Hcfc1a regulates neural precursor proliferation and asxl1 expression in the developing brain

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Abstract

Background: Precise regulation of neural precursor cell (NPC) proliferation and differentiation is essential to ensure proper brain development and function. The HCFC1 gene encodes a transcriptional co-factor that regulates cell proliferation, and previous studies suggest that HCFC1 regulates NPC number and differentiation. However, the molecular mechanism underlying these cellular deficits has not been completely characterized.

Methods: Here we created a zebrafish harboring mutations in the hcfc1a gene (the hcfc1a<sup>co60/+</sup> allele), one ortholog of HCFC1, and utilized immunohistochemistry and RNA-sequencing technology to understand the function of hcfc1a during neural development.

Results: The hcfc1a<sup>co60/+</sup> allele results in an increased number of NPCs and increased expression of neuronal and glial markers. These neural developmental deficits are associated with larval hypomotility and the abnormal expression of asxl1, a polycomb transcription factor, which we identified as a downstream effector of hcfc1a. Inhibition of asxl1 activity and/or expression in larvae harboring the hcfc1a<sup>co60/+</sup> allele completely restored the number of NPCs to normal levels.

Conclusion: Collectively, our data demonstrate that hcfc1a regulates NPC number, NPC proliferation, motor behavior, and brain development.

Keywords: HCFC1, Neural precursor cells (NPCs), Brain development, asxl1

Background

Neural precursor cells (NPCs) give rise to the differentiated cells of the central nervous system and defects in the number produced, their proliferation, and/or survival can result in a variety of neural developmental disorders. These disorders include intellectual disability [1], cognitive dysfunction [2], behavioral impairment [3], microcephaly [4], epilepsy [5], autism spectrum disorders [6], and cortical malformations [7]. Previous studies have demonstrated a complex network of transcription factors that are responsible for modulating NPC function including SOX2 [8], SOX1 [9], NESTIN [10], and PAX transcription factors [11]. Recent evidence suggests that the HCFC1 gene, which encodes a transcriptional cofactor, is essential for stem cell proliferation and metabolism [12, 13] in a variety of different tissue types, including NPCs [14–17]. These data strongly suggest that HCFC1 is part of a more global transcriptional program modulating NPC proliferation and differentiation.

HCFC1 regulates a diverse array of target genes and has been shown to bind to the promoters of more than 5000 unique downstream target genes [18]. Consequently, the molecular mechanisms by which HCFC1 regulates NPC proliferation and differentiation are complex. Mutations...
in HCFC1 cause methylmalonic acidemia and homocysteinemia, cblX type (cblX) (309541). cblX is an X-linked recessive disorder characterized by defects in cobalamin (vitamin B12) metabolism, nervous system development, neurological impairment, intractable epilepsy, and failure to thrive [19]. Functional analysis of cblX syndrome has provided a platform whereby the function HCFC1 in discrete organs and tissues can be elucidated. For example, in vitro analysis has demonstrated that HCFC1 regulates metabolism indirectly by regulating the expression of the MMACHC gene [12, 14, 15, 19]. These data are further supported by in vivo analysis using transient knockdown in the developing zebrafish [16, 20]. Additional mouse models exist and have demonstrated a function for HCFC1 in diverse cell populations [21, 22], including a subset of NPCs [17]. However, although it is clear that HCFC1 is essential for NPC function [17], previous studies have not yet determined a mechanistic basis for the cellular phenotypes observed. Thus, additional studies examining the function of HCFC1 in NPCs are warranted.

We have created a zebrafish harboring a mutation in the hcfc1a gene (hcfc1a<sup>co60/+</sup> allele) using CRISPR/Cas9. Zebrafish have two paralogues of HCFC1 and in previous studies, we demonstrated that hcfc1b is associated with increased NPC production [16]. The two zebrafish paralogues have been shown to have divergent functions, as the knockdown of hcfc1b causes facial dysmorphism, but knockdown of hcfc1a does not [20]. Therefore, we asked whether germine mutations in the hcfc1a gene cause defects in neural development. Our results demonstrate that the hcfc1a<sup>co60/+</sup> allele results in increased numbers of proliferating NPCs (Sox2<sup>+</sup>) and hypomotility. Subsequent RNA sequencing on whole brain homogenates obtained from the hcfc1a<sup>co60/+</sup> allele identified increased expression of assx1, which encodes a transcription factor known to modulate the cell cycle [23, 24]. Furthermore, inhibition of assx1 expression in larvae carrying the hcfc1a<sup>co60/+</sup> allele restored the number of NPCs to normal levels. Collectively, our study demonstrates a molecular mechanism by which hcfc1a regulates NPC proliferation and brain development.

**Methods**

**Experimental model and subject details**

The experimental model used in this study is the zebrafish, *Danio rerio*. Zebrafish were obtained from the University of Colorado, School of Medicine or the Zebrafish International Resource Center (ZIRC). The hcfc1a<sup>co60/+</sup> allele was produced using CRISPR/Cas9 methodology as described [25]. The hcfc1a<sup>co60/+</sup> allele was produced at the University of Colorado, School of Medicine by the corresponding author and obtained according to protocols from the University of Texas El Paso. Briefly, a guide RNA (GGTTCATACAGCGGTTCGT) was designed using publicly available software (ZiFit) [26]. Oligonucleotides from the forward and reverse strand were annealed and ligated into the DR274 vector as described [26]. Guide template DNA was synthesized using PCR amplification with primers (DR274 FWD: TTGTAGACGGCCAGCATG and DR274 Rev: TTCTGCTATGGAAGGTCAGGT) and RNA was synthesized using the MEGAscript T7 in vitro transcription kit. Cas9 was synthesized using the T7 mMessage machine after linearization with PmeI (New England Biolabs) of the Cas9 vector (pMLM3613). A solution (0.2 M KCl with phenol red indicator) containing a final concentration of 500 ng Cas9 and 70 ng of guide RNA was injected at a volume of 2 nl at the single cell stage and embryos were grown to adulthood. The hcfc1a<sup>co60/+</sup> allele was generated from a single founder (F<sub>0</sub>), which was outcrossed with 3 independent wildtype (AB) fish to generate 3 families of F1 carriers. Each family consisted of approximately 20 total fish with equal numbers of males and females. To generate subsequent generations, we outcrossed a minimum of 3 F1 individuals with wildtype (Tupfel Long Fin) to obtain a minimum of 3 families of F2 carriers. We subsequently outcrossed F2 carriers (minimum of 3) with wildtype (AB) fish to produce an F3 generation of approximately 3 total families with equal numbers of males and females. Sanger sequencing confirmed mutation and experiments were initiated in the F3 generation.

The *Tg(hsp701:HCFC1)* was created using Gateway cloning technology. Briefly, the p5e-<i>hsp701</i>, pME-<i>HCFC1</i> (created from <i>pcDNA6.1</i> reported in [20]), p3E-polyA, and the pDestTol2PA were recombined via LR recombination. The resultant vector was co-injected with transposase mRNA synthesized from the pCS2FA vector as previously described [27]. The experiments described herein were performed in the F2 generation, which was produced from a single founder (F<sub>0</sub>). The positive F<sub>0</sub> carrier was outcrossed with wildtype (AB) to produce 2 families of F1 individuals and a minimum of 3 carriers of the F1 generation were outcrossed to produce 3 families of F2 carriers that were utilized for the experiments described.

For all experiments, embryos (prior to sexual dimorphism) were obtained by crossing AB wildtype, Tupfel Long Fin wildtype, *Tg(hsp701:HCFC1)*, or hcfc1a<sup>co60/+</sup>. Experiments were performed at developmental stages only [0–5 days post fertilization (DPF)]. All embryos were maintained in embryo medium at 28 °C. All adults beyond the age of peak breeding age (>1.5 years) were euthanized using a 10 g/l buffered solution of pharmaceutical grade MS 222 (Tricaine). Fish were emerged in solution for 30 min at room temperature. All euthanized...
adults underwent secondary euthanasia using a cold ice bath (2–4 °C). Fish were monitored for operculum movement during euthanasia and cessation of movement was indicative of euthanasia. Embryos (<7 days old) were euthanized using 1–10% sodium hypochlorite solution after being anesthetized in cold ice bath. Prior to fin clipping and before fixation, all fish, adults and larvae, respectively, were anesthetized using MS 222 (150 mg/l for adults and 300 mg/l for embryos). The degree of anesthesia was monitored by operculum movement of adults and cessation of movement for larvae. These techniques are approved and advised by the American Veterinary Medical Association and approved by the University of Texas El Paso Institutional Animal Care and Use Committee.

**Genotyping**

Genotyping of the \(hcfc1a^{co60/+}\) allele was performed by lysing excised larval tissue or fin clips (adults) in lysis buffer (10 mM Tris pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, and 200 μg/ml proteinase K) for 3 h at 55 °C. DNA was isolated according to standard phenol chloroform: ethanol precipitation procedures. Primers pairs were developed that specifically bind to and amplify the mutated allele, which did not amplify the wildtype allele. The fragment of interest was amplified by standard PCR at an annealing temperature of 64° (FWD: CCA GTTCGCCCTTTTTGTGT and REV: ACCGGTGTT ATGAACACTGGC). Positive amplification indicates positive carriers of the allele (Fig. 1). Genotyping of the \(Tg(hsp701:HCFC1)\) allele was performed with the following primers: forward primer (TGAAACAATGGC ACCATAAATTG) present in the \(hsp701\) promoter and reverse primer in the \(HCFC1\) open reading frame (CGT CACACACAAGCCCATAG). Amplification indicates the genotype of interest.

**Immunohistochemistry**

Embryos/larvae were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for minimum of 1 h

![Fig. 1](image)
at room temperature (RT). For each time point, a small piece of caudal tissue was excised for genotyping and the remaining rostral tissue was embedded in 1.5% agarose (Fisher) produced in 5% sucrose (Fisher) and embryo medium. Embedded blocks were incubated overnight in 30% sucrose (Fisher) and then snap frozen with dry ice and cryosectioned (12–20 μM). Sections were washed twice in 1× phosphate buffered saline (PBS) pH 7.4 at RT for 30 min each and blocked for 1 h in blocking buffer (2 mg/ml bovine serum albumin (Fisher), 2% goat serum (Fisher) diluted in 1× PBS). Primary antibody [1:200 anti-Sox2 (Abcam) or 1:500 anti-HuC/D (Fisher)] was incubated overnight at 4 °C and then washed twice in 1× PBS for 30 min each at RT. Alexa fluor antibodies (Fisher) were diluted 1:200 and incubated on each slide for 1 h at RT. All slides were cover slipped using Vectashield (Vector Laboratories) and imaged on a Zeiss LSM 700 at 20×–63× magnification. For cell proliferation, larvae were pulsed in 20 mM 5-ethynyl-2′-deoxyuridine (EdU) (Fisher) diluted in 10% dimethyl sulfoxide (DMSO) (Fisher) for 30 min at RT prior to fixation. EdU was detected using the EdU Click-It technology (Fisher) according to manufacturer protocol.

**Cell quantification**

For cell quantification, sections were first divided into forebrain, midbrain, and hindbrain regions using two zebrafish brain atlases; (1) Atlas of Zebrafish Development [28] and (2) Atlas of Early Zebrafish Brain Development [29]. To specify brain regions, major hallmark sub-divisions of each brain section were separated based on age of larvae and the published sections and demarcations present in [29]. For example, sections of the developing brain are organized by letters: A–F indicate forebrain sections, G–L are midbrain, and M–R are hindbrain according to the zebrafish brain atlas. After standardization of the brain region by atlas, cells from each section (12–20 μM) were counted using the ImageJ cell counter. The ImageJ cell counter allows for the manual counting of cells by marking each cell with a colored square and adds the tallied cell to the quantification sheet. The cell counter allows for the tally of 4 independent groups separately with a different color square. Cells were easily visible across all replicates. Only biological replicates with high tissue integrity were quantified. For comparison of representative images, equivalent sections were standardized from the zebrafish brain atlas. For bar graphs, the average number of cells across each brain region were utilized for quantification using approximately 10–20 equivalent sections/brain region/fish. The number of animals per group is described in each figure legend. To determine the relative increase/decrease in total cell number, the number of total cells/section was divided by the average number of cells present in wildtype siblings for each brain region analyzed and multiplied by 100. All statistical analysis was performed using total numbers of cells/section/brain region. All immunohistochemistry was validated with quantitative real time PCR (QPCR) of each gene analyzed.

**QPCR and in situ hybridization**

Whole mount in situ hybridization (ISH) was performed as described by Thisse and Thisse [30]. Briefly, embryos were harvested and dechorionated at the indicated time point and fixed in 4% paraformaldehyde (PFA) (ElectroN Microscope Sciences) for 1 h at RT. Embryos were permeabilized with proteinase K (10 μg/ml) for the time indicated [30]. Permeabilized embryos were prehybridized in hybridization buffer (HB) [50% deionized formamide (Fisher), 5× SSC (Fisher), 0.1% Tween 20 (Fisher), 50 μg/ml heparin (Sigma, St. Louis), 500 μg/ml of RNase free tRNA (Sigma), 1 M citric acid (Fisher) (460 μl for 50 ml of HB)] for 2–4 h and then incubated overnight in fresh HB with probe (50–100 ng) at 70 °C. Samples were washed, blocked in 2% sheep serum, and incubated with anti-DIG Fab fragments (1:10,000) (Sigma) overnight at 4 °C. Samples were developed with BM purple AP substrate (Sigma) and images were collected with a Zeiss Discovery Stereo Microscope fitted with Zen Software. The asxl1 cDNA probe sequence was amplified using the following primers: FWD: CATCAACACACGGACCTTG and REV: CAGTGAGTTGGGTGAAGTT; purified using a DNA purification kit (Fisher), then ligated into the pGEM-T easy vector using the pGEM T-easy Plasmid Ligation Kit (Promega).

For QPCR, RNA was isolated from embryos at the indicated time point using Trizol (Fisher) according to manufacturer’s protocol. Reverse transcription was performed using Verso cDNA synthesis (Fisher) and total RNA was normalized across all samples. PCR was performed in technical triplicates for each sample using an Applied Biosystem’s StepOne Plus machine with Applied Biosystem’s software. Sybr green (Fisher) based primer pairs for each gene analyzed are as follows: mmachc fwd: GCTTCCAGGTTTACCCTTTC, mmachc rev: ACGGCAAGGGTGCTCTGA, asxl1a fwd: ACAGGCGGCTAAGACGATGTT, asxl1a rev: TCCTGTGACTGTGGCGGAA, asxl1 fwd: CCAGAAGTCGAAGAAGGCT, asxl1 rev: ACAAACGCTCCTCAGATT, rpl13a fwd: TCCCCAGCTGCTCAGAATT, rpl13a rev: TTCTTGGAATAGCCGAGCT, sox2 fwd: AACTCTCCTCGGGAACACCA, sox2 rev: ATCCGGGATGCTCTCTCATGT, elavl3 fwd: TAAAGCGCCCTGTCATAGCA, elavl3 rev: CGTGGTGTATAGCCTGTGG, grfp fwd: GGC...
CAACTCTAACATGCAGG, 
gap rev: ATTCAGGTT
CACAGGTCAGG, olig2 fwd: TTCTGTAGGCACAC
ACCAG, and olig2 rev: TTAACTCGGTGGAGAAT
CG. Analysis was performed using 2ΔΔct.

For RNA sequencing analysis, total RNA was iso-
lated from brain homogenates (N = 12/group from 3
biological replicates), analyzed for RNA integrity, and
sequenced at The University of Texas El Paso Border
Biomedical Research Center Genomics Core Facility.
RNA sequencing was performed in biological triplicate.
RNA integrity was assessed with a TapeStation 2200 and
the library was prepared with a TruSeq stranded mRNA
library preparation kit. Sequencing was performed on a
NextSeq 500 (Illumina) using a high output kit V2 (150
cycles). For analysis, the sequences were quality trimmed
using Trimmomatic [31] and aligned to the Danio rerio
genome (build GRCz11) obtained from Ensembl v95
using TopHat2 [32]. Cufflinks [33] was used to determine
the differential expression patterns between mutant and
wildtype samples.

Tg(hsp701:HCFC1) analysis and rescue experiments
F2 carriers of the Tg(hsp701:HCFC1) were incrossed and
grown at 28°C for 24 h and then split into two groups, non-
heat shock and heat shock. Heat shock was performed
for 30 min at 38°C and then allowed to acclimate at RT for
20 min. Heat shock was initiated at 24 h post fertiliza-
tion (HPF) and performed every 8 h until 2 or 5 DPF. For
LY294002 (Selleck Chemicals) rescue, the drug was dis-
solved in 100% DMSO (Fishier) and embryos were treated
at 24 HPF with a 12uM concentration for a period of
24 h. Media was removed and embryos were dechorion-
ated (if necessary) and fixed for immunohistochemistry.

For morpholino rescue, 2 nl of a 0.1 mM solution of
asxl1 targeting translation inhibiting morpholinos (GTT
TGTCCTCATTTCTCAGTGGTT) or random control
morpholinos (Gene-Tools) were injected into offspring
of the hcfc1Δa- allele (Additional file 1: Figure S1). Full length Hcfc1a
is approximately 1778 amino acids in length. Genotyp-
ing of the hcfc1aΔa- allele was developed according
to the materials and methods section, using a reverse
primer unique to the mutant allele in the amplification
strategy (Fig. 1a). Positive amplification was indica-
tive of positive carriers (Fig. 1b), as the primers did not
bind to or amplify the wildtype allele. Initial crosses
between heterozygous carriers of the hcfc1aΔa- allele
integration period of 300 s. Data was measured as total
distance traveled (mm) and total swim speed (mm/s)
(Swim Speed = [Total distance traveled in large and small
movements] /[Total duration spent by
the animal in small and large movements (smldur + lardur)). Statistical significance was determined according
to a T-test. All experiments were performed in biological
triplicate.

Quantification and statistical analysis
For all assays, statistical significance was calculated using
a T-test to compare the means of two groups. All assays
were performed in biological duplicate and triplicate and
all QPCR was performed in technical and biological tri-
plicate or duplicate, respectively. For each assay, the total
number of animals (N) is indicated in the figure legend.
Number of animals was determined based on power
analysis conducted from preliminary studies. For all
graphs, statistical significance between groups and the
p-value is shown in the figure legend. For cell quantifica-
tion, the number of Sox2+ or EdU+ cells were counted
to section and normalized according to the methods
section above. All sections were sub-divided based on
landmarks in the Atlas of Early Zebrafish Brain Develop-
ment, 2nd Edition and then separated into specific brain
regions (forebrain, midbrain, and hindbrain). All graphs
represent error bars as standard error of the mean (SEM).

Results
Production of the hcfc1aΔa- allele
Previous studies suggest that HCFC1 regulates the
number of NPCs in vitro and in mouse models [14–
17]. HCFC1 is highly conserved across species [19]
and zebrafish have been used as a model system to
understand the mechanisms by which mutations in
HCFC1 cause disease [16, 20]. Therefore, we devel-
oped a zebrafish harboring a germline mutation in the
hcfc1a gene using CRISP/Cas9 technology. We de-
veloped a specific guide RNA (sgRNA) that targets exon
3 of the hcfc1a gene (Fig. 1a). The sgRNA was injected
at the single cell stage and resulted in the net inser-
tion of 13 nucleotides (Fig. 1a). The introduction of
these nucleotides is predicted to introduce a premature
stop codon and encode a peptide of 94 amino acids in
length (Additional file 1: Figure S1). Full length Hcfc1a
is approximately 1778 amino acids in length. Genotyp-
ing of the hcfc1aΔa- allele was developed according
to the materials and methods section, using a reverse
primer unique to the mutant allele in the amplification
strategy (Fig. 1a). Positive amplification was indica-
tive of positive carriers (Fig. 1b), as the primers did not
bind to or amplify the wildtype allele. Initial crosses
between heterozygous carriers of the hcfc1aΔa- allele

failed to generate homozygous progeny and did not obey Mendelian inheritance patterns. These results indicate that Hcfc1a is required for early development, which is consistent with previously published studies [21, 22], however the mechanism for embryonic lethality of the homozygous allele was not explored further here. Importantly, heterozygous carriers survived to adulthood with a lifespan equivalent to wildtype adult zebrafish. Adult heterozygous carriers did not show any gross morphological phenotypes associated with the allele. Larval heterozygous carriers did not show overt morphological phenotypes during early development, which is consistent with previous studies using anti-sense morpholinos targeting the hcfc1a gene [20]. However, based on previous studies [14, 15] we surmised that heterozygous carriers of the hcfc1a co60/+ allele would have defects in overall hcfc1a expression and potential defects in brain development. Therefore, we measured the expression of hcfc1a in carriers of the hcfc1a co60/+ allele and their wildtype siblings using QPCR. We designed primers to detect hcfc1a expression downstream of Exon 3 that span exons 15 and 17 to ensure that the primers were capable of detecting changes in total mRNA expression and not truncated N-terminal transcripts. As shown in Fig. 1c, at 2 DPF carriers of the hcfc1a co60/+ allele had a 50% decrease in total hcfc1a mRNA (p < 0.05).

**Hcfc1a regulates NPC number in vivo**

Several studies suggest that mutation or abnormal expression of Hcfc1 (mouse) mRNA disrupts the number of NPCs, their proliferation, and/or their differentiation. Moreover, we have previously published that hcfc1a is expressed in the developing brain across the forebrain, midbrain, and hindbrain [20]. Therefore, we asked whether the decrease in hcfc1a mRNA expression in carriers of the hcfc1a co60/+ allele resulted in abnormal numbers of NPCs in vivo. To test this, we first measured the expression of sox2 and pax6 using QPCR at 5 DPF. We used sox2 and pax6 as a readout for NPCs because they are co-localized and established markers of NPCs in vivo [35–37]. At 5 DPF, the expression of both sox2 and pax6 were up-regulated in hcfc1a co60/+ larvae relative to their wildtype siblings (Fig. 2a). We next compared the total number of Sox2+ cells in hcfc1a co60/+ larvae and their wildtype siblings over the course of development at 1 (Fig. 2b), 2, and 5 DPF. Increased NPCs were not observed until 2 DPF (Fig. 2c), but this increase was
sustained until 5 DPF (Fig. 2d). *hcfc1a* co60/+ larvae had increased numbers of NPCs in the forebrain, midbrain, and hindbrain regions, with NPCs highly enriched in the ventricular region of the developing brain (Fig. 3a–d and a′–d′; arrowheads indicate cells). There was approximately 25–30% more Sox2+ cells per brain region based upon our quantification (Figs. 2, 3).

The *hcfc1a* co60/+ allele disrupts cell proliferation
Mutation of *Hcfc1* in mice is associated with increased cell death in a subpopulation of NPCs [17], therefore, we hypothesized that the excess NPCs produced undergo cell death. To measure cell death, we performed immunohistochemistry with anti-active caspase 3 antibodies and anti-Sox2 antibodies at 5 DPF. As shown in Fig. 4a, a′, we detected approximately 1–2 Caspase+ Sox2+ cells in sibling controls (white arrowhead), however larvae harboring the *hcfc1a* co60/+ allele had on average approximately 4–5 co-localized cells per section (white arrowheads in Fig. 4a′). As we detected very few total Caspase+ NPCs, we next quantified the total number of NPCs in each group to determine the total number of NPCs surviving. As shown in Fig. 4b, while the *hcfc1a* co60/+ allele led to an increase in the number of caspase positive NPCs (red bars), the vast majority of NPCs in both wildtype and *hcfc1a* co60/+ larvae were not caspase positive (gray bars), indicating that a significant fraction of NPCs survive. Because of this survival, we next analyzed cell proliferation in *hcfc1a* co60/+ larvae and their wildtype siblings using EdU click-it technology. The *hcfc1a* co60/+ allele resulted in a statistically significant increase in the number of EdU positive cells in both the midbrain and hindbrain regions (Fig. 4c–d, c′–d′ and quantified in Fig. 4e). We observed an increase in the number of EdU positive cells in the forebrain, although the increase in the forebrain was not significant across multiple biological replicates (p = 0.06). Collectively, these data suggest the *hcfc1a* co60/+ allele results in an increase in NPC proliferation, whereby a sub-population of these NPCs undergo cell death, while the majority of those NPCs produced, survive.

The *hcfc1a* co60/+ allele is associated with abnormal expression of pro-neural and pro-glial genes
The *hcfc1a* co60/+ allele is associated with increased proliferation and an increased number of NPCs. Importantly,
the majority of these cells do not undergo cell death and therefore, we asked if the expression of genes associated with either neuronal or glial differentiation was abnormal. We measured the expression of two established markers of neurons and radial glial cells, *elavl3* and *gfap*, by immunohistochemistry. As shown in the Fig. 5a', b', the expression of both Gfap and Elavl3 expression was increased in *hcfc1a*<sup>co60/+</sup> larvae at 5 DPF. Next, we
Castro et al. BMC Neurosci           (2020) 21:27

quantified the level of expression of each marker and one additional marker of differentiation (olig2) using QPCR at 5DPF. QPCR demonstrated an increase in the level of mRNA expression of each marker (Fig. 5c) in hcfc1a co60/+ larvae relative to their wildtype siblings (p < 0.05).

**Overexpression of HCFC1 reduces the number of NPCs and decreases neural and glial gene expression**

Our data demonstrates that haploinsufficiency of hcfc1a is associated with increased proliferation and increased numbers of Sox2+ cells. Previous studies have shown that over-expression of Hcfc1 (mouse) in vitro is associated with reduced NPC proliferation/growth and reduced NPC proliferation [15].

We tested the effects of HCFC1 over expression in zebrafish by creating a transgenic zebrafish expressing human HCFC1 under the control of the heat shock promoter, hsp701. The efficacy of the hsp701 promoter in zebrafish has been widely established in previous studies [27, 38–42]. We activated expression of HCFC1 by performing a heat shock as described in the methods section for a period of 5 days. We first measured the mRNA expression of sox2, elavl3 (HuC/D), gfap, and olig2 by QPCR. Activation by heat shock of the Tg(hsp701:HCFC1) allele resulted in decreased expression of all markers analyzed (Fig. 6a; p < 0.05). We next analyzed the number of Sox2+ cells in the presence and absence of heat shock. Activation of the Tg(hsp701:HCFC1) was associated with a decreased number of NPCs across the forebrain (p = 4.88568E−05), midbrain (p = 0.004359), and hindbrain (p = 0.0776) (Fig. 6b, c–c′).

**Asxl1 is overexpressed in animals with the hcfc1a co60/+ allele**

HCFC1 is known to regulate metabolism and craniofacial development via the modulation of MMACHC expression [14, 19, 20]. Therefore, we hypothesized the neural phenotypes associated with mutations in hcfc1a were the direct consequence of defects in mmachc expression. We measured the expression of mmachc in hcfc1a mutants and their wildtype siblings. As shown in Fig. 7a, QPCR analysis demonstrated that mmachc expression was unchanged by the hcfc1a co60/+ allele. Based upon these data, we hypothesized that mutation...
of \textit{hcfc1a} does not regulate brain development by modulating \textit{mmachc} expression. To better understand the mechanisms downstream of \textit{hcfc1a}, we performed RNA-sequencing at 2 DPF using whole brain homogenates from wildtype and \textit{hcfc1a} co60/+ larvae (Additional file 2: Table S1). Using literature analysis, we identified the \textit{asxl1} gene as one possible downstream effector of \textit{Hcfc1a} in the developing brain. \textit{asxl1} encodes a transcriptional regulator that is essential for proper cell proliferation and whose deletion causes cellular senescence [24, 43]. More importantly, mutations in \textit{ASXL1} have been associated with Boring Opitz Syndrome (605039), which has been characterized by profound intellectual disability [44]. According to in situ hybridization, \textit{asxl1} expression is restricted to the developing zebrafish brain as indicated by the purple stain in panel b' (Fig. 7b, b'). The sense negative control was absent of this purple stain as shown in Fig. 7b. Additional, QPCR analysis of brain homogenates validated a 14-fold increase of \textit{asxl1} expression at 2 DPF in \textit{hcfc1a} co60/+ larvae relative to wildtype siblings (Fig. 7c).

\textbf{Inhibition of \textit{asxl1} restores the NPC phenotype in \textit{hcfc1a} co60/+ larvae}

Deletion of \textit{Asxl1} in mouse embryonic fibroblasts (MEFs) causes growth retardation because \textit{Asxl1} regulates the cell cycle via activation of the AKT-E2F axis [24]. Based upon these data, we hypothesized that over-expression of \textit{asxl1} in \textit{hcfc1a} co60/+ larvae promotes proliferation of NPCs. To test this hypothesis, we designed a translational blocking morpholino to inhibit \textit{asxl1} expression in \textit{hcfc1a} co60/+ larvae. We determined the concentration for injection empirically and selected the highest concentration that promoted > 70% survival of injected embryos. We injected \textit{asxl1} morpholinos or random control morpholinos into \textit{hcfc1a} mutants and their wildtype siblings at the single cell stage and then analyzed the number of EdU positive cells at 2 DPF. As shown in Fig. 7d, the injection of random control morpholinos into \textit{hcfc1a} mutants and their wildtype siblings had no detrimental effects and recapitulated the NPC phenotype previously observed (i.e. increased NPCs, Figs. 2 and 3). However, the injection of \textit{asxl1} morpholinos completely
restored the number of NPCs to wildtype levels in all brain regions (Fig. 7d, red bars).

Morpholinos can display off-target effects [45] and therefore we sought an alternative route of asxl1 inhibition in hcfc1a co60/+ larvae. Youn and colleagues have demonstrated that ASXL1 (mouse) expression promotes cell proliferation by binding to AKT kinase and promoting AKT phosphorylation [24]. However, this function can be inhibited with PI3K/AKT inhibitors. Therefore, we treated hcfc1a co60/+ larvae and their wildtype siblings with LY294002, a PI3K inhibitor as described in [24]. hcfc1a co60/+ embryos were treated at 24HPF with 12 μM LY294002 or vehicle control (DMSO). Vehicle treatment of wildtype and hcfc1a co60/+ larvae recapitulated the NPC phenotype (i.e. increased NPCs) present in hcfc1a co60/+ larvae (Fig. 8a–a″, b–b″ and c–c″, white arrowheads), which was consistent across all brain regions as both the number of Sox2+ cells (Fig. 9a) and the number of EdU+ cells (Fig. 9b) were increased in hcfc1a co60/+ larvae. In contrast, treatment with LY294002 reduced the number of cycling cells (EdU+) and the number of NPCs (Sox2+) in hcfc1a co60/+ larvae, consistent with our hypothesis (Fig. 9a, b, red bars). To complement these data, we next performed mRNA expression analysis of sox2, asxl1, and cyclin E (ccne1) in treated and untreated hcfc1a co60/+ larvae at 5 DPF. As shown in Fig. 9c, treatment with LY294002 resulted in decreased expression of sox2 and asxl1, which was correlated with decreased ccne1 expression (p < 0.05).

Defects in neural development are associated with larval hypomotility

The functional consequences of the defects in brain development in the hcfc1a co60/+ allele are not completely understood. However, mutation of HCFC1 in patients with cblX syndrome is associated with movement disorders [19]. Therefore, we performed larval behavioral...
assays to determine if defects in the number of NPCs were associated with abnormal swim patterns. To test this, we monitored swim behavior of 5 DPF larvae using Zebrabox technology. Carriers of the *hcfc1a*<sup>co60/+</sup> allele exhibited reduced overall distance swam in response to light stimulus as described in [34], but overall speed was not affected (Fig. 10a, b). These behavioral deficits are consistent with a hypomotility phenotype [46]. Importantly, *hcfc1a*<sup>co60/+</sup> responded normally to dark–light–dark transitions (Fig. 10c) as has been previously demonstrated [47].

**Discussion**

Here we demonstrate that the *hcfc1a*<sup>co60/+</sup> allele results in an increase in the number of NPCs during early brain development. This increase in NPC number is a direct consequence of over proliferation of NPCs. Mutations in *HCFC1* cause *cblX* syndrome, a multiple congenital anomaly syndrome, associated with cobalamin deficiencies and significant neurological deficits, among other phenotypes [15, 19]. *HCFC1* encodes for a transcriptional co-activator that regulates genes important for metabolism and proliferation [12]. It is suggested that *HCFC1* binds to and regulates the expression of >5000 different genes [18], with various different interacting partners including THAP11 [13] and ZNF143 [48], where mutation of either can cause a *cblX* like disorder [16, 49].

Previous reports suggest that HCFC1 regulates NPC function [14–17]. In vitro, decreased *Hcfc1* (mouse) expression increases the number of NPCs and reduces the expression of markers associated with differentiation [14]. These data are consistent with the known function of *Hefc1* in cell proliferation [50–52]. Mutation of *Hefc1* (mouse) is embryonically lethal [21] and consequently, the in vivo function of HCFC1 has been difficult to characterize. Our results are consistent with this observation,

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**Fig. 8** Inhibition of Asxl1 activity restores the NPC deficits in the *hcfc1a*<sup>co60/+</sup> allele. *a*-a″, b–b″ and c–c″ Representative 20X images of Sibling wildtype (*a–a″*), *hcfc1a*<sup>co60/+</sup> larvae (Co60/+ (*b–b″*)), or Co60/+ larvae treated with 12 μM LY294002 (*c–c″*) at 2 days post fertilization (DPF).
as we did not detect viable homozygous larvae or adults. We genotyped larvae from an incross of \textit{hcfc1a}^co60/+ adults at 2, 4, and 8 HPF and even at the earliest time points, we detected few to zero homozygous larvae. We did not explore this mechanism further, however in previous studies the murine Hcfc1 mutant allele was subject to compensatory mechanisms and only the paternally inherited allele was viable [21].

Recently a cell-type specific mutant allele was created in which \textit{Hcfc1} (mouse) was deleted from a subpopulation of neural precursors (NKX2.1+). This cell type specific deletion of \textit{Hcfc1} (mouse) induces cell death and defects in differentiation without affecting proliferation. These results differ from in vitro assays, whereby decreased \textit{Hcfc1} (mouse) results in an increase in the number of precursors. The discrepancy between these results might be explained by many factors, including the propagation of neurospheres in vitro and the inability to decipher how the developing microenvironment affects normal physiology. However, some studies have helped to shed light on the latter explanation because the in vivo knockdown of \textit{hcfc1b}, one of the zebrafish orthologs of \textit{HCFC1}, resulted in increased NPC proliferation [16].

Thus, the function of HCFC1 is complex, with several unknown cell-type specific functions that can be affected by the surrounding microenvironment.

Here we developed a zebrafish harboring a germline mutation in the \textit{hcfc1a} gene. We analyzed the effects of this mutation on NPC number and proliferation. Consistent with the literature [14–16], our allele resulted in an increase in the number of Sox2+ cells and increased cellular proliferation without significant deficits in cell survival. Increased cell proliferation was observed across all brain regions. However, our studies were limited to detection of NPCs using the Sox2 marker. A significant fraction of these cells co-localize with EdU positive cells in the Co60/+ allele, but not all of them. These data strongly suggest that \textit{hcfc1a} is important for the proliferation of Sox2 positive NPCs. However, the NPC population is heterogeneous in nature and can include Sox2+ and Sox2− cells. We do not yet understand the effects of \textit{hcfc1a} mutations on the Sox2− population or other NPC sub-populations (actively proliferating versus not proliferating). However, we did observe increased \textit{pax6} expression. Sox2 and \textit{Pax6} are known to be co-expressed in a subpopulation of NPCs so it is possible these two markers label similar populations of NPCs [37].

The increased numbers of NPCs in the Co60 allele was not associated with increased apoptosis of NPCs, but instead was associated with increased expression of markers associated with neurons and glia. The effects of this increased expression are yet to be elucidated, however studies that provide information on brain volume and the ratio of gray and white matter in the central nervous system are warranted; particularly given
that the increases in neurons and glia we observed are unique from the differentiation defects observed in previous studies [14, 17]. However, we suspect the difference in phenotype between previous studies and our own is likely associated with the type of mutation introduced, the brain microenvironment, the cell population analyzed, and the region of the brain of interest. For example, Minocha and Herr [17] deleted exons 2 and 3 of \textit{Hcfc1} using a Cre-Lox system with a cell type specific promoter. The resulting approach introduces the formation of a truncated protein, whereas, our system results in decreased overall expression (Fig. 1), which may be more consistent with previous in vitro assays and haploinsufficiency. However our haploinsufficient allele advances the field because unlike the previous in vitro assays [14, 15], our allele accounts for the broad expression of Hcfc1 which was recently documented by Minocha and colleagues [17].

\textit{HCFC1} regulates a myriad of downstream target genes [12, 18] and therefore, the mechanisms by which \textit{HCFC1} regulates NPC function are not clear. The majority of the literature focuses primarily on the function of

Fig. 10 The \textit{hcfc1a}^{c060+} is associated with hypomotility. Total distance (a) and average speed (b) of wildtype (Sibling) and heterozygous carriers of the \textit{hcfc1a}^{c060+} (Co60/+) allele were tracked at 5 days post fertilization (DPF) using ZebraBox technology. Distance and speed were monitored during light stimulus for a 5 min duration. Top panel shows representative tracking patterns from Sibling wildtype and heterozygous carriers of the Co60/+ allele. *p < 0.001. N = 52 Sibling wildtype and 56 Co60/+ individual larvae. c At 5 DPF larvae were monitored for total distance swam in alternating dark-light conditions.
HCFC1 at the MMACHC promoter in the human syndrome, cblX. cblX disorder is the result of mutations in the HCFC1 gene and these mutations disrupt protein function causing a decrease the expression of MMACHC and a metabolic disorder [14, 19]. Interestingly, mutations in MMACHC cause cblC disorder, which has many overlapping phenotypes with cblX including neurodevelopmental defects [53]. These data led us to hypothesize that HCFC1 regulates NPC function by modulating MMACHC expression. However, the hfc1a<sup>coe60/+</sup> allele did not disrupt mmachc expression. However, whether our allele causes other metabolic deficits is not known and was not explored further here. For example, mutation of the human HCFC1 gene has been associated with non-ketotic hyperglycinemia [54]. Collectively the data presented here suggests that the hfc1a<sup>coe60/+</sup> allele disrupts NPC proliferation by a novel molecular mechanism. In addition, these data suggest some divergent function between hfc1a and hfc1b, as the latter has been shown to regulate mmachc expression and craniofacial development [20].

Our results strongly suggest an mmachc independent mechanism underlying the neural developmental phenotypes associated with the hfc1a<sup>coe60/+</sup> allele, but we cannot completely rule out that hfc1b regulates brain development via mmachc expression or that other factors including cobalamin, homocysteine, or methylmalonic acid accumulation. However, RNA-sequencing of brain homogenates provided us a list of potential downstream effectors of hfc1a. Of those candidates, the asxl1 gene was afforded high priority because of its known role regulating cellular proliferation [24, 43, 55] and for its documented function in mouse embryonic stem cells and neural differentiation [23]. Interestingly, the hfc1a<sup>coe60/+</sup> allele causes a 14-fold induction of asxl1 expression. In mouse embryonic fibroblasts, the deletion of Asxl1 causes cellular senescence. We observed increased cellular proliferation and therefore, postulated that an increased level of Asxl1 protein was promoting NPC proliferation in hfc1a mutant larvae. Consistent with the known function of asxl1 and our hypothesis, knockdown of asxl1 in hfc1a mutant larvae restored the defects in cellular proliferation, resulting in normal numbers of Sox2<sup>+</sup> cells.

Interestingly, ASXL1 has been shown to regulate cell proliferation in other cell types [55, 56] and this activity has been associated with activation of AKT. Based upon these data, we attempted to restore the phenotypes present in the hfc1a<sup>coe60/+</sup> allele by inhibiting ASXL1 activity downstream of PI3K [24]. Consistent with this role, the inhibition of ASXL1 activity using pharmacological inhibition completely restored the NPC deficits present in hfc1a mutants. Thus, our data suggest a mechanism whereby hfc1a regulates the expression of Asxl1 and the cell cycle during early brain development. However, whether hfc1a regulates asxl1 by directly binding to the asxl1 promoter is still not known, but interestingly ASXL1 and HCFC1 interact with one another in myeloid cells to regulate proliferation and differentiation [57]. Thus, these two proteins may regulate the activity of one another at multiple levels.

We observed changes in the expression of various markers of neurons and glia. However, the physiological consequences of these changes are not currently known. Zebrafish have emerged as a model for neurodevelopmental disorders [58] and behavioral assays for seizure [59] and motor deficits [60] have been described. Therefore, we characterized the locomotion of hfc1a mutants in response to light stimulus [61]. Our results, using Zebrafish technology, demonstrated reduced motility as indicated by decreased distance travelled. Importantly, the decreased motility during a light stimulus was not due to an overall defective response to light, as our analysis demonstrated that carriers of the hfc1a<sup>coe60/+</sup> allele responded to normally to light stimulus, as indicated by the "V" like pattern in dark–light–dark conditions [62]. Decreased distance travelled has been previously defined as hypolocomotion and is associated with motor incoordination [63]. Hypolocomotion has been demonstrated in zebrafish models of ALS [60, 64] and fetal akinesia [65], two disorders characterized by motor deficits. Interestingly, the hypolocomotion we observed was correlated with increased NPCs and defects in the expression of various markers associated with differentiation. We did not observe short convulsions or whirlpool like behaviors in mutant larvae, which would have been indicative of a seizure phenotype and it is likely that the protocol we used to detect behavioral deficits does not stimulate a seizure like phenotype. Future studies that use multiple stimuli, including low dose convulsants will likely shed light on the epileptic phenotypes associated with mutations in hfc1a.

Conclusions
Our study focuses on the function of hfc1a, one ortholog of HCFC1, during brain development. Specifically, we focus on the function of hfc1a in modulating NPC number and proliferation. We demonstrate that HCFC1 is essential for the proliferation of NPCs. Importantly we connect these cellular deficits to a molecular mechanism whereby hfc1a indirectly or directly regulates asxl1 expression to control cellular proliferation. Thus, we propose that our system has the potential to inform about the transcriptional program regulating NPC function.
Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12868-020-00577-1.

Additional file 1: Figure S1. The Co60 allele is predicted to produce a premature stop codon. The predicted protein would cause an N-terminal truncation. ClustalOmega alignment of the allele encoded protein and the full length Hcfc1a is depicted.

Additional file 2: Table S1. RNA-sequencing reveals 36 upregulated and downregulated genes.

Abbreviations
NPC: Neural precursor; cbIX: Methylmalonic acidemia and homocysteinemia, cbIX type; F0: Founder; DPF: Days post fertilization; HPF: Hours post fertilization; NPC: Neural precursor; cblX: Methylmalonic acidemia and homocysteinemia, cbIX type; F0: Founder; DPF: Days post fertilization; HPF: Hours post fertilization; RT: Room temperature; DMISO: Dimethylsulfoxide; QPCR: Quantitative real time PCR; ISH: Whole mount in situ hybridization; SEM: Standard error of the mean; Edu: 5-ethyl-2'-deoxyuridine; mM: Millimolar; μM: Micromolar; PFA: Paraformaldehyde; SSC: Saline sodium citrate; DIG: Digoxigenin; AP: Alkaline phosphatase; CRISPR/Cas9: Clustered Regularly Interspaced short palindromic repeats/Cas9 nuclease; ng: Nanograms; nl: Nanoliters; EDTA: Ethylenediaminetetraacetic acid; NaCl: Sodium chloride; SDS: Sodium dodecyl sulfate; μg: Microgram; PCR: Polymerase chain reaction; HB: Hybridization buffer; MEF: Mouse embryonic fibroblasts.

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Authors’ contributions
AMQ synthesized the hypothesis, wrote the manuscript, analyzed data, performed statistical analysis, genotyped, performed QPCR maintained zebrafish lines. VLC cryosectioned, imaged, counted cells, performed immunohistochemistry, performed injections, drug treatments, RNA analysis and QPCR, and aided in the study design. JFR performed cryosectioning, immunohistochemistry, genotyping, and cell counts. AWQ produced the germline mutant and transgenic heat shock animal. NGRN performed cell counts, imaging, and genotyping. DP performed genotyping. All authors read and approved the final manuscript.

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Availability of data and materials
The RNA-sequencing data sets generated during this study have been deposited into the GEO database with Accession number GSE132864 and a summary is included within the article. All files are also available from the corresponding author upon request. All files are accessible at the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132864 and available as of May 6, 2020.

Ethics approval and consent to participate
All experiments were performed according to Protocol 811689-5 approved by The University of Texas El Paso Institutional Animal Care and Use Committee (IACUC).

Consent to publish
Not applicable.

Competing interests
Authors report no competing financial interests.

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