Hcfc1b, a zebrafish ortholog of HCFC1, regulates craniofacial development by modulating \textit{mmachc} expression

Anita M. Quintana\textsuperscript{a}, Elizabeth A. Geiger\textsuperscript{a}, Nate Achilly\textsuperscript{b}, David S. Rosenblatt\textsuperscript{c}, Kenneth N. Maclean\textsuperscript{a,d}, Sally P. Stabler\textsuperscript{e}, Kristin B. Artinger\textsuperscript{f}, Bruce Appel\textsuperscript{a}, Tamim H. Shaikh\textsuperscript{b,d,e}

\textsuperscript{a} Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO 80045, USA
\textsuperscript{b} Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA
\textsuperscript{c} Department of Human Genetics, McGill University, Montreal, Quebec, Canada H3A 1B1
\textsuperscript{d} Department of Genetics, University of Colorado, School of Medicine, Aurora, CO 80045, USA
\textsuperscript{e} Department of Medicine, University of Colorado School of Medicine, CO 80045, USA
\textsuperscript{f} Department of Craniofacial Biology, School of Dental Medicine, University of Colorado, CO 80045, USA

**A R T I C L E  I N F O**

Article history:
Received 19 September 2014
Accepted 24 September 2014
Available online 2 October 2014

Keywords:
HCFC1
Cobalamin
Craniofacial defects
Facial dysmorphia
MMACHC

**A B S T R A C T**

Mutations in HCFC1 (MIM300019), have been recently associated with \textit{cblX} (MIM309541), an X-linked, recessive disorder characterized by multiple congenital anomalies including craniofacial abnormalities. HCFC1 is a transcriptional co-regulator that modulates the expression of numerous downstream target genes including \textit{MMACHC}, but it is not clear how these HCFC1 targets play a role in the clinical manifestations of \textit{cblX}. To begin to elucidate the mechanism by which HCFC1 modulates disease phenotypes, we have carried out loss of function analyses in the developing zebrafish. Of the two \textit{HCFC1} orthologs in zebrafish, \textit{hcfc1a} and \textit{hcfc1b}, the loss of \textit{hcfc1b} specifically results in defects in craniofacial development. Subsequent analysis revealed that \textit{hcfc1b} regulates cranial neural crest cell differentiation and proliferation within the posterior pharyngeal arches. Further, the \textit{hcfc1b}-mediated craniofacial abnormalities were rescued by expression of human \textit{MMACHC}, a downstream target of HCFC1 that is aberrantly expressed in \textit{cblX}. Furthermore, we tested distinct human \textit{HCFC1} mutations for their role in craniofacial development and demonstrated variable effects on \textit{MMACHC} expression in humans and craniofacial development in zebrafish. Notably, several individuals with mutations in either \textit{HCFC1} or \textit{MMACHC} have been reported to have mild to moderate facial dysmorphia. Thus, our data demonstrates that \textit{HCFC1} plays a role in craniofacial development, which is in part mediated through the regulation of \textit{MMACHC} expression.

© 2014 Elsevier Inc. All rights reserved.

**Introduction**

Mutations in \textit{HCFC1} recently have been associated with an X-linked, recessive disorder characterized by defects in cobalamin metabolism, nervous system development, neurological impairment, and failure to thrive (Yu et al., 2013). The cellular and biochemical manifestations associated with this disorder, methylmalonic acidemia and homocysteinemia \textit{cblX} type (\textit{cblX}; MIM309541), overlap those observed in individuals with methylmalonic acidemia and homocysteinemia \textit{cblC} type (\textit{cblC}; MIM277400). \textit{cblC} is caused by mutations in \textit{MMACHC} (Lerner-Ellis et al., 2009, 2006; Liu et al., 2010), which codes for a protein involved in cobalamin metabolism (Hannibal et al., 2009; Kim et al., 2009, 2008). HCFC1 is a transcriptional co-regulator that interacts with multiple proteins including transcription factors to regulate different biological processes, such as cell proliferation, migration and cell death (Luciano and Wilson, 2003; Machida et al., 2009; Van den Berg et al., 2010, p. 4; Yu et al., 2010). The \textit{MMACHC} promoter contains conserved binding motifs for HCFC1 and its transcription factor binding partner THAP11 (Dejosez et al., 2010). Furthermore, \textit{cblX} patients with mutations in \textit{HCFC1} had markedly reduced levels of \textit{MMACHC} expression (Yu et al., 2013). These and other published data have demonstrated that HCFC1 regulates
MMACHC expression and that the metabolic defects characteristic of cbIX result from aberrant regulation of MMACHC expression. However, the molecular mechanisms underlying the phenotypic spectrum observed in cbIX remain to be elucidated.

HCFC1 was originally discovered as a host transcription factor during latent reactivation of Herpes Simplex Virus (HSV) infection (Kristie and Sharp, 1993; La Boissière et al., 1999; Lu et al., 1998). However, HCFC1 is not a traditional transcription factor because it lacks a DNA binding domain (Dejosez et al., 2010) and requires interaction with other proteins to regulate the expression of downstream effectors. The kelch domain of HCFC1 serves as a docking site for protein–protein interactions and is highly conserved across species (Li et al., 2004). Mutations in HCFC1 that lead to cbIX cluster within the kelch domain, thereby potentially disrupting its interactions with protein partners (Yu et al., 2013) and the expression of critical downstream effector molecules. Based on recent data from genome wide ChiP-Seq, HCFC1 binds the promoters of over 5000 genes in conjunction with various protein partners (Michaud et al., 2013). Thus, it is likely that HCFC1 regulates the development of the organs and tissues affected in cbIX patients via its regulation of multiple genes including MMACHC.

cbIC and cbIX are highly similar in that both diseases are characterized by the accumulation of methylmalonic acid and homocysteine in blood and/or urine. However, because of the myriad of genes downstream of HCFC1, the role of MMACHC in the formation and progression of other overlapping clinical manifestations associated with cbIX remains unclear. For example, although craniofacial abnormalities are not a cardinal feature associated with cbIC or cbIX patients, some individuals with mutations in either HCFC1 or MMACHC have exhibited mild dysmorphic features (Cerone et al., 1999; D’Alessandro et al., 2010; Yu et al., 2013). What remains in question is whether the facial dysmorphia observed in cbIX patients is caused by defects in MMACHC expression or by aberrant expression of some other HCFC1 target gene. To begin to address this question we performed transient loss of function analysis in the developing zebrafish. Zebrafish have two HCFC1 orthologs, hcfc1a and hcfc1b, whose protein products both share high levels of sequence identity with human HCFC1. We show that antisense morpholin oligonucleotide knockdown of hcfc1b, but not hcfc1a, leads to craniofacial defects in the zebrafish embryo. Further analysis using molecular markers associated with neural crest cells (NCCs), which give rise to the cartilaginous structures of the face, suggested that hcfc1b regulates the division and differentiation of NCCs. In order to determine if MMACHC plays a role in the craniofacial phenotype in morphant zebrafish, we co-injected human MMACHC mRNA into the hcfc1b morphants. This rescued the craniofacial defects resulting from hcfc1b loss of function, which is consistent with the possibility that hcfc1b regulates craniofacial development by modulating the expression of the zebrafish ortholog of human MMACHC. Our results implicate both HCFC1 and MMACHC as key mediators of craniofacial development.

Materials and methods

Zebrafish maintenance

For all experiments, embryos [TAB (obtained by crossing adult Tupfel long fin (TL) with adult AB), Tg(prdm1a:EGFP) or Tg(sox10: mRFP)] were maintained in embryo medium at 28.5 °C. Zebrafish (Danio rerio) were maintained at the University of Colorado Anschutz Medical Campus according to the Institutional Animal Care and Use Committee (IACUC) guidelines (protocol #B-85411 (08)1D).

Cells and culture conditions

All human subject samples used in this study were collected at McGill University Health Centre, Montreal, Quebec, Canada after obtaining informed consent in compliance with the Helsinki Declaration. Primary fibroblasts were cultured at 37 °C/5% CO2 in Dulbecco’s modified Eagle’s Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Grand Island, NY).

MO and mRNA injections

MOs for each paralog were designed by targeting unique, paralog-specific sequences. MOs (2 nl) targeting either hcfc1a (15.28 ng) mRNA splicing (5’-ACCATAAAGAAACTTTCT- TACCTGT-3’), hcfc1b (3.8 ng) translation (5’-ACCGAAGTGGCCTGCCT- GAAGAAGCCATGT-3’), or mmachc (2 ng, 4 ng, 8 ng, or 16 ng) (5’- GGTTTACTGAGATCGCCATTTT-3’) (GeneTools, LLC, Philomath, OR USA) were injected into the single cell of newly fertilized embryos. Co-injection with a morpholino targeting the tsp53 gene was performed as a control for off target effects. For mRNA rescue, embryos were co-injected (2 nl) with MO targeting hcfc1b (3.8 ng), or MO targeting mmachc (8 ng) and either full length HCFC1 (RefSeq NM_005334.2) mRNA (100, 400, or 800 pg/embryo) or MMACHC (RefSeq NM_015506.2) mRNA (400, 800, 1600, or 2000 ng/embryo). Concentrations for sub-optimal concentrations are listed in the supplementary material. Injected embryos were maintained in embryo water at 28.5 °C and harvested according to dpf or hpf.

In vitro mRNA synthesis, cloning, and production of HCFC1 mutations

Full length HCFC1 cDNA (Fisher Scientific, Waltham, MA) was cloned into the pcDNA 6.2 vector using LR recombination (Life Technologies, Grand Island, NY). Plasmid encoding full length HCFC1 was linearized with Sall (Fisher Scientific, Waltham, MA) and plasmid encoding full length MMACHC:Mycl:DDK (Origene, Rockville, MD) was linearized with PciI (New England Biolabs, Ipswich, MA) mRNA was synthesized with the T7 Ultra mMessage mMachine kit (Life technologies, Grand Island, NY) according to manufacturer’s instructions. All HCFC1 mutations were induced using the QuikChange Site Directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to manufacturer’s instructions. All primer pairs can be found in Supplementary Table 6. All riboprobes were PCR amplified from 1 day old embryos and cloned into the pGem T-easy (Promega, Madison, WI) vector. Probe synthesis was performed as previously described (Thissè and Thissè, 2008). All primers used for probe synthesis are included in Supplementary Table 6.

Staining of cartilage and in situ hybridization

Larvae were collected at 4 or 5 dpf and fixed for 1 h in 2% paraformaldehyde (Boston BioProducts, Ashland, MA) at room temperature. Larvae were washed in wash buffer [100 mM Tris pH 7.5 (Boston BioProducts, Ashland, MA), 10 mM MgCl2 (Boston BioProducts, Ashland, MA)] and incubated overnight at room temperature in Alcian blue [0.4% Alcian blue (Sigma, St. Louis, MO), 100 mM Tris pH 7.5 (Boston BioProducts, Ashland, MA)]. Cells and culture conditions...
(Sigma, St. Louis, MO)/0.1% KOH (Boston BioProducts, Ashland, MA) at room temperature for 10 min. Alizarin red stain (0.01%) (Sigma, St. Louis, MO) in 25% glycerol (Sigma, St. Louis, MO) and 100 mM Tris pH 7.5 (Boston BioProducts, Ashland, MA) was added to the larvae for 30 min at room temperature. Larvae were destained in 50% glycerol (Sigma, St. Louis, MO)/0.1% KOH (Boston BioProducts, Ashland, MA) for 10 min and stored at 4 °C. All in situ hybridization was performed as previously described (Thissen and Thissen, 2008). Statistical analysis was performed using a Fisher’s exact test.

**Western blot and immunohistochemistry**

Total protein was isolated from embryos (20) staged at 1 dpf. Embryos were homogenized in cell lysis buffer (Cell Signaling, Danvers, MA) and protein was quantitated using Precision Red (Cytoskeleton Inc., Denver, CO) according to manufacturer’s instructions. Western blotting was performed with approximately 50 μg of protein using 1.25 μg of anti-HCFC1 antibody (Sigma, St. Louis, MO) and developed with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). For immunohistochemistry, embryos were fixed in 4% paraformaldehyde for 2 h at room temperature. Embryos were washed in PBS and embedded in 1.5% agar and 5% sucrose. Embedded blocks were incubated in 30% sucrose overnight, dried, frozen with dry ice, and sectioned with a cryostat (20 μM). Sections were rehydrated in 1 × PBS, blocked for 1 h at room temperature in 2% goat serum (Life technologies, Grand Island, NY) and 2 mg/ml BSA (Sigma, St. Louis, MO). Slides were incubated with primary antibody (anti-phospoSer10 histone H3, 1:500 (Cell Signaling, Danvers, MA),) overnight, washed in PBS, and incubated with appropriate AlexaFlour secondary antibody (Life technologies, Grand Island, NY) at 1:200 for 1 h at room temperature. Samples were washed, cover slipped with VectaShield (Vector Laboratories Burlingame, CA), and imaged with appropriate filter. Statistical analysis of immunohistochemistry was performed using a standard T-test.

**RNA isolation, PCR analysis, and chromatin immunoprecipitation**

For RNA isolation, larvae were lysed in Trizol (Life Technologies, Grand Island, NY) (200 μl), and processed according to the manufacturer’s recommendations. Total RNA (500 ng) was converted to cDNA and the level of mmmachc was determined using Sybr green based real time PCR. For RNA isolation from fibroblasts obtained from healthy donors or patients, approximately 1 × 10^6 cells were lysed in Trizol (Life Technologies, Grand Island, NY) and processed according to manufacturer’s instructions. Total RNA (1 μg) was converted into cDNA with Superscript III Reverse Strand Synthesis system (Life technologies, Grand Island, NY) according to manufacturer’s protocol. Semi-quantitative PCR was performed with GoTaq mastermix (Promega, Madison, WI). Quantitative real time PCR (QPCR) was performed as previously described (Ye et al., 2013) or with Fast Brilliant III Mastermix (Agilent Technologies, Santa Clara, CA). ChiP assays were performed as previously described with anti-HCFC1 antibodies (Sigma, St. Louis, MO) (Quintana et al., 2011). Sequences of primers are listed in Supplementary Table 6.

**Whole embryo metabolic profile**

At 5 days post fertilization pooled embryos (50) were homogenized in molecular grade water and centrifuged for 5 min at 10,000XG at 4 °C. Total protein concentration was measured using the Precision Red Assay (Cytoskeleton Inc. Denver, CO). Supernatant was removed and metabolites were measured by capillary stable isotope dilution gas chromatography/mass spectrometry as previously described (Allen et al., 1993a, 1993b; Stabler et al., 1993). All metabolic concentrations were normalized according to total protein content. Metabolites were measured in biological replicates each consisting of 50 pooled embryos per group, except for homocystine, methylmalonic acid, and cystathionine, which were performed in biological triplicate. Statistics was performed using a student’s T-test.

**Results**

*hcfc1b* regulates craniofacial development.

In order to carry out functional analysis of HCFC1 in an *in vivo* model, we performed transient knockdown of the zebrafish orthologs, *hcfc1a* and *hcfc1b*. We injected antisense morpholino oligonucleotides (MO) that specifically interfere with either the mRNA splicing of *hcfc1a* or the translation of *hcfc1b* mRNA into freshly fertilized zebrafish eggs. Western blot analysis using anti-HCFC1 antibodies, which are predicted to recognize a kelch domain cleavage product of both Hcfc1a and Hcfc1b, confirmed that the simultaneous inhibition of Hcfc1a and Hcfc1b reduced the amount of total HCFC1 protein (Fig. 1A). Based upon amino acid sequence, Hcfc1a and Hcfc1b are predicted to be different sizes, however HCFC1 is proteolytically cleaved into multiple N-terminal and C-terminal fragments. The single band detected by our HCFC1 antibody specifically recognizes a highly conserved epitope within the kelch domain. This conserved epitope is present in the N-terminal cleavage products of both Hcfc1a and Hcfc1b. Therefore, the intensity of the band shown in Fig. 1A represents the expression levels of both Hcfc1a and Hcfc1b. Zebrafish gene duplication can in some cases result in paralogs with independent functions. In order to properly attribute phenotypes to each paralog we performed independent knockdown of both *hcfc1a* and *hcfc1b* with separate morpholinos (Fig. 1B and Supplementary Fig. 2E). Reverse transcription coupled with polymerase chain reaction (RT-PCR) using primers flanking the targeted splice junction of *hcfc1a* or *hcfc1b* confirmed aberrant mRNA splicing at 1 or 2 days post fertilization (dpf) (Fig. 1B and Supplementary Fig. 2E).

Although we do not have comprehensive phenotypic information on all of the 14 individuals diagnosed with cbDX, at least 2 (including the index case), have been reported to have mild to moderate facial dysmorphism (Yu et al., 2013). We phenotyped *hcfc1* morphants for defects in craniofacial development to determine if there is a correlation between aberrant HCFC1 function and craniofacial dysmorphism observed in *cbDX*. *hcfc1a* morphants appeared normal in size and overall development with any...
obvious craniofacial defect. However, at 5 dpf *hcfc1b* morphants had an abnormally small lower jaw. To confirm that knockdown of *hcfc1b* resulted in craniofacial defects, we stained larvae with Alcian blue: Alizarin red to visualize developing facial features. At 5 dpf, knockdown of *hcfc1b* resulted in a shortening of Meckel’s cartilage, inversion of the ceratohyal, and a complete loss of the ceratobranchial arches (Fig. 2B and B’). Approximately 60% of the embryos injected with *hcfc1b* MO presented with a craniofacial phenotype (Supplementary Table 1). Interestingly, although *hcfc1a* and *hcfc1b* were expressed similarly, both temporally and spatially, *hcfc1a* morphants did not exhibit craniofacial abnormalities (Fig. 2C and C’ and Supplementary Table 1).

Human HCFC1 and zebrafish Hcfc1b share a high degree of sequence identity, but the level of functionality shared between these orthologs has not been determined. To determine if the human and zebrafish proteins are functionally equivalent we performed rescue experiments with *in vitro* synthesized human HCFC1 mRNA. Co-injection of *hcfc1b* MO with HCFC1 mRNA rescued the *hcfc1b*-mediated craniofacial phenotype (Fig. 2D and D’ and Supplementary Table 1) showing that the human protein

---

**Fig. 2.** Loss of *hcfc1b* causes defects in craniofacial development. (A–D) Alcian:Alizarin staining was performed to visualize the developing cartilage in non-injected controls (NI), *hcfc1a* morphants (*hcfc1a* MO), *hcfc1b* morphants (*hcfc1b* MO), or embryos co-injected with *hcfc1b* MO and *in vitro* synthesized human HCFC1 mRNA. Embryos were stained at 5 days post fertilization and manual dissection of the viscerocranium and neurocranium was performed. Neurocranium is depicted in A–D and the viscerocranium is depicted in A’–D’.
can substitute for the zebrafish protein. An appropriate rescue included an appropriately formed Meckel’s cartilage, the appropriate directionality of the ceratohyal, and the formation of ceratobranchial arches. Additionally, these data provide strong evidence that the craniofacial phenotype caused by hcfc1b MO injection results specifically from loss of hcfc1b function and not off-target MO effects. To further establish that craniofacial defects were the result of loss of hcfc1b and not hcfc1a or other off target effects, we performed loss of function analysis with an independent hcfc1b targeting MO, which interfered with normal mRNA splicing. Consistent with our previous results, injection of the hcfc1b splice site targeting morpholino (hcfc1b SMO) resulted in craniofacial defects (Supplementary Fig. 1B and B’). Taken together these data demonstrate that the craniofacial defects associated with the hcfc1b MO are the direct result of loss of hcfc1b expression and not some other off target effect.

hcfc1b does not regulate the specification or migration of NCCs.

Neural crest cells (NCCs) give rise to multiple different tissues including the cartilaginous structures of the viscerocranium. Because the loss of Hcfc1b function causes defects in the cartilaginous structures of the viscerocranium we hypothesized that hcfc1b may regulate the specification or migration of developing NCCs. To address this hypothesis, we first assessed specification of NCCs using the Tg(sox10:memRFP) transgenic reporter line, which marks NCCs with membrane-tethered RFP expressed under the control of sox10 regulatory DNA (Kucenas et al., 2008). sox10 is a transcription factor expressed in NCCs and has been used as a molecular marker to visualize NCC location in vivo (Honore et al., 2003, p. 10). Expression of sox10 was similar in control and hcfc1b MO-injected embryos at the 12 somite stage demonstrating that hcfc1b does not regulate the specification of neural crest cells (Fig. 3 A and B).

hcfc1b regulates the expression of CNCC specific genes in the posterior pharyngeal arches.

A subset of NCCs are further fated to become cranial NCCs (CNCCs) and migrate to the pharyngeal arches before forming the cartilaginous structures of the viscerocranium. One possible explanation for the craniofacial abnormalities observed in hcfc1b morphants could be that NCCs fail to migrate into the pharyngeal arches, which are populated by the cells that give rise to the cartilaginous structures of the face. To confirm that CNCCs migrate normally to the pharyngeal arches, we performed static fluorescent imaging using the Tg(sox10:memRFP) reporter fish. sox10 expression in the pharyngeal arches demonstrated that CNCCs were localized to arches 1–5 (Fig. 3 C and D). Our data have demonstrated that NCC specification and migration are normal in morphant animals raising the possibility that hcfc1b regulates an alternative cellular process. One possibility is that CNCCs fail to express dlx2a, a transcription factor needed for CNCC maintenance (Akimenko et al., 1994; Sperber et al., 2008). To test this possibility, we assayed the expression of dlx2a by RNA in situ hybridization. dlx2a expression was localized to the forebrain and pharyngeal arch regions in both wild type and hcfc1b morphants at 1 dpf consistent with the hypothesis that CNCC specification is normal in morphant embryos (Fig. 3 E and F). Morphant embryos are able to develop cartilage structures consistent with Meckel’s cartilage and the ceratohyal, however, they do not develop ceratobranchial cartilages (Fig. 2). Based upon these results, we hypothesized that hcfc1b is essential for posterior arch development and maintenance. prdm1a is an essential mediator of posterior arch development, and loss of prdm1a results in a partially inverted ceratohyal and the loss of ceratobranchial cartilages 4–7 (Birkholz et al., 2009, p. 1). This phenotype is consistent with the phenotype we observe after loss of hcfc1b. Therefore, to test our hypothesis, we measured the expression of prdm1a, in non-injected and morphant animals using the Tg(prdm1a:EGFP) transgenic line at 1 dpf. Morphant embryos demonstrated a significant decrease in EGFP expression relative to non-injected controls consistent with the hypothesis that hcfc1b regulates posterior arch development (Fig. 3 G and H). Taken together, these data suggest that hcfc1b does not play a significant role in the specification or migration of NCCs, but alternatively that there is a reduction in the differentiation of craniofacial cartilage in the posterior arches, which would result in a loss of ceratobranchial cartilage.

hcfc1b is essential for the production of chondrocyte progenitors.

Chondrocytes are one of the differentiated derivatives of CNCCs and undergo endochondral ossification to form the skeletal structures of the viscerocranium. A possible explanation for the craniofacial abnormalities we observed in morphant animals, which is consistent with a reduced level of prdm1a expression, is
that CNCCs in the posterior arches fail to differentiate. To address this, we performed RNA in situ hybridization at 3 dpf to detect the expression of col2a1, a major component of type II collagen whose expression marks differentiated chondrocytes actively producing collagen mRNA. We observed that hcfc1b morphants lacked col2a1 expression within the ceratobranchial arches (Fig. 4A and A'). These data are consistent with decreased prdm1a expression in the posterior arches, with the loss of Alcian blue in the ceratobranchial cartilages, and with previously published results (Lee et al., 2007). Taken together these data suggest that hcfc1b regulates the differentiation of chondrocyte progenitors, which subsequently leads to the observed craniofacial defects.

Expression of col2a1 is tightly regulated at the transcriptional level during facial development and the expression of specific transcription factors is essential for downstream col2a1 expression and chondrocyte differentiation. Sox9a and Sox10 are important regulatory transcription factors associated with CNCC differentiation and both have been implicated in the regulation of col2a1 expression. The Sox9a transcription factor has been shown to bind to the enhancer element of the col2a1 (Lefebvre et al., 1997, p. 9) and over-expression of sox10 is sufficient to promote col2a1 expression in avian neural crest cells (Suzuki et al., 2006). Hence, these two genes can serve as markers for appropriate CNCC differentiation and defects in col2a1 expression could be caused by defects in the expression of either gene. In order to determine if defects in col2a1 are downstream of sox9a and sox10, we measured the expression of each gene either by RNA in situ hybridization or via injection of the hcfc1b MO into freshly fertilized embryos carrying the Tg(sox10:memRFP) reporter, respectively. RNA in situ hybridization demonstrated a loss of sox9a expression in the ceratobranchial cartilages (Fig. 4B and B'). Furthermore, visualization of sox10 via the transgenic memRFP reporter animal demonstrated that sox10 expression was absent from areas that give rise to the ceratobranchial cartilages (Fig. 4C and C'). These data are consistent with the hypothesis that hcfc1b regulates the differentiation of CNCCs and that a loss of col2a1 is due to defects in both sox9a and sox10 expression, both of which are necessary for appropriate col2a1 expression.

hcfc1b regulates cell division in the pharyngeal arches.

We have shown that mRNA levels of prdm1a, sox9a, and col2a1 are reduced in morphant animals, all of which mark CNCCs and their derivatives. However, this reduction is not due to defects in CNCC migration or specification because sox10 expressing cells appropriately populate the pharyngeal arches. One possible explanation for the reduction in expression of the markers tested is that cells within the pharyngeal arches fail to divide. To test the hypothesis that hcfc1b regulates CNCC cell division within the pharyngeal arches we performed immunohistochemistry to detect the level of phosphorylated histone H3 at serine 10 (p-H3), a
Hcfc1b regulates craniofacial development by modulating mmachc expression.

Mutation of HCFC1 has been shown to reduce the expression of MMACHC (Yu et al., 2013). Additionally, mutation of MMACHC causes cblC disease (Lerner-Ellis et al., 2006), a multiple congenital anomaly characterized primarily by defects in cobalamin metabolism. Notably, mild facial dysmorphia has been associated with early onset cblC [18,19,25–28]. Based upon these observations we hypothesized that HCFC1 regulates craniofacial development by modulating the expression of MMACHC. To begin to address this hypothesis, we first used real time PCR to examine if loss of hcfc1b resulted in reduced mmachc expression. Consistent with our hypothesis, hcfc1b MO demonstrated significantly reduced levels of mmachc mRNA at 1 dpf to approximately 10% of the normal levels (Fig. 6). The hcfc1a MO also reduced mmachc mRNA levels, but only by about 40% of normal (Fig. 6). Simultaneous knockdown of hcfc1a and hcfc1b reduced mmachc mRNA levels more than the individual knockdowns (Fig. 6), indicating that both hcfc1a and hcfc1b promote mmachc expression.

Our data show that loss of Hcfc1b function causes craniofacial abnormalities and that both Hcfc1a and Hcfc1b promote mmachc expression, raising the possibility that Mmachc function contributes to craniofacial abnormalities. To test this possibility we performed transient knockdown of mmachc with a morpholino designed to inhibit the translation of mmachc mRNA. Knockdown of mmachc resulted in craniofacial abnormalities similar in severity to those observed after loss of hcfc1b (Fig. 7A–C and Supplementary Table 2), consistent with the idea that Hcfc1 function in craniofacial development is mediated, at least in part, by Mmachc function. If this is true, then restoring mmachc expression in the absence of Hcfc1b function should rescue part or all of the defects that result from hcfc1b deficiency. To address this hypothesis, we injected in vitro synthesized human Mmachc mRNA with or without hcfc1b MO into single cell stage embryos and visualized the developing cartilage at 4 dpf using Alcian blue. Human MMACHC

had no adverse effect on craniofacial development of wild-type control embryos (Figs. 7D and D’) but efficiently rescued the craniofacial defects resulting from hcfc1b knockdown (Figs. 7E and E’). Furthermore, injection of MMACHC effectively rescued the mmachc-MO phenotype providing strong evidence that the zebrafish and human MMACHC orthologs share some functional equivalence (Figs. 7F and F’). Consistent with our previous results the co-injection of sub-optimal levels of hcfc1b-MO and mmachc-MO induced craniofacial defects (Supplementary Fig. 2C and C’). Sub-optimal concentrations for each morpholino are defined as concentrations that do not induce a craniofacial phenotype independently. The exact concentrations can be found in Supplementary Tables 2 and 3. Taken together, these data support the hypothesis that MMACHC is a functionally relevant target for HCFC1 in craniofacial development.

Mutations in either HCFC1 or MMACHC result in the accumulation of toxic metabolites such as homocysteine and methylmalonic acid, and high levels of both metabolites have been associated with the risk of developmental defects (Murphy and Fernandez-Ballart, 2011; Nyhan et al., 1989). To investigate if the facial abnormalities associated with knockdown of hcfc1b could result from the accumulation of toxic metabolites, we measured the level of the major metabolites associated with cobalamin metabolism in morphant animals. Morphant animals did not have increased homocysteine or methylmalonic acid levels relative to wildtype nor did they have reduced methionine levels, indicating that cobalamin metabolism is unperturbed in these animals (Supplementary Table 4). The absence of elevated homocysteine in morphant animals is likely not the result of compensatory homocysteine metabolism as there was no indication of elevated cystathionine or cysteine, where high levels of either metabolite would suggest increased transsulfuration. Similarly, the absence of any significant increase in either dimethylglycine, methylglycine (also known as sarcosine), glycine or methionine indicates that there has not been any increase in the remethylation of homocysteine mediated by betaine-homocysteine S-methyltransferase. Therefore, hcfc1b and mmachc might influence craniofacial development by mechanisms that operate independently of one carbon metabolism.

Distinct HCFC1 mutations have variable effects on MMACHC expression and craniofacial development

Our data so far have demonstrated that HCFC1 regulates craniofacial development by modulating the expression of MMACHC. Also, we have previously demonstrated that two different HCFC1 mutations identified in individuals with cblX lead to variable levels of reduction in MMACHC expression (Yu et al., 2013). This led us to hypothesize that individual HCFC1 mutations have a different impact on its activity at downstream promoters, including the MMACHC promoter. Thus, some mutations might lead to a moderate reduction in MMACHC expression and to different phenotypic penetrance. To address this possibility, we measured the level of MMACHC expression by quantitative real time PCR (qPCR) in cell lines derived from patient fibroblasts. The p.Ala115Val (c.344C>T) variant resulted in a significant reduction in the level of MMACHC, while the p.Ala73Val (c.218C>T) variant resulted in a more moderate reduction in MMACHC expression (Fig. 8A). These data are consistent with the hypothesis that different mutations in HCFC1 have different effects on the expression of MMACHC. Furthermore, these data are consistent with our qPCR results measuring mmachc expression in morphant animals, where moderate changes in mmachc expression do not cause facial dysmorphia.

HCFC1 binds to the MMACHC promoter region in both humans and mice (Dejosez et al., 2010; Michaud et al., 2013) and our results demonstrate the different mutations have a different effect...
on \textit{MMACHC} expression. Therefore, it is plausible that some mutations in HCFC1 completely abrogate binding to the \textit{MMACHC} promoter, but others do not and have an impact on the activity of the protein rather than the specificity and binding. To test this idea we performed chromatin immunoprecipitation (ChIP) with anti-HCFC1 antibodies to determine if HCFC1 binds to the \textit{MMACHC} promoter in fibroblast lines. qPCR analysis demonstrated that HCFC1 was significantly enriched at the \textit{MMACHC} promoter in fibroblasts derived from normal healthy individuals (Fig. 8B). Mutation of p.Ala115Val (c.344C>T) abrogates binding completely, but in contrast, HCFC1 harboring mutations affecting p. Ala73Val (c.218C>T) remains enriched at the \textit{MMACHC} promoter, although the level of enrichment is severely reduced relative to healthy individuals (Fig. 8B). These data suggest that the different HCFC1 variants have different binding affinities for the \textit{MMACHC} promoter, which may explain the different effects on phenotypes mediated by modulation of \textit{MMACHC} expression.

These observations led us to ask if distinct HCFC1 mutations have variable effects on craniofacial phenotypes. There are 5 distinct missense mutations associated with \textit{cblX}. Of the 5, the p. Ala115Val (c.344C>T) and p.Ala115Thr (c.343G>A) affect the same amino acid and are the most common (Yu et al., 2013). Two of the remaining 3, the p.Ala73Val (c.218C>T) and p. Ala73Thr (c.217G>A) also affect the same amino acid and are less common. Thus far we have demonstrated a link between p. Ala115Val (c.344C>T) mutation, severely reduced \textit{MMACHC} expression, and the presence of facial dysmorphia. In contrast the p.Ala73Val (c.218C>T) is associated with only moderate changes in \textit{MMACHC} expression and a lack of facial dysmorphia. Thus, HCFC1 mutations which result in a significant reduction in the expression of \textit{MMACHC}, appear to be associated with facial dysmorphia, while those that only moderately affect \textit{MMACHC} expression are not. Due to the limited clinical data available on the remaining individuals with \textit{cblX}, it was not possible to determine the potential variability in craniofacial phenotypes associated with all of the observed HCFC1 mutations.

In order to further test the hypothesis that different HCFC1 mutations may have a different effect on craniofacial phenotype we again utilized the zebrafish model. We performed loss of function rescue experiments with 1 of 4 independent mutant alleles [p.Ala115Val (c.344C>T), p.Ala115Thr (c.343G>A), p. Ala73Val (c.218C>T) and p.Ala73Thr (c.217G>A)] in the
developing zebrafish. We characterized the morphant phenotypes with Alcian blue: Alizarin red staining at 5 dpf and observed that the p.Ala73Val (c.218C>T) and p.Ala115Thr (c.343G>A) were able to rescue hcf1b mediated craniofacial defects to the same extent as wildtype HCFC1. However, the p.Ala115Val (c.344C>T) and p.Ala115Thr (c.343G>A) did not rescue the craniofacial defects (Fig. 9 and Supplementary Table 5). Taken together our data provide evidence that individual mutations within the HCFC1 protein differentially affect HCFC1 function, which results in different degrees of phenotypic penetrance.

Our results in patient derived human cell lines suggest that significant decreases in MMACHC expression lead to facial dysmorphia, while more moderate decreases do not. Furthermore, our work in zebrafish has demonstrated that knockdown of hcf1a results in moderate changes in mmachc expression without any facial defects, but in contrast loss of hcf1b results in drastic changes in mmachc expression and facial dysmorphia. Based upon these results we hypothesized that moderate changes in mmachc expression are not sufficient to cause craniofacial abnormalities. To test this hypothesis we co-injected sub-optimal levels of the hcf1b-MO (those that do not cause a phenotype) with sub-optimal levels of the hcf1a-MO. Consistent with our hypothesis, we observed craniofacial phenotypes similar to those observed with optimal levels of the hcf1b-MO alone (Supplementary Fig. 2D and D’).

Discussion

Whole exome sequencing (WES) has recently identified mutations in HCFC1 that result in cbIX, a multiple anomaly disorder and we have previously shown that the biochemical manifestations of cbIX are mediated via the modulation of MMACHC expression by HCFC1 (Yu et al., 2013). However, the role of HCFC1 in other phenotypic manifestations of cbIX remains to be elucidated. Because HCFC1 regulates the expression of thousands of genes (Michaud et al., 2013) it is difficult to tease out the cellular and molecular mechanisms underlying each individual phenotypic manifestation. In order to understand the pathogenicity associated with mutation of HCFC1 at the cellular and molecular level, we performed loss of function studies in the developing zebrafish.

We observed that knockdown of hcf1b, one of the two zebrafish orthologs of HCFC1, resulted in craniofacial abnormalities in zebrafish embryos. Furthermore, we have shown that these craniofacial defects are mediated in part by reduction in MMACHC expression. This novel role for MMACHC in facial development is supported by the observation of dysmorphic features in patients with MMACHC mutations (Biancheri et al., 2001; Carrillo-Carrasco et al., 2012; Cerone et al., 1999; D’Alessandro et al., 2010; Martinelli et al., 2011). However, we know that not all mutations in MMACHC or HCFC1 lead to craniofacial abnormalities. The spectrum of MMACHC mutations is quite large (Lerner-Ellis et al., 2009), including 42 different mutations identified in 204 individuals with cbIX (Martinelli et al., 2011). The effect of each of these mutations on the levels of MMACHC expression and function is not known. Thus, it is quite possible that some mutant forms of MMACHC retain residual activity, resulting in variable degrees of phenotypic penetrance. The spectrum of HCFC1 mutations is not as large and we have demonstrated that distinct mutations in HCFC1 have variable effects on the level of MMACHC function and hence, the occurrence of facial dysmorphia. Based upon these observations, we suggest that craniofacial abnormalities in individuals with cbIX and cbIX may be directly correlated to the effect of the underlying mutation on the level of MMACHC expression and function.

Furthermore, mutations in HCFC1 that result in increased levels of HCFC1 mRNA lead to a very different phenotypic spectrum, characterized mainly by intellectual disability and no reported craniofacial abnormalities or metabolic defects (Huang et al., 2012). Thus, it is likely that these mutations do not result in decreased MMACHC expression and therefore, do not affect its function. This is further supported by observations by several groups that mutations affecting different amino acids within the same gene can lead to different phenotypes. For example, mutations that truncate the C-terminus of KAT6B result in Genitopatallean Syndrome (MIM606170), whereas mutations that lead to non-sense mediated decay of KAT6B mRNA lead to Say-Barber-Bieseker-Young-Simpson syndrome (MIM603736) (Campeau et al., 2012). Recently these phenotypes have been expanded to include Blepharophimosis–ptosis–epicanthus inversus syndrome (MIM101010) (Yu et al., 2014). These data can also be expanded to other genes such as ADAR, where mutations can cause Aicardi-Goutieres syndrome (MIM615010) (Rice et al., 2012) or Dyschromatosis symmetrica hereditaria (MIM127400) (Miyamura et al., 2003; Müller et al., 2012). Future studies aimed at characterizing the functional consequence of distinct HCFC1 mutations, likely using model organisms, will help improve our understanding of its
Fig. 9. Mutations in HCFC1 differentially affect craniofacial development. (A–F) Staining of cartilage with Alcian blue on non-injected (NI), hcfc1b MO, or hcfc1b MO co-injected with the indicated mutated version of HCFC1. Numbers of animals affected are presented in Supplementary Table 5. Embryos were stained at 5 days post fertilization and manual dissection of the viscerocranium and neurocranium was performed. Neurocranium is depicted in A–F and the viscerocranium is depicted in A′–F.
role in specific cell types during development and the effect of different mutations on downstream effectors and subsequently their phenotypic outcomes.

An important question that remains to be answered is how might imbalances in MMACHC expression and function lead to craniofacial abnormalities? Do mutations in MMACHC cause facial dysmorphia because they result in the perinatal accumulation of toxic metabolites such as homocysteine? Homocysteine is teratogenic and can induce neural tube defects in chick embryos (Rosenquist et al., 1996). However, case controlled studies measuring homocysteine levels during gestation have failed to correlate high levels of this metabolite with cleft lip with and without cleft palate (Shaw et al., 2009), suggesting that mutation of MMACHC might not cause facial deformities via the accumulation of toxic metabolites. MMACHC is expressed developmentally and is a putative cobalamin transport protein with enzymatic capabilities (Hannibal et al., 2009; Papavac et al., 2011). Therefore defects in MMACHC expression or function result in cobalamin deficiencies (Liu et al., 2010). The presence of craniofacial defects in the absence of any apparent changes in homocysteine, methionine or methylnalonic acid levels in the developing zebrafish and the fact that those defects can be prevented by forced MMACHC expression strongly suggest that some aspects of pathogenesis in cblC and cblX disease are not solely due to impaired cobalamin metabolism. Our own observations that individuals with cblX have variable levels of homocysteine and methylnalonic acid in blood and urine further support this possibility (Yu et al., 2013). In addition, cblC exhibits a number of unique clinical sequelae that are not present in other forms of homocystinuria or methylnalonic acidemia despite having biochemical markers in common with them (Weisfeld-Adams et al., 2013). Similarly, a previous report has shown progressive neurological deterioration and head MRI abnormalities in a patient with cblC disease despite successful lowering of methylnalonic acid levels by early intervention with hydroxycobalamin treatment (Enns et al., 1999). Thus, the accumulation of toxic metabolites may not be the cause of at least some of the observed phenotypes.

Together these data suggest that MMACHC either has a yet to be identified secondary function, which is distinct from its role in cobalamin metabolism or that it mediates the observed craniofacial abnormalities through another pathway. One such pathway might be the folate pathway because there is interplay between cobalamin and folate pathways that could result in folate trapping, impaired DNA synthesis, and impaired methylation (Chanarin et al., 1985; Varela-Moreiras et al., 2009). Folate trapping occurs when cobalamin levels are low causing an increased amount of methyl tetrahydrofolate that cannot be metabolized which mimics folate deficiency. This is significant as folate deficiency has been associated with cleft lip/palate and other facial abnormalities (Hartridge et al., 1999; Johnson and Little, 2008; Schubert et al., 2002; Wilcox et al., 2007). More specifically, polymorphisms in genes within the folate pathway such as MTHFR, have been associated with risk and susceptibility of facial anomalies (Brouns et al., 2008; Chevrier et al., 2007; Mills et al., 2008; Mostowska et al., 2010). The biochemical data from our zebrafish experiments indicate that the hfc1 morpholino model is not exhibiting impaired cobalamin metabolism, suggesting that the occurrence of the methylfolate trap is unlikely. However, a future stable mutant model may reveal more consistent changes in the folate cobalamin pathway. It would therefore be interesting to investigate if mutation of hfc1b exhibits any of the hematological sequelae, cellular folate, or global DNA hypomethylation that has previously been proposed as a consequence of the methylfolate trap. These possibilities have not yet been fully explored and could provide useful data regarding the role of both folate and cobalamin metabolism in facial development.

Developmentally, the craniofacial abnormalities observed in our zebrafish morphants are likely mediated by the regulation of neural crest cells (NCCs), a multipotent progenitor cell population. Our results suggest that HCFC1 regulates the proliferative capacity of cranial NCCs and highlight an essential role for HCFC1 during craniofacial development. The role of HCFC1 in cell proliferation has been previously established, especially in embryonic stem cells (Dejosez et al., 2010; Van den Berg et al., 2010, p. 4) as well as other cell lines (Mangone et al., 2010; Parker et al., 2012; Tyagi et al., 2007). Further, HCFC1 interacts with THAP11, a protein that regulates stem cell self-renewal (Dejosez et al., 2008) and with OCT4, a protein that is at the center of a protein network that is essential for pluripotency (Ding et al., 2012; Van den Berg et al., 2010, p. 4). Together, these results suggest that HCFC1 regulates stem cell pluripotency through its interaction with its protein partners. This idea is further supported by the fact that mutations in HCFC1 cluster within the kelch protein interaction domain, which likely abrogates binding to THAP11, OCT4, other protein partners as well as DNA. Future efforts focused on understanding normal and mutant HCFC1 activity will be necessary in order to gain a better understanding of how single point mutations in HCFC1 lead to various biochemical and developmental defects.

In summary, the functional analysis in a model organism of HCFC1, a gene associated with human disease, provides direct evidence for its role in early vertebrate development. The observation of defects in the development of craniofacial structures in zebrafish, highlight facial dysmorphia, a previously under-reported phenotype, as a potential outcome of mutations in both HCFC1 and its downstream target, MMACHC. Further, our data sheds new light on how distinct mutations in HCFC1 may lead to variable phenotypes. These results provide a foundation for future studies to further elucidate the downstream effectors regulated by HCFC1 and the developmental pathways affected by its mutation. The elucidation of developmental mechanisms underlying phenotypic outcomes associated with HCFC1 mutations has the potential to provide future therapeutic targets and treatment options to alleviate some or all of the disease symptoms.

Author contributions

A.M.Q., B.A., and T.H.S. designed the study. A.M.Q. performed the zebrafish experiments. E.A.G. and N.A. generated cDNA clones used in rescue experiments. K.N.M. and S.P.S. measured and interpreted metabolic data from zebrafish embryos. D.S.R. provided patient derived material for expression analysis and downstream ChIP assays. K.B.A. provided expertise in experimental design and interpreted data. T.H.S and B.A. supervised the overall project. A.M.Q., B.A., and T.H.S. wrote the manuscript with input and approval from all coauthors.

Acknowledgements

A.M.Q was supported by an Institutional Postdoctoral Research Training Grant T32MH015442. This work was supported in part by an NIH Grant GM081519/S1 and institutional funds to T.H.S; NIH Grant NS062717 and a gift from the Gates Frontiers Fund to B.A. The University of Colorado Anschutz Medical Campus Zebrafish Core Facility is supported by NIH P30 NS048154. We thank Alison Brenner for her help in organizing patient derived material.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.09.026.


