodelers, identified as subfamily II [3–5], have been strongly linked to determination of developmental identity. All ATP-dependent remodelers possess an ATPase domain that powers the remodeling of nucleosomes [6,7]. CHD family members possess two additional domains of sequence homology: a chromodomain region that contributes to recognition of the nucleosomal substrate [8–10] and a Myb-like DNA binding domain that has been demonstrated to possess DNA-binding activity for some family members [6,7,11,12]. CHD remodelers have been further broken into subfamilies I, II, and III, based on additional conservation of domain architecture [3–5,8–10]. In particular, almost all members of subfamily II have 1–2 plant homeodomain (PHD) zinc fingers, which contribute to recognition of histones with specific methylation states [13–18].

Three members of subfamily II of CHD remodelers are found in vertebrates: CHD3, CHD4, and CHD5 [5,19]. CHD3 and CHD4 have been extensively characterized biochemically and genetically as members of the Mi-2/NuRD complex that promotes histone deacetylation and gene inactivation [20–22]. This complex is the most abundant histone deacetylase complex in mammalian cells and can be targeted to regions of DNA enriched for methylation or hydroxymethylation [23] as well as by protein-protein interactions [22,24] and plays a critical role in silencing developmentally regulated genes. In addition, this class of remodelers has been linked to gene activation [25–28], demonstrating that these remodelers can contribute to both positive and negative regulation of gene expression.

CHD5 exhibits identical domain architecture and high sequence similarity to CHD3 and CHD4 and is also a component of a Mi-2/NuRD-like complex [29,30], raising the prospect that it contributes to highly similar remodeling events. Several lines of evidence suggest, however, that *CHD5* plays a functional role that is distinct from that of *CHD3* and *CHD4*. *CHD3* and *CHD4* are expressed fairly ubiquitously in mammals whereas *CHD5* is preferentially expressed in the nervous system of adult mammals as well as in the testes [13,29,31–34]. Investigation of a role for *CHD5* during brain development revealed that *CHD5* is necessary for differentiation of embryonic neurons [13]. The authors also show that CHD5 preferentially associates with the repressive epigenetic modification trimethylation of histone H3 lysine 27 (H3K27me3) and that this interaction is mediated by its chromodomains [13]. This interaction is likely to contribute to the function of CHD5; depletion of *CHD5* in mouse embryonic stem cells (ESCs) undergoing differentiation to neurons results in increased expression of a cohort of H3K27me3-repressed genes that are normally expressed in other lineages. Further, induction of neuronal genes is impaired in *CHD5*-depleted ESCs, indicating that *CHD5* is also necessary for activation of these genes. Chromatin immunoprecipitation (ChIP) analysis revealed that CHD5 associates with the promoters of both the affected neuronal genes and H3K27me3-repressed genes, suggesting that CHD5 directly contributes in some fashion to both gene activation and repression much like CHD3 and CHD4 remodelers. Similarly, *CHD5* contributes to both gene activation and repression during spermiogenesis [35].

Obtaining a detailed understanding of the role of *CHD5* in gene expression and development is strongly motivated by the importance of *CHD5* as a tumor suppressor in mammals. Loss or reduced expression of *CHD5* is associated with formation of a wide range of tumors including colorectal cancer, lung cancer, and leukemia [36–41]. Loss of *CHD5* plays a particularly significant role in development of neuroblastoma [31,42–45]. Neuroblastoma arises during embryogenesis and is derived from precursor cells of the peripheral sympathetic nervous system [46,47]. The recent demonstration of a requirement for *CHD5* in specification of neural identity [13] provides a compelling rationale for why loss of *CHD5* contributes to neuroblastoma.

The etiology of neuroblastoma suggests that understanding the role of *CHD5* during embryogenesis may be critical to understanding its role as a tumor suppressor. In addition, although the role of *CHD5* has been explored in the developing brain of mouse embryos [13], it remains to be determined if *CHD5* remodelers contribute to other developmental programs during embryogenesis. Here we use biochemistry, expression analysis, and morpholino knock-down to characterize the role of *chd5* in the zebrafish model system, which offers distinct advantages for study of both embryogenesis and oncogenesis [48–51]. We find that many aspects of *CHD5* remodelers are conserved in zebrafish and mammals indicating that zebrafish are indeed a robust model system for functional analysis of *CHD5* remodelers. In particular, our data support a role for *chd5* in both brain and eye development during zebrafish embryogenesis and also reveal that *CHD5* remodelers play a more global role during embryogenesis than previously appreciated.

2. Materials and Methods

2.1. Restriction enzyme accessibility assays

Accessibility assays were based on Smith et al., 2005 and carried out as described in Ho et al., 2013. In brief, 15 µl reactions with mononucleosomes reconstituted on fluorescently labeled 343-bp DNA. The reactions were prepared on ice using the corresponding buffer (New England Biolabs), 0.1 mg/ml bovine serum albumin, 2.67 mM ATP, 10 nM *Chd5* and 48 nM mononucleosomes and initiated with the addition of the individual restriction enzymes (1U/ul). After incubating in a thermocycler at 30 °C for 30 min, reactions were deproteinated with 7.5 µl of stop buffer (1.5% SDS, 100 mM EDTA, 15% glycerol and 300 ng proteinase K) and kept at 55 °C for 25 min. Cut and uncut DNA were separated on 8% non-denaturing acrylamide/bisacrylamide (37.5:1, 2.6% C) Tris Tricine-gel. Gels were visualized on a Typhoon 8600 Imager (GE Healthcare) and quantified using ImageJ.

2.2. Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Purdue Animal Care and Use Committee (Permit Number: 120100592)

2.3. Zebrafish Maintenance and Embryo Collection

Zebrafish wild-type lines were maintained according to standard procedures [52]. Embryos were collected after breeding the parental fish in pairs for 15 min, maintained in E3 medium

at 280C, and staged as described in Kimmel et al, 1995. For embryos that were collected for *in situ* hybridization, 0.003% PTU (Sigma, St. Louis, MO) in E3 medium was applied between 12 and 23 hours post fertilization (hpf) to inhibit melanization [53].

2.4. Quantitative Reverse-Transcription PCR (qRT-PCR)

Total RNAs were extracted from either adult tissues or developing embryos. Tissues were derived from two adults or twenty embryos per sample for each developmental time point. RNA was extracting using TRIZOL (Ambion, #15596026). qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and run on a StepOnePlus real-time PCR system (Applied Biosystems), as described by the manufacturer. Sequences of primers used in this analysis are provided in Supplemental Table 1. β -actin was utilized as an internal control. All primers were designed and purchased from Integrated DNA Technologies (IDT, Coralville, IA). Three technical replicates of each of two biological replicates were included for each sample.

2.5. Morpholino (MOs) and Full Length RNA Injection

To knock down *Chd5* (NCBI Reference Sequence: XM_003199497.2 and sequenced full-length *chd5*), two nonoverlapping translation-blocking morpholinos (MO) (5 ng *chd5*MO1: CGGGCATCTTTACCGCATAACAAGA; 5ng *chd5*MO2: AAATCACATCCGAGA) were used. Morpholinos were individually injected into the zebrafish embryos at the one cell stage [54]. The corresponding amount of a standard control MO (CoMO) (CCTCTTACCTCAGTTACAATTTATA) was injected into the embryos to control for non-specific effects resulting from microinjection. All MOs were purchased from Gene Tools (Philomath, OR). Full-length *chd5* for injection was produced from plasmid pJO1301, in which *chd5* is cloned downstream of the Sp6 promoter. Full-length capped *chd5* mRNA was synthesized using the mMessage mMachine Kit (Ambion, Product #AM1340). Embryos were injected with 90 pg of *chd5* mRNA at the one-cell stage with and without 5ng of *chd5*MO1. Full-length *mCherry* for injection was produced from plasmid pJO1344, in which *mCherry* is cloned downstream of the Sp6 promoter. Full-length capped *mCherry* mRNA was synthesized as described above. Embryos were injected with 90pg of *mCherry* mRNA at the one-cell stage with and without 5ng of *chd5*MO1.

2.6. In Situ Hybridization

In situ hybridization was conducted as described previously in Hensley et al., 2011. The riboprobes used in this study are as follows: *chd5* (this study), *fgf8* (this study), *otx2* [55], *pax6a* [56], and *th* [57]. To prepare a probe for *chd5*, a fragment of the *chd5* gene was amplified from cDNA prepared from embryos with the following primers (*chd5*-1F: GATGCCCCGGATCGCTCAGCA; *chd5*-1R: TGGACGACTTTGGTTCCTTC) To prepare a probe for *fgf8*, a fragment of the *fgf8* gene was amplified from cDNA prepared from embryos with the following primers (*fgf8*-1F: TCCTTCACCTCTTTGCGTTT; *fgf8*-1R: TGCGTTTAGTCCGTCTGTTG). The resulting PCR fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI) for propagation. The riboprobes were synthesized using the DIG RNA labeling Kit (SP6/T7) (Roche, #11175025910).

2.7. Alcian Blue and Acridine Orange Staining

Alcian blue analysis was performed as previously described [58]. In brief, embryos were fixed in acid-ethanol when they were 72 hpf. These embryos were subsequently stained overnight in 0.1% Alcian Blue dissolved in acidic ethanol (70% ethanol, 5% concentrated hydrochloric acid) prior to analysis. The embryos were washed extensively in acidic ethanol, dehydrated and stored in 80% glycerol. Acridine orange analysis was performed as previously described in Tucker and Lardelli 2007. In brief, live embryos were immersed in 1mL of 2 µg/mL acridine orange in E3 medium (Sigma, <http://www.sigmaaldrich.com>) for 1 hour, then visualized and imaged for less than 60 s.

2.8. Image Acquisition of all embryos

All embryos were immobilized in 3% methylcellulose for image acquisition. Bright-field and fluorescent images were acquired by a SPOT-RT3™ color slider camera (Diagnostic Instruments) mounted on an Olympus BX51 fluorescence compound microscope. For morphological analysis, the head and eye lengths of the 72 hpf embryos were measured using i-Solution (IMT i-Solution). Subsequent data analyses including ANOVA and Student's *t*-tests were performed in Microsoft Excel.

3. Results

3.1. The zebrafish Chd5 protein is an ATP-dependent chromatin remodeler

A previous comprehensive phylogenetic analysis of CHD remodelers from animals and plants clearly places the zebrafish Chd5 protein in the same clade as other vertebrate CHD5 remodelers [19]. Based on its similarity to other known chromatin remodelers, we hypothesized that the zebrafish Chd5 protein exhibits ATP-dependent chromatin remodeling activity. In order to test this hypothesis, we performed a standard assay for remodeling activity: we used a restriction endonuclease to measure the accessibility of DNA incorporated within a mononucleosome [59,60]. We generated recombinant zebrafish Chd5 that was tagged with the FLAG epitope at the C-terminus using the baculovirus expression system. Mononucleosomes were reconstituted on a fluorescently end-labeled 343-bp fragment of DNA containing the 601 nucleosome positioning sequence and a unique P1mI site (Fig. 1A). The presence of recombinant zebrafish Chd5 and ATP greatly increased digestion of the nucleosomal fragment by P1mI by 40% compared to a Chd5-only control (appearance of cut band in Fig. 1B), demonstrating that the zebrafish Chd5 protein exhibits ATP-dependent chromatin remodeling activity.

3.2. The expression of *chd5* in adult zebrafish resembles that of *CHD5* in adult mammals

Phylogenetic analysis suggests that CHD5 remodelers are unique to vertebrates [19] raising the possibility that CHD5 remodelers have a conserved function in this phylum. In mammals, *CHD5* promotes neural differentiation [13] and is preferentially expressed in the brain and to a lesser extent in the testes and eye [13,29,32]. To begin examining whether *chd5* plays a role in zebrafish that is similar to the role that *CHD5* plays in mammals, we examined whether the pattern of expression of *chd5* in adult zebrafish is similar to that of *CHD5* genes in adult mammals. We used quantitative reverse transcription (qRT)-PCR to

determine the relative transcript level of *chd5* in different tissues of adult zebrafish. As previously observed in adult mammals, *chd5* is preferentially expressed in the brain, testes, and eyes of adult zebrafish relative to the tail fin with the greatest relative expression in the brain (Fig. 2). In contrast, the closely related CHD remodeler genes *chd3* (XM_005172235.1), *chd4a* (NM_001044858), and *chd4b* (XM_680607) are expressed at largely similar levels in these tissues relative to the tail fin. These expression data thus indicate that *chd5* remodelers exhibit a distinct expression pattern from *chd3* and *chd4* remodelers in fish as previously observed in mammals [29,32,61].

3.3. The pattern of *chd5* expression changes during embryogenesis

Characterization of *CHD5* in mammals strongly suggests that *CHD5* plays one or more roles during mammalian embryogenesis [13,46,52]. We used both qRT-PCR and *in situ* hybridization to examine expression of *chd5* during zebrafish embryogenesis to begin addressing when and where *chd5* might act. qRT-PCR analysis shows that the transcript level of *chd5* increases dramatically during embryogenesis until leveling off around 72 hours post fertilization (hpf) (Fig. 3A). Total expression of *chd3*, *chd4a*, and *chd4b*, in contrast, remains relatively constant during these times.

Intriguingly, *in situ* hybridization revealed that *chd5* was expressed relatively ubiquitously throughout the developing organism at 10 and 15 hpf, suggesting that *chd5* plays a global role during early embryogenesis (Fig. 3B and 3C). At 24 hpf and thereafter, expression of *chd5* became preferentially localized toward the anterior portion of the embryo in regions corresponding to the brain (Fig. 3D – 3F), likely reflecting the acquisition of the brain-specific expression observed in adults. The sense probe for *chd5* did not show any *in situ* staining, suggesting that the observed staining of the antisense probe was not the result of non-specific hybridization (Fig. 3G).

3.4. *Chd5* is necessary for proper craniofacial development during embryogenesis

To explore the role of *chd5* during zebrafish embryogenesis, we knocked down expression of *chd5* protein using two non-overlapping antisense morpholino oligonucleotide (MO) designed to inhibit translation of *chd5* mRNA. The predicted binding site of *chd5*MO1 overlaps the initiation codon of the *chd5* transcript whereas the predicted binding site of *chd5*MO2 is immediately 5' of *chd5*MO1 in the 5' untranslated region of the *chd5* transcript. Embryos injected with *chd5*MO1 possess small eyes, small heads, and small bodies (225 larvae examined, quantitative measurement of 8 representative samples from this population; Fig. 4A – 4D). Embryos injected with *chd5*MO2 are visually indistinguishable from embryos injected with *chd5*MO1, (data not shown). Quantitative analysis of *chd5*MO1 embryos reveals that both the head and eye lengths are approximately 81% and 73% the size of uninjected embryos or embryos injected with a control morpholino (CoMO) (Fig. 4A and 4B). We performed an analysis of variance (ANOVA) on these data, which indicated a statistically significant difference in both mean head length and mean eye length between the populations. Student's two-tailed *t*-test was also used to specifically compare the head length and eye length of *chd5*MO1-injected embryos with CoMO-injected embryos. Both head length and eye length were significantly reduced in the *chd5*MO1-injected embryos ($p < 0.05$). In order to address the possibility that the reductions in head

length and eye length are the consequence of a nonspecific reduction in overall body size, we normalized the head and eye length to whole body length for each morphant. These normalized values were statistically analyzed as described above and found to be significant, indicating that the head and eye size are preferentially reduced in Chd5 morphants.

To determine if the small head/eye phenotypes of chd5MO1 larvae are due to reduced expression of Chd5 rather than non-specific or off-target effects resulting from injection with chd5MO1, we examined whether injection of *in vitro* transcribed full-length *chd5* mRNA could rescue these phenotypes. For this analysis, we utilized a *chd5* transcript that lacks the chd5MO1 target sequence and therefore should be translated even in the presence of the morpholino. Previous work in mice suggested that this type of rescue could be problematic as a result of deleterious phenotypes triggered by elevated levels of *chd5* transcript [53,61]. Nevertheless, we observed that co-injection of *chd5* transcript with chd5MO1 resulted in a modest but significant reduction in the number of fish exhibiting abnormal chd5MO1-associated phenotypes (Fig. 5). Using a chi-square analysis at a significance threshold of 0.05, the null hypothesis of no difference from the morphant-only distribution is rejected ($p < 0.001$). Importantly, embryos injected with *chd5* mRNA alone were indistinguishable from embryos injected with CoMO (Fig. 5). We repeated this experiment several times to demonstrate that the rescue was reproducible (data not shown). To investigate if the rescue of the morphant phenotype by *chd5* mRNA is due to a non-specific effect resulting from addition of an exogenous transcript, we examined whether addition of full-length *mCherry* mRNA could similarly rescue the morphant phenotype. Unlike co-injection of *chd5* transcript, co-injection of *mCherry* transcript with chd5MO1 failed to rescue chd5MO1-associated phenotypes (Supp. Fig. 1). Taken together, our analyses indicate that the phenotypes observed in Chd5 morphant embryos are specific and result from depletion of the Chd5 protein.

3.5. Loss of Chd5 leads to a defect in cartilage formation during embryogenesis

The preferential impairment of head and eye development in larvae injected with chd5MO1 or chd5MO2 is consistent with the expression pattern of *chd5* observed at 24 hpf and thereafter (Fig. 3). To examine whether there are other defects in craniofacial development, we stained 72hpf larvae with Alcian blue to examine cartilage development [54,58]. This analysis revealed greatly reduced cartilage formation in both chd5MO1 and chd5MO2 larvae ($n=25$) relative to uninjected and CoMO larvae ($n=25$). In particular, the mandibular cartilage is substantially reduced, and there is a decrease in cartilage in the chd5MO1 and chd5MO2 larvae (2–3 craniofacial cartilages in CoMO larvae versus 0–1 craniofacial cartilages in chd5MO larvae, Fig. 6).

Co-injection of *chd5* transcript with chd5MO1 resulted in modest rescue of cartilage formation as observed for the chd5MO1-induced small head/eye phenotype. Importantly, this and every other rescue experiment were carried out independently, and fish were sorted and scored based on the specific phenotype of interest in each rescue experiment (e.g. cartilage formation in Fig. 6). Thus we observe that modest rescue of chd5MO1-dependent phenotypes by *chd5* transcript is reproducible for a spectrum of phenotypes.

3.6. Loss of Chd5 increases cell death during embryogenesis

One possible explanation for reduced organ size is increased cell death. To test the hypothesis that Chd5 morphants exhibit increased cell death, we stained 24 hpf embryos with acridine orange to identify dead cells. We observed a substantial increase in staining in chd5MO1- and chd5MO2-injected embryos versus the uninjected and CoMO injected embryos (Fig. 7). Importantly, we observed increased staining in both the head and trunk of the embryo, suggesting that the decreased size of both regions of the body is due in part to increased cell death in those regions leading to a reduction in cell number. It is worth noting that these data also provide evidence that *chd5* acts in a widespread fashion in the developing embryo as suggested by the *in situ* analysis of 10 and 15 hpf embryos (Fig. 3B and 3C). Co-injection of *chd5* transcript with chd5MO1 suppresses the observed increase in acridine orange positive cells (Fig. 7) indicating that the increase in cell death is a specific effect due to reduced levels of Chd5 in the embryos. Rescue of this phenotype is particularly noteworthy in light of the observation that cell death is a common off-target effect in response to injection with morpholinos [55,63].

3.7. Chd5 is necessary for proper expression of neural markers in the brain

The preceding analyses along with previously published data strongly suggest that loss of *chd5* leads to altered brain development in zebrafish, most likely as a result of altered neural differentiation [13,56]. To investigate the possibility that loss of *chd5* affects brain development, we examined whether the expression pattern of well-characterized neural markers was perturbed in the developing brain of chd5MO1-injected zebrafish embryos. We used *in situ* hybridization to characterize the expression of three genes that promote normal brain and/or eye development and are expressed in distinct regions of the brain of 24 hpf embryos: fibroblast growth factor *fgf8* [57,64], homeobox gene *otx2* [58,65,66], and homeobox gene *pax6a* [19,67,68].

We found that the expression of all three genes was altered in chd5MO1 embryos relative to uninjected embryos or CoMO embryos (Fig. 8). Expression of *pax6a* was reduced in both the telencephalon of the forebrain as well as in the hindbrain whereas expression of *otx2* was dramatically reduced in the midbrain. Similarly, the domain of *pax6a* expression is reduced in the eye. In contrast, we observed that expression of *fgf8* was modestly but consistently increased in the telencephalon, the dorsal diencephalon, and the optic stalk. Thus, our *in situ* analysis reveals that the fore-, mid-, and hindbrain all exhibit altered gene expression, indicating that *chd5* acts throughout the brain. Importantly, such a widespread effect on gene expression in Chd5 morphants is consistent the observed pattern of expression of this remodeler in developing embryos (Fig. 3).

These analyses also identify candidates for regulatory pathways that are perturbed by reduced expression of *chd5* and may thereby contribute to altered neural development. *fgf8* and *otx2* play important roles in brain development [59,60,64,69,70] whereas *otx2* and *pax6a* have similarly been shown to be necessary for normal eye development [19,55,70–73]. As a result, reduced expression of *otx2* and *pax6a* in chd5MO embryos may contribute to altered eye and brain development in these lines.

3.8. Loss of Chd5 impairs differentiation of dopaminergic amacrine cells

In light of the significant role that loss of *CHD5* plays in development of neuroblastoma in mammalian systems [13,42,44,45], we examined whether our Chd5 morphants exhibit phenotypic similarity to morphants of a zebrafish homolog to another mammalian gene causally linked to neuroblastoma: *phox2b* [13,29,32,57]. PHOX2B is a homeodomain transcription factor that has been shown to act as a critical regulator of sympathetic neuron differentiation in murine and avian models [29,32,61,74]. Neuroblastoma is thought to result from aberrant differentiation of progenitors of sympathetic neurons, and loss or mutation of *PHOX2B* is strongly associated with development of neuroblastoma [75]. *phox2b* morphants exhibit impaired differentiation of sympathetic neurons [57], indicating that the role of this transcriptional regulator in differentiation of these cells is conserved in zebrafish [57]. We similarly analyzed the contribution of *chd5* to differentiation of sympathetic neurons by undertaking *in situ* analysis of tyrosine hydroxylase (*th*), a marker for terminal differentiation of sympathetic neurons. Our data indicated that Chd5 morphants are not impaired in differentiation of sympathetic neurons in the superior cervical ganglion (SCG) (Fig. 9) in contrast to the defect observed in *phox2b* morphants [57]. Expression of another differentiation marker, dopamine beta hydroxylase (*dbh*), was similarly indistinguishable from uninjected and CoMO injected embryos in these cells (data not shown).

Strikingly, however, we did observe a dramatic decrease in *th*-expressing cells in the eyes of Chd5 morphants and also found that this phenotype was rescued by co-injection of *chd5* transcript (Fig. 9) but not by co-injection of *mCherry* transcript (Supp. Fig. 2). *th*-expressing cells correspond to the dopaminergic amacrine cells in the retina [76,77], which are interneurons that play a role in determining the sensitivity of other retinal neurons during light adaptation in other species [78–80]. Thus Chd5 morphants exhibit a highly penetrant defect in differentiation of at least one type of neuron, consistent with the recently established role for *CHD5* in neurogenesis in mammalian systems [13].

4. Discussion

CHD5 is a vertebrate-specific ATP-dependent chromatin remodeler that belongs to a subfamily of CHD proteins that contribute to both repression and activation of gene expression [8–10]. In particular, CHD5 and related remodelers play a key role in expression of genes that contribute to tissue identity during development. In mammals, *CHD5* is preferentially expressed in neural tissue and in testes, and loss of *CHD5* leads to defects in both neurogenesis and spermatogenesis in this class of animals [13,34,35]. In addition, *CHD5* acts as a potent tumor suppressor in a variety of tissues in mammals [37], suggesting a more global role in facilitating differentiation. Here we find that zebrafish *chd5* shares several characteristics with mammalian *CHD5* and uncover new potentially conserved roles for CHD5 remodelers during vertebrate embryogenesis based on our analyses in this model.

A biochemical hallmark of ATP-dependent chromatin remodelers is the ability to alter nucleosomal structure [6,7]. In the presence of ATP, recombinant zebrafish Chd5 protein increases the accessibility of DNA incorporated within a nucleosome to a restriction enzyme [59,60] and is thus a bona fide ATP-dependent chromatin remodeler (Fig. 1). Demonstration of biochemical activity for *chd5* protein sets the stage for further characterization of its

remodeling activity using existing assays such as determining whether it preferentially positions nucleosomes toward the center of DNA fragment in a similar fashion to other CHD proteins [19,81–83] or carrying out assays designed to test new models of action for CHD5 remodelers that emerge from ongoing studies.

There are three subclass II remodelers found in vertebrates based on phylogenetic analysis: CHD3, CHD4, and CHD5 [5,19]. All three types of remodelers are found in zebrafish, with the addendum that there are two CHD4 remodelers, most likely as a result of a genome duplication that occurred in the lineage leading to modern teleosts [84–88]. In mammals, CHD5 remodelers exhibit a distinct pattern of expression compared to CHD3 and CHD4: they are preferentially expressed in the neural tissue and in the testes [13,29,31–34]. We find that this pattern of expression is conserved in zebrafish: *chd5* is preferentially expressed in the brain, eye, and testes whereas *chd3* and both *chd4* genes are more ubiquitously expressed (Fig. 2). These data thus suggest that the respective patterns of expression of these CHD remodelers was established relatively early during vertebrate evolution.

Given the previously characterized roles for *CHD5* in promoting neurogenesis in mouse embryos [13] and in suppression of the embryonal tumor neuroblastoma [37], we also examined expression of *chd5* during zebrafish embryogenesis. *In situ* analysis revealed that *chd5* is preferentially expressed in the brain during the later stages of embryogenesis (24 hpf and beyond) (Fig. 3), which is consistent with the pattern of expression observed in adults. Our *in situ* analysis also revealed, however, that *chd5* is expressed much more extensively during early embryogenesis, suggesting a more widespread role for *chd5* during this stage of development in zebrafish. It remains to be determined if *CHD5* genes are similarly ubiquitously expressed during embryogenesis in other vertebrates including mammals.

To investigate the role of *chd5* during embryogenesis, we undertook transient knockdown of *Chd5* expression using morpholinos that block translation. Two independent non-overlapping morpholinos were used for all analyses to address the possibility that a given phenotype was a result of non-specific effects [63,89]. In addition, we confirmed that the identified phenotypes were specific by demonstrating that co-injection of *chd5* transcript led to a modest but significant rescue of each of the observed morpholino-induced phenotypes (Figs. 5–7 and 9). We observed strong craniofacial defects in *chd5*MO1 and *chd5*MO2 larvae including reduced head size, reduced eye size, and reduced cartilage formation (Figs. 4 and 6). These phenotypes are consistent with the observed preferential expression of *chd5* in the anterior region of developing zebrafish embryos (Fig. 3). Further, we also observed altered expression of markers of neural identity in the brain at 24 hpf (Fig. 8), suggesting that *chd5* plays a role in differentiation of neural tissue in zebrafish much as *CHD5* does in mammals.

Intriguingly, we also observed increased cell death in *Chd5* morphants (Fig. 7). Increased staining by acridine orange was observed in developing brain and eye tissue relative to other tissue. In addition, more cells that exhibit positive acridine orange staining are detected throughout developing *Chd5* morphants relative to embryos injected with a control morpholino. These data raise the prospect that it is increased cell death in *Chd5* morphants that contributes to the observed reduction in head and body size (Figs. 4 and 5). The

observation that cell death is elevated throughout the body of the embryo suggests that the ubiquitous expression of *chd5* during early development is necessary for cell viability throughout the body, and that *chd5* has a similar if somewhat more substantial role in promoting cell viability in the developing brain and eye.

Another phenotype of interest associated with Chd5 morphants is the substantially reduced number of fully differentiated dopaminergic amacrine cells in the retina. We analyzed expression of *th*, a marker for sympathetic neuron differentiation [90], in Chd5 morphants. It was previously shown that reduced expression of another gene linked to neuroblastoma, *phox2b*, leads to impaired differentiation of sympathetic neurons based in part on analysis of *th* expression [57]. Although our *in situ* analysis of Chd5 morphants did not reveal a defect in *th* expression in sympathetic neurons, it did uncover an almost complete absence of *th* signal in the retina (Fig. 9). Cells that express *th* in the retina have previously been shown to correspond to dopaminergic amacrine cells, a type of neuron [76], thus revealing that reduced expression of *chd5* impedes production of fully-differentiated dopaminergic amacrine neurons. Thus our Chd5 morphants are defective in at least some aspect of differentiation of at least one type of neuron. This phenotype is consistent with the defect in neurogenesis in murine *CHD5*-deficient neural progenitor cells [13] and thus indicates that vertebrates have harnessed *CHD5* remodelers to promote neurogenesis throughout evolution

It has been reported that mice lacking *CHD5* develop normally with minimal phenotypic consequence [34,35]. In particular, it was reported that there was no gross phenotypic defect observed at birth of *Chd5*^{-/-} mice and that subsequent growth and development was normal, although modest defects in neuro-behavioral development were observed. No data were shown with regards to this phenotypic characterization as the emphasis of both studies was on characterization of the effect of loss of *CHD5* on spermiogenesis. Thus the possibility remains that zebrafish and mice lacking *chd5* are phenotypically similar. For example, it is not known if *Chd5*^{-/-} mice exhibit phenotypes during early embryogenesis and subsequently recover. Similarly, it is not known if transgenic fish that lack *chd5* will exhibit phenotypes similar to Chd5 morphants during early embryogenesis and subsequently recover as larva or juveniles. Alternatively, it is possible that loss of *chd5* results in a stronger mutant phenotype in zebrafish than in mice, as has been previously observed in zebrafish mutants of dystrophin, which corresponds to the *Duchenne muscular dystrophy* (*DMD*) gene in humans [91]. The observed alterations in expression of well-defined neural markers in the brain as well as the decrease in dopaminergic amacrine cells of Chd5 morphants (Figs. 8 and 9) indicate that *CHD5* remodelers play a role in differentiation of neural tissue in fish that is similar to that played in mammals. Thus, even if loss of *chd5* does result in a more dramatic phenotype in fish, we hypothesize that the remodelers play similar roles in gene expression and cellular differentiation in fish and mammals.

Our analyses lay the foundation for use of the zebrafish model system for further functional characterization of the vertebrate-specific remodeler *CHD5*. Importantly, our findings raise the prospect of additional conserved roles for *CHD5* remodelers during vertebrate embryogenesis, which may in turn shed additional light on the contribution of these remodelers to cellular differentiation and tumor suppression. Development of transgenic animals by technology such as the type II prokaryotic clustered regularly interspaced short

palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) system [92,93] will be necessary to fully investigate the role of *chd5*. Whether or not *chd5* does play such a role in fish, further functional characterization of these remodelers using the unique experimental toolset available in the zebrafish model is likely to greatly illuminate how they contribute to gene expression and determination of developmental identity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank James Lister for providing plasmids for *in situ* probes for *otx2* and *pax6a*, Monica Hensley and Skye Brown for contributing to fish maintenance, and Zeran Li and Liyun Zhang for their help with technical support. We would also like to thank the other members of the Ogas lab for their thoughtful discussions and critical reading of the manuscript. This work was supported by the National Institutes of Health (R21CA182197 to J.O.), the Ralph W. and Grace M. Showalter Research Trust, and Purdue University. Sequencing data were acquired by the Purdue Genomics Core Facility supported by P30 CA023168.

References

1. Chen T, Dent SYR. Chromatin modifiers and remodellers: regulators of cellular differentiation. *Nat Rev Genet.* 2014; 15:93–106. [PubMed: 24366184]
2. Ho L, Crabtree GR. Chromatin remodelling during development. *Nature.* 2010; 463:474–484. [PubMed: 20110991]
3. Hall JA, Georgel PT. CHD proteins: a diverse family with strong ties. *Biochem Cell Biol.* 2007; 85:463–476. [PubMed: 17713581]
4. Marfella CGA, Imbalzano AN. The Chd family of chromatin remodelers. *Mutat Res.* 2007; 618:30–40. [PubMed: 17350655]
5. Sims JK, Wade PA. SnapShot: Chromatin Remodeling: CHD. *Cell.* 2011; 144:626–626.e1. [PubMed: 21335242]
6. Clapier CR, Cairns BR. The biology of chromatin remodeling complexes. *Annu Rev Biochem.* 2009; 78:273–304. [PubMed: 19355820]
7. Narlikar GJ, Sundaramoorthy R, Owen-Hughes T. Mechanisms and Functions of ATP-Dependent Chromatin-Remodeling Enzymes. *Cell.* 2013; 154:490–503. [PubMed: 23911317]
8. Bouazoune K, Mitterweger A, Längst G, Imhof A, Akhtar A, Becker PB, et al. The dMi-2 chromodomains are DNA binding modules important for ATP-dependent nucleosome mobilization. *Embo J.* 2002; 21:2430–2440. [PubMed: 12006495]
9. Flanagan JF, Mi L-Z, Chruszcz M, Cymborowski M, Clines KL, Kim Y, et al. Double chromodomains cooperate to recognize the methylated histone H3 tail. *Nature.* 2005; 438:1181–1185. [PubMed: 16372014]
10. Pray-Grant MG, Daniel JA, Schieltz D, Yates JR, Grant PA. Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature.* 2005; 433:434–438. [PubMed: 15647753]
11. Ryan DP, Sundaramoorthy R, Martin D, Singh V, Owen-Hughes T. The DNA-binding domain of the Chd1 chromatin-remodelling enzyme contains SANT and SLIDE domains. *Embo J.* 2011; 30:2596–2609. [PubMed: 21623345]
12. Sharma A, Sharma A, Jenkins KR, Jenkins KR, Héroux A, Héroux A, et al. Crystal structure of the chromodomain helicase DNA-binding protein 1 (Chd1) DNA-binding domain in complex with DNA. *J Biol Chem.* 2011; 286:42099–42104. [PubMed: 22033927]
13. Egan CM, Nyman U, Skotte J, Streubel G, Turner S, O'Connell DJ. CHD5 Is Required for Neurogenesis and Has a Dual Role in Facilitating Gene Expression and Polycomb Gene Repression. *Developmental Cell.* 2013; 26:223–236. [PubMed: 23948251]

14. Mansfield RE, Musselman CA, Kwan AH, Oliver SS, Garske AL, Davrazou F, et al. Plant homeodomain (PHD) fingers of CHD4 are histone H3-binding modules with preference for unmodified H3K4 and methylated H3K9. *J Biol Chem.* 2011; 286:11779–11791. [PubMed: 21278251]
15. Musselman CA, Mansfield RE, Garske AL, Davrazou F, Kwan AH, Oliver SS, et al. Binding of the CHD4 PHD2 finger to histone H3 is modulated by covalent modifications. *Biochem J.* 2009; 423:179–187. [PubMed: 19624289]
16. Musselman CA, Ramírez J, Sims JK, Mansfield RE, Oliver SS, Denu JM, et al. Bivalent recognition of nucleosomes by the tandem PHD fingers of the CHD4 ATPase is required for CHD4-mediated repression. *Proc Natl Acad Sci USA.* 2012; 109:787–792. [PubMed: 22215588]
17. Oliver SS, Musselman CA, Srinivasan R, Svaren JP, Kutateladze TG, Denu JM. Multivalent recognition of histone tails by the PHD fingers of CHD5. *Biochemistry.* 2012; 51:6534–6544. [PubMed: 22834704]
18. Shi X, Shi X, Hong T, Hong T, Walter KL, Walter KL, et al. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature.* 2006; 442:96–99. [PubMed: 16728974]
19. Ho KK, Zhang H, Golden BL, Ogas J. PICKLE is a CHD subfamily II ATP-dependent chromatin remodeling factor. *Biochim Biophys Acta.* 2013; 1829:199–210. [PubMed: 23128324]
20. McDonel P, Costello I, Hendrich B. Keeping things quiet: roles of NuRD and Sin3 co-repressor complexes during mammalian development. *Int J Biochem Cell Biol.* 2009; 41:108–116. [PubMed: 18775506]
21. Wade PA, Geggion A, Jones PL, Ballestar E, Aubry F, Wolffe AP. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat Genet.* 1999; 23:62–66. [PubMed: 10471500]
22. Wolffe AP, Urnov FD, Guschin D. Co-repressor complexes and remodelling chromatin for repression. *Biochem Soc Trans.* 2000; 28:379–386. [PubMed: 10961924]
23. Yildirim O, Li R, Hung J-H, Chen PB, Dong X, Ee L-S, et al. Mbd3/NURD complex regulates expression of 5-hydroxymethylcytosine marked genes in embryonic stem cells. *Cell.* 2011; 147:1498–1510. [PubMed: 22196727]
24. Aguilera C, Nakagawa K, Sancho R, Chakraborty A, Hendrich B, Behrens A. c-Jun N-terminal phosphorylation antagonises recruitment of the Mbd3/NuRD repressor complex. *Nature.* 2011; 469:231–235. [PubMed: 21196933]
25. Mathieu E-L, Mathieu E-L, Finkernagel F, Finkernagel F, Murawska M, Murawska M, et al. Recruitment of the ATP-dependent chromatin remodeler dMi-2 to the transcribed region of active heat shock genes. *Nucleic Acids Res.* 2012; 40:4879–4891. [PubMed: 22362736]
26. Murawska M, Hassler M, Renkawitz-Pohl R, Ladurner A, Brehm A. Stress-induced PARP activation mediates recruitment of Drosophila Mi-2 to promote heat shock gene expression. *PLoS Genet.* 2011; 7:e1002206. [PubMed: 21829383]
27. Shimono K, Shimono Y, Shimokata K, Ishiguro N, Takahashi M. Microspherule protein 1, Mi-2beta, and RET finger protein associate in the nucleolus and up-regulate ribosomal gene transcription. *J Biol Chem.* 2005; 280:39436–39447. [PubMed: 16186106]
28. Williams CJ, Naito T, Arco PG-D, Seavitt JR, Cashman SM, De Souza B. The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development. *Immunity.* 2004; 20:719–733. [PubMed: 15189737]
29. Potts RC, Zhang P, Wurster AL, Precht P, Mughal MR, Wood WH, et al. CHD5, a brain-specific paralog of Mi2 chromatin remodeling enzymes, regulates expression of neuronal genes. *PLoS ONE.* 2011; 6:e24515. [PubMed: 21931736]
30. Quan J, Adelmant G, Marto JA, Look AT, Yusufzai T. The Chromatin Remodeling Factor CHD5 Is a Transcriptional Repressor of WEE1. *PLoS ONE.* 2014; 9:e108066. [PubMed: 25247294]
31. Garcia I, Mayol G, Rodriguez E, Sunol M, Gershon TR, Rios J, et al. Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma. *Mol Cancer.* 2010; 9:277. [PubMed: 20950435]
32. Thompson P, Gotoh T, Kok M, White P, Brodeur G. CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. *Oncogene.* 2003; 22:1002–1011. [PubMed: 12592387]

33. Vestin A, Mills AA. The tumor suppressor Chd5 is induced during neuronal differentiation, in the developing mouse brain. *Gene Expr Patterns*. 2013
34. Zhuang T, Hess RA, Kolla V, Higashi M, Raabe TD, Brodeur GM. CHD5 is required for spermiogenesis and chromatin condensation. *Mech Dev*. 2013
35. Li W, Wu J, Kim S-Y, Zhao M, Hearn SA, Zhang MQ, et al. Chd5 orchestrates chromatin remodelling during sperm development. *Nat Commun*. 2014; 5:3812. [PubMed: 24818823]
36. Fatemi M, Paul TA, Brodeur GM, Shokrani B, Brim H, Ashktorab H. Epigenetic silencing of CHD5, a novel tumor-suppressor gene, occurs in early colorectal cancer stages. *Cancer*. 2013
37. Kolla V, Zhuang T, Higashi M, Naraparaju K, Brodeur GM. Role of CHD5 in Human Cancers: 10 Years Later. *Cancer Res*. 2014
38. Maser RS, Choudhury B, Campbell PJ, Feng B, Wong K-K, Protopopov A, et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature*. 2007; 447:966–971. [PubMed: 17515920]
39. Wood LD, Wood LD, Parsons DW, Parsons DW, Jones S, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007; 318:1108–1113. [PubMed: 17932254]
40. Zhao R, Meng F, Wang N, Ma W, Yan Q. Silencing of CHD5 Gene by Promoter Methylation in Leukemia. *PLoS ONE*. 2014; 9:e85172. [PubMed: 24454811]
41. Zhao R, Yan Q, Lv J, Huang H, Zheng W, Zhang B, et al. CHD5, a tumor suppressor that is epigenetically silenced in lung cancer. *Lung Cancer*. 2012; 76:324–331. [PubMed: 22186629]
42. Fujita T, Igarashi J, Okawa ER, Gotoh T, Manne J, Kolla V, et al. CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. *J Natl Cancer Inst*. 2008; 100:940–949. [PubMed: 18577749]
43. Garcia I, Mayol G, Rios J, Domenech G, Cheung N-KV, Oberthuer A, et al. A three-gene expression signature model for risk stratification of patients with neuroblastoma. *Clinical Cancer Research : an Official Journal of the American Association for Cancer Research*. 2012; 18:2012–2023. [PubMed: 22328561]
44. Koyama H, Zhuang T, Light JE, Kolla V, Higashi M, McGrady PW, et al. Mechanisms of CHD5 Inactivation in neuroblastomas. *Clinical Cancer Research : an Official Journal of the American Association for Cancer Research*. 2012; 18:1588–1597. [PubMed: 22294723]
45. Okawa ER, Gotoh T, Manne J, Igarashi J, Fujita T, Silverman KA, et al. Expression and sequence analysis of candidates for the 1p36.31 tumor suppressor gene deleted in neuroblastomas. *Oncogene*. 2008; 27:803–810. [PubMed: 17667943]
46. Jiang M, Stanke J, Lahti JM. The connections between neural crest development and neuroblastoma. *Curr Top Dev Biol*. 2011; 94:77–127. [PubMed: 21295685]
47. Maris JM, Maris JM, Hogarty MD, Hogarty MD, Bagatell R, Bagatell R, et al. Neuroblastoma. *Lancet*. 2007; 369:2106–2120. [PubMed: 17586306]
48. Feitsma H, Cuppen E. Zebrafish as a cancer model. *Mol Cancer Res*. 2008; 6:685–694. doi:6/5/685 [pii]. [PubMed: 18505914]
49. Mione M, Meijer AH, Snaar-Jagalska BE, Spaink HP, Trede NS. Disease modeling in zebrafish: cancer and immune responses--a report on a workshop held in Spoleto, Italy, July 20–22, 2009. *Zebrafish*. 2009:445–451. [PubMed: 20047471]
50. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. *J Clin Invest*. 2012; 122:2337–2343. [PubMed: 22751109]
51. Ablain J, Zon LI. Of fish and men: using zebrafish to fight human diseases. *Trends Cell Biol*. 2013; 23:584–586. [PubMed: 24275383]
52. Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Brachydanio rerio*). 1993
53. Li Z, Ptak D, Zhang L, Walls EK, Zhong W, Leung YF. Phenylthiourea specifically reduces zebrafish eye size. *PLoS ONE*. 2012; 7:e40132. [PubMed: 22761952]
54. Nusslein-Volhard C, Gilmour DT, Dahm R. Nüsslein-Volhard: Introduction: zebrafish as a system... - Google Scholar. *Zebrafish: a Practical Approach*. 2002

55. Lane BM, Lane BM, Lister JA, Lister JA. Otx but not Mitf transcription factors are required for zebrafish retinal pigment epithelium development. *PLoS ONE*. 2012; 7:e49357. [PubMed: 23139843]
56. Püschel AW, Püschel AW, Gruss P, Gruss P, Westerfield M, Westerfield M. Sequence and expression pattern of pax-6 are highly conserved between zebrafish and mice. *Development*. 1992; 114:643–651. [PubMed: 1352238]
57. Pei D, Luther W, Wang W, Paw BH, Stewart RA, George RE. Distinct Neuroblastoma-associated Alterations of PHOX2B Impair Sympathetic Neuronal Differentiation in Zebrafish Models. *PLoS Genet*. 2013; 9:e1003533. [PubMed: 23754957]
58. Walker MB, Kimmel CB. A two-color acid-free cartilage and bone stain for zebrafish larvae. *Biotech Histochem*. 2007; 82:23–28. [PubMed: 17510811]
59. Polach KJ, Polach KJ, Widom J, Widom J. Mechanism of protein access to specific DNA sequences in chromatin: a dynamic equilibrium model for gene regulation. *J Mol Biol*. 1995; 254:130–149. [PubMed: 7490738]
60. Smith CL, Peterson CL. A conserved Swi2/Snf2 ATPase motif couples ATP hydrolysis to chromatin remodeling. *Mol Cell Biol*. 2005; 25:5880–5892. [PubMed: 15988005]
61. Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D, et al. CHD5 is a tumor suppressor at human 1p36. *Cell*. 2007; 128:459–475. doi:S0092-8674(07)00053-0 [pii]. [PubMed: 17289567]
62. Gupta T, Mullins MC. Dissection of organs from the adult zebrafish. 2010
63. Bedell VM, Westcot SE, Ekker SC. Lessons from morpholino-based screening in zebrafish. *Briefings in Functional Genomics*. 2011; 10:181–188. [PubMed: 21746693]
64. Reifers F, Bohli H, Walsh E, Crossley P, Stainier D, Brand M. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrainhindbrain boundary development and somitogenesis. *Development*. 1998; 125:2381–2395. [PubMed: 9609821]
65. Mercier P, Simeone A, Cotelli F, Boncinelli E. Expression pattern of two otx genes suggests a role in specifying anterior body structures in zebrafish. *Int J Dev Biol*. 1995; 39:559–573. [PubMed: 8619954]
66. Mori H, Miyazaki Y, Morita T, Nitta H, Mishina M. Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res. Mol. Brain Res*. 1994; 27:221–231. [PubMed: 7898305]
67. Li HS, Yang JM, Jacobson RD, Pasko D, Sundin O. Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. *Dev Biol*. 1994; 162:181–194. [PubMed: 8125186]
68. MacDonald R, Xu Q, Barth KA, Mikkola I, Holder N, Fjose A, et al. Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron*. 1994; 13:1039–1053. [PubMed: 7946344]
69. Foucher I, Mione M, Simeone A, Acampora D, Bally-Cuif L, Houart C. Differentiation of cerebellar cell identities in absence of Fgf signalling in zebrafish Otx morphants. *Development*. 2006; 133:1891–1900. doi:dev.02352 [pii]. [PubMed: 16611693]
70. Joyner A, Liu A, Millet S. Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr Opin Cell Biol*. 2000; 12:736–741. doi:S0955-0674(00)00161-7 [pii]. [PubMed: 11063941]
71. MacDonald R, MacDonald R, Barth KA, Barth KA, Xu Q, Xu Q, et al. Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development*. 1995; 121:3267–3278. [PubMed: 7588061]
72. Nornes S, Nornes S, Clarkson M, Clarkson M, Mikkola I, Mikkola I, et al. Zebrafish contains two pax6 genes involved in eye development. *Mech Dev*. 1998; 77:185–196. [PubMed: 9831649]
73. Bellipanni G, Bellipanni G, Murakami T, Murakami T, Doerre OG, Doerre OG, et al. Expression of Otx homeodomain proteins induces cell aggregation in developing zebrafish embryos. *Dev Biol*. 2000; 223:339–353. [PubMed: 10882520]
74. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature*. 1999; 399:366–370. [PubMed: 10360575]

75. McConville C, Reid S, Baskcomb L, Douglas J, Rahman N. PHOX2B analysis in nonsyndromic neuroblastoma cases shows novel mutations and genotype-phenotype associations. *Am J Med Genet A*. 2006; 140:1297–1301. [PubMed: 16691592]
76. Jang Y-J, Yu S-H, Lee E-S, Jeon C-J. Two types of tyrosine hydroxylase-immunoreactive neurons in the zebrafish retina. *Neurosci. Res.* 2011; 71:124–133. [PubMed: 21784111]
77. Arenzana FJ, Arévalo R, Sánchez-González R, Clemente D, Aijón J, Porteros A. Tyrosine hydroxylase immunoreactivity in the developing visual pathway of the zebrafish. *Anat. Embryol.* 2006; 211:323–334. [PubMed: 16506065]
78. Gustincich S, Feigenspan A, Wu DK, Koopman LJ, Raviola E. Control of dopamine release in the retina: a transgenic approach to neural networks. *Neuron*. 1997; 18:723–736. [PubMed: 9182798]
79. Feigenspan A, Gustincich S, Bean BP, Raviola E. Spontaneous activity of solitary dopaminergic cells of the retina. *J Neurosci.* 1998; 18:6776–6789. [PubMed: 9712649]
80. Meng S, Ryu S, Zhao B, Zhang D-Q, Driever W, McMahon DG. Targeting retinal dopaminergic neurons in tyrosine hydroxylase-driven green fluorescent protein transgenic zebrafish. *Mol Vis.* 2008; 14:2475–2483. [PubMed: 19112533]
81. Brehm A, Brehm A, Langst G, Langst G, Kehle J, Kehle J, et al. dMi-2 and ISWI chromatin remodelling factors have distinct nucleosome binding and mobilization properties. *Embo J*. 2000; 19:4332–4341. [PubMed: 10944116]
82. Guschin D, Guschin D, Wade PA, Wade PA, Kikyo N, Kikyo N, et al. ATP-dependent histone octamer mobilization and histone deacetylation mediated by the Mi-2 chromatin remodeling complex. *Biochemistry*. 2000; 39:5238–5245. [PubMed: 10819992]
83. Rippe K, Rippe K, Schrader A, Schrader A, Riede P, Riede P, et al. DNA sequence- and conformation-directed positioning of nucleosomes by chromatin-remodeling complexes. *Proc Natl Acad Sci USA*. 2007; 104:15635–15640. [PubMed: 17893337]
84. Woods IG, Woods IG, Kelly PD, Kelly PD, Chu F, Chu F, et al. A comparative map of the zebrafish genome. *Genome Res.* 2000; 10:1903–1914. [PubMed: 11116086]
85. Postlethwait JH, Postlethwait JH, Woods IG, Woods IG, Ngo-Hazelett P, Ngo-Hazelett P, et al. Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res.* 2000; 10:1890–1902. [PubMed: 11116085]
86. Amores A, Amores A, Force A, Force A, Yan YL, Yan YL, et al. Zebrafish hox clusters and vertebrate genome evolution. *Science*. 1998; 282:1711–1714. [PubMed: 9831563]
87. Postlethwait JH, Yan Y-L, Gates MA, Horne S, Amores A, Brownlie A, et al. Vertebrate genome evolution and the zebrafish gene map. *Nat Genet.* 1998; 18:345–349. [PubMed: 9537416]
88. Taylor JS, Taylor JS, Braasch I, Braasch I, Frickey T, Frickey T, et al. Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res.* 2003; 13:382–390. [PubMed: 12618368]
89. Kok FO, Shin M, Ni C-W, Gupta A, Grosse AS, van Impel A, et al. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev Cell*. 2015; 32:97–108. [PubMed: 25533206]
90. Alam G, Cui H, Shi H, Yang L, Ding J, Mao L, et al. MYCN promotes the expansion of Phox2B-positive neuronal progenitors to drive neuroblastoma development. *Am J Pathol.* 2009; 175:856–866. [PubMed: 19608868]
91. Bassett D, Currie PD. Identification of a zebrafish model of muscular dystrophy. *Clin. Exp. Pharmacol. Physiol.* Blackwell Science Pty. 2004:537–540.
92. Jao L-E, Wenthe SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci USA*. 2013; 110:13904–13909. [PubMed: 23918387]
93. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. 2014; 157:1262–1278. [PubMed: 24906146]

Highlights

- Recombinant zebrafish *chd5* protein exhibits chromatin remodeling activity.
- Expression of *chd5* in adult zebrafish mirrors expression of *CHD5* in adult mammals.
- *chd5* promotes craniofacial development during embryogenesis.
- *chd5* is necessary for proper expression of neural markers in the brain of embryos.
- *chd5* morphants exhibit increased cell death in both the brain and trunk of embryos.
- *chd5* is necessary for proper differentiation of dopaminergic amacrine cells.

Fig. 1. Recombinant zebrafish Chd5 increases the accessibility of DNA in mononucleosomes to the restriction enzyme Plm1 in an ATP-dependent fashion

(A) Schematic of the mononucleosomal template used in this analysis. The position of the PlmI site is indicated. (B) Native PAGE gel analysis of mononucleosomal template depicted in (A) after incubation for 30 minutes with components specified below each lane. Position of uncut and cut DNA is indicated to right of gel.

Fig. 2. *chd5* is preferentially expressed in the brain, eye, and testes of adult zebrafish
qRT-PCR analysis of *chd5* relative transcript levels in different adult tissues dissected from one-year-old zebrafish. The closely related remodelers *chd3*, *chd4a*, and *chd4b* were included for comparison. The tissues were dissected as previously described [62]. The actin *actb1* was used as a standardization control and expression levels of all tissues were normalized to expression in tailfin. Data are the average of two biological replicates that each includes three technical replicates. Error bars represent standard deviation.

Fig. 3. The pattern of *chd5* expression changes during embryogenesis

(A) qRT-PCR analysis of relative transcript levels of *chd5* during embryogenesis. *chd3*, *chd4a*, and *chd4b* were included for comparison. *actb1* was used as a standardization control, and all data were normalized to 6 hpf. Data are the average of two biological replicates that each includes three technical replicates. Error bars represent standard deviation calculated from the six replicates. (B–F) *In situ* analysis of *chd5* during different stages of embryogenesis. *chd5* transcript is detected throughout the embryo at 10 hpf (B) and 15 hpf (C) and then becomes progressively restricted to the anterior portion of the embryo during 24 hpf (D), 48 hpf (E), and 72 hpf (F). All embryos are oriented such that anterior is on the left and dorsal is up. Embryos were developed for different times. No detectable staining occurred when 48 hpf embryos were hybridized with a *chd5* sense probe under identical conditions to the antisense probe (G). Scale bar = 100 μ m.

Fig. 4. Injection with chd5MO impairs development of craniofacial features at 72 hpf
 Phenotypic analysis of 72 hpf embryos after no injection (uninjected), injection with a control morpholino (CoMO), or injection with a morpholino designed to block translation of *chd5* mRNA (chd5MO) (A–D) Data are based on measurements of 8 representative embryos selected at random from 225 larvae examined. Raw data is graphed in A and B while the ratio of eye or head size relative to body size is graphed in C and D. Error bars denote standard deviation. Asterisks denote statistical significance between embryos injected with CoMO and chd5MO1 by Student's two-tailed *t*-test ($p < 0.05$).

Fig. 5. Full-length *chd5* mRNA can rescue the phenotype of *Chd5* morphants

Embryos were injected with indicated morpholino and/or full-length *chd5* mRNA and then scored as “normal” or “abnormal” based on eye length phenotype. (A) Representative 72 hpf embryos of indicated morpholino injection and phenotypic class are shown. Scale bars = 300 μ m. (B) Quantitative scoring of phenotypes, as based on eye length phenotype. The difference between *chd5MO1* and *chd5* + *chd5MO1* is significant as determined by chi-square analysis ($p < 0.001$). Numbers in parentheses under each treatment indicate total number of embryos scored in each category.

Fig. 6. Injection with *chd5*MO impairs development of craniofacial features at 72 hpf
 (A) Cartilage development was characterized in twenty-five 72 hpf larvae by staining with Alcian blue. Each photo is a representative sample of the specified injection (listed above panel). Scale bar = 100 μ m. (B) Quantitative scoring of phenotypes, as based on presence/absence of craniofacial cartilages in each set of injections. The difference between *chd5*MO1 and *chd5* + *chd5*MO1 is significant as determined by chi-square analysis ($p < 0.01$). A total of 30 embryos were scored for each injection.

Fig. 7. Reduced expression of Chd5 leads to an increase in cell death

Acridine orange was used to assess cell viability in 24 hpf embryos. Each photo is a representative sample of the specified injection (listed above panel). The top image of each panel shows the lateral view of the head of the embryo, and the bottom image shows a magnified view of the trunk. Scale bars for both set of panels= 200 μ m. (B) Quantitative scoring of phenotypes in each set of injections, based on the abundance of positive acridine orange cells in the head and trunk. The difference between *chd5*MO1 and *chd5* + *chd5*MO1 is significant as determined by chi-square analysis ($p < 0.01$). A total of 30 embryos were scored for each injection.

Fig. 8. Chd5 knockdown leads to changes in expression of brain-specific markers in 24 hpf embryos

In situ hybridization was used to examine expression of *fgf8*, *otx2*, and *pax6a* in uninjected embryos (A,D,G), CoMO embryos (B,E,H), and *chd5*MO embryos (C,F,I) at 24 hpf. MHB – midbrain-hindbrain boundary, DD – dorsal diencephalon, T – telencephalon, OS – optic stalk, MB – midbrain, HB – hind brain, ASC – anterior spinal cord, E – eye. Scale bar = 100 μ m.

Fig. 9. Chd5 knockdown leads to a defect in *th*-positive amacrine cells in the zebrafish eye in 96 hpf embryos

In situ hybridization was used to examine expression of *th* in uninjected embryos and embryos injected with a morpholino and/or *chd5* transcript (A) *in situ* of *th* expression in the superior cervical ganglion (indicated by arrow). The nature of the injection is indicated above each representative embryo (N=30). Scale bar = 100 μ m. (B) *in situ* of *th* expression in the eye. The nature of the injection is indicated above each representative embryo (N=30). Scale bar = 150 μ m. (C) Quantitative scoring of phenotypes for each set of injections depicted in (B), based on the abundance of *th* expressing cells in the eye. The difference between chd5MO1 and chd5 + chd5MO1 is significant as determined by chi-square analysis ($p < 0.001$). A total of 30 embryos were scored for each injection.