Modeling Cartilage-Hair Hypoplasia in zebrafish through modulation of the rmrp locus

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Abstract

Cartilage-Hair Hypoplasia (CHH) is an autosomal recessive genetic disorder caused by mutations in RMRP (RNA Component of the mitochondrial RNA Processing Complex). Although extremely rare in the general population, CHH is highly prevalent in the Amish (~1:1,300 births) and Finnish (~1:20,000 births), and can manifest in dwarfism, bone dysplasia, hypotrichosis, gastrointestinal dysfunction, anemia, and predisposition to certain cancers. Currently, no animal models exist for CHH; dysfunctional RMRP is embryologically lethal in mice. The goal of this project was to develop a model of rmrp dysfunction in zebrafish, Danio rerio, to study the developmental mechanisms underlying CHH. We designed and injected four CRISPR-Cas9 guide RNAs targeting the zebrafish ortholog, rmrp, and isolated DNA from F0 mutants at two days post-fertilization (dpf). Three of these guide RNAs induced genome editing as evidenced by heteroduplexing on a PAGE (polyacrylamide gel electrophoresis) analysis. We quantified mosaicism through cloning and sequencing analysis of embryos corresponding to these three guides, which demonstrated an estimated 50.24%, 100%, 95.99% mosaicism respectively. Next, we characterized phenotypes relating to cartilage dysplasia, anemia, and lack of enteric neurons (Hirschsprung Disease). We used automated imaging of a collagen-fluorescent reporter line (-1.4coll1a1:egfp) to examine cartilage dysplasia, using ceratohyal angle as a proxy. We determined erythrocyte count using a fluorescent transgenic line (gata1:dsRed) to test for anemia, and we used HuC/D immunostaining, which allowed us to quantify enteric neurons. The F0 rmrp mutants recapitulate cartilage dysplasia and gastrointestinal deficiency, as indicated by a significantly more obtuse ceratohyal angle in mutants compared to controls for guides 2 and 3 (ex., p= 7.5 x 10^-11, p= 1.9 x 10^-4, respectively) and a significant reduction of enteric neurons in guide 2 F0 mutants (p=0.016 vs controls). We did not observe an anemia phenotype. This model will provide us with a deeper understanding of the cellular and molecular mechanism of RMRP, guide novel therapeutic avenues, and will likely uncover broader insight into the treatment of other phenotypically overlapping ribosomopathies in the long term.
Introduction

Cartilage-Hair Hypoplasia (CHH): Symptoms & Clinical Observations

Cartilage-Hair Hypoplasia (CHH) is an autosomal recessive disorder within the Metaphyseal Dysplasia without Hypotrichosis (MDWH)-Anauxetic Dysplasia (AD) spectrum. This disease continuum is characterized by an array of symptoms across multiple body systems, which include severely short limbs and stature, joint hypermobility, hypotrichosis, immunodeficiency, anemia, gastrointestinal problems, and increased risk of malignancy [4]. On this spectrum, MDWH is the least severe, exhibiting only skeletal and joint dysplasia. AD is the most severe, with all of the associated hallmark symptoms in addition to cognitive disability [4]. CHH falls in between these clinical entities with short stature and skeletal dysplasia, hypotrichosis, Hirschsprung-like gastrointestinal deficiencies (absence of neurons in the small intestine resulting in limited to no peristalsis [5]), immunodeficiency, and hematological abnormalities [2, 6]. Additionally, 6-10% of individuals with CHH present with malignancies, primarily lymphomas; leukemia; skin, eye and liver neoplasms [1]. Phenotypic variability has been reported to exist between cases. Most cases of CHH in the US are in the Old Order Amish, at a frequency of 1:1000 live births, with ancestry traceable to John Miller and Jacob Hochstetler, immigrants in the 1700s [7]. The highest European concentration of this disease is within the Finnish population, where the prevalence is estimated at 1:23,000 births [6, 7]. Crucially, the Amish carrier frequency could be as high as 1:19, while the Finnish carrier frequency is approximately 1:76 [8].
Little is currently known about the developmental mechanisms underlying symptoms associated with CHH. In terms of phenotypic onset, some characteristics, such as anterior angulation of the sternum, are visible neonatally [7]. Most CHH-related phenotypes present in infancy; for example, slightly widened and scalloped metaphyses, (the wide portion of bone between the shaft and epiphysis of long bones containing the growth plate). This effect becomes pronounced by two years of age [7, 9]. Case studies have shown further skeletal dysplasias ranging from pediatric shortness of limbs, increased upper:lower body segment ratios due to shortening of long bones compared to normal pediatric growth, hyper-extensible fingers and wrists, and limited elbow extension [6, 7]. There is a marked shortening of distal bones, including the carpals, metacarpals, and phalanges, and studies have noted a weak or absent pubertal growth spurt correlated with reduced bone growth [10] (Figure 1). However, CHH patients demonstrate normal bone density, a mostly normal vertebral column, and normal psychomotor development; genu varum (bow-leggedness) is the most common reason for orthopedic consultation [6]. The most prevalent skeletal hallmark of CHH is the short and thickened long bones, which also become visible in infancy, even as early as one month [7, 9].

Dwarfism is a predominant phenotype of CHH (Figure 1). Adult heights range typically from 104-149cm [1]. Accordingly, a study by Le Merrer and Maroteaux (1991) found that by six months old, patients were five standard deviations below average height of same-age children.
Though bone density is normal in these patients, bones showed advanced ossification, more appropriate for a matured age. For one patient of 18 months of age, Le Merrer and Marroteaux recorded a bone age of 2 years and 8 months [9]. Treatments for such skeletal conditions associated with CHH are currently limited to surgical lengthening and repair, as well as growth hormone (GH) therapy; the latter has demonstrated success as a non-invasive procedure as indicated by a reduction in height difference between cases and age group average for healthy controls of up to two standard deviations [11].

Immunological symptoms exclusive of a predisposition to certain cancers have been linked with cell-mediated immunity, but not antibody deficiency in CHH [12]. Recurrent infections are known to occur in up to 60% of patients, as well as defective T cell proliferation and function [6, 7]. Such immunodeficiency has been explored in relation to vaccine effectiveness: case studies with normal polio vaccine have shown that some CHH patients develop severe, vaccine-related paralytic poliomyelitis [12]. The patients who experienced these symptoms were those who also exhibited Hirschsprung-like symptoms. These findings suggested that live vaccines should not be administered to CHH patients until the variant of the patient’s CHH can be determined, to prevent risk of unintentional infection [12].

Hematological abnormalities of CHH result from abnormal erythrogenesis, leading to anemia and macrocytosis [6]. Previous clinical studies focused on the hematological aspects of CHH and include an iron chelation study by Taskinen et al. (2013). Twenty-three Finnish CHH patients dependent on regular blood cell transfusions were examined; these patients possessed a mean cumulative iron overload of 4640 mg from normal range. Twenty-two of 23 cases tolerated typical iron chelation therapies, though one individual with Hirschsprung-like symptoms did not respond to treatment [13]. Based on these observations, these authors concluded that iron
Chelation is well tolerated in CHH patients, though possibly not for those with Hirschsprung-like symptoms. Thus, the anemia associated with CHH mandates intensive transfusion dependency with iron chelation, but patients with Hirschsprung disease may not tolerate iron chelation therapy well [13]. These findings, supported by variable vaccination outcomes, demonstrate the variability of CHH phenotypic manifestation. Phenotypic variability has been shown among individuals harboring the same causal genotype as well [14]. However, anemia, and iron overload in particular, have been shown to correlate with severity of immunodeficiency and growth failure [6].

A prevailing hypothesis potentially explaining the physiological source of these symptoms was proposed by Rogler et al. (2014) as the alteration of stem cells, particularly mesenchymal stem/progenitor cells (MSPC). These cells differentiate into chondrocytes and osteoblasts in bone growth plates, as well as stem cells for hematopoiesis and hair follicles [1]. Such affected areas would lead to the symptoms observed with respect to dwarfism, anemia, and hypotrichosis; similarly, bone marrow failure, often reported in CHH patients, would result in an inability to maintain the hematopoietic stem cell pool [1].

**Genetic Causes & Implications**

Cartilage-Hair Hypoplasia is caused by autosomal recessive mutations in a non-protein coding region on human chromosome 9p13.3 known as *RMRP*, or the RNA Component of the Mitochondrial RNA Processing Complex (Figure 2) [6]. Mutations result in decreased abundance or instability of RNA transcript, confirmed by assays in cultured cells [15]. In a study by Ridanpää et al. (2001) a cohort of 70 CHH patients and their family members underwent mutational analysis; all 26 parents studied were heterozygous for one of the pathogenic
mutations found in the affected children, but showed no phenotypes themselves [8] supporting a recessive inheritance paradigm. The founder mutation is a 70 A→G change identified through genotyping of initially characterized clinical cases; this mutation is most commonly observed in Finnish cases (92%) and in non-Finnish published cases (48%), and is the most common mutation in the Old Order Amish [2, 6]. Early diagnosis for these at-risk populations is possible, though its frequency of use has not been reported. Diagnoses have been made around 17.5 weeks gestation using ultrasonography to detect visible skeletal abnormalities such as shortened femurs, and genetic testing and predictions are available from private organizations [16].

RMRP is highly conserved across multiple species, from human and mouse to Arabidopsis and Saccharomyces [8]. This includes the transcriptional start site, as well as regions 61-81 and 241-258, which are typically conserved due to their interactions as tertiary elements in the enzymatic sites of the protein complex involved with RNA cleavage (Figure 3) [2, 3]. Thirty-three of the 46 known pathogenic single-nucleotide mutations are in these regions [2, 14]. The properly transcribed RNA interacts with ten protein subunits, designated hPop1, hPop4, hPop5,
Rpp14, Rpp20, Rpp21, Rpp25, Rpp30, Rpp38 and Rpp40 (Figure 3). A study by Thiel et al. (2007) showed that the founder variant, 70 A→G, is likely linked to ribosome assembly and cell cycle regulation specifically, whereas other Anauxetic Dysplasia-specific mutations are not (Figure 2). This was shown using meta-analysis of patient cohorts with published clinical and genetic data, by scoring phenotype and degree of severity with known mutations. While the 70A→G mutation is the most frequent worldwide, mutational screening of ethnically heterogeneous CHH cases have shown that compound heterozygosity, or different mutations contained on both of the alleles, with or without the 70 A→G change can also manifest as CHH [2, 17].

Additional pathogenic mutations recorded in the literature include 25 different insertion or duplication events between the TATA box and transcriptional start site, as well as over 62 mutational events in the transcribed region [1, 2]. Mutations in the region between the TATA box and transcriptional start are considered highly detrimental, and are likely embryologically lethal. This hypothesis has been supported through functional studies in yeast, and the observation that only two patients with biallelic mutations in this region have ever been reported.
Furthermore, 5’ region insertions have also been shown to reduce RMRP expression levels with CHH-patients producing 35-40% of RMRP transcript in comparison to that of control cells [8]. This is hypothesized to be a result of lengthening of the region, and thus silencing of the allele due to improper transcription factor binding.

The RMRP transcript is intronless, 276 base pairs in length and expressed ubiquitously [1, 2]. It is transcribed by RNA Polymerase III in the nucleus and is untranslated. Major functions of the RMRP complex include a role in cell cycle progression and chromosomal segregation at the end of mitosis, RNA cleavage such as that of 5.8s RNA, and ribosome assembly [2, 8]. Additionally, RMRP is among the first nuclear-synthesized RNAs shown to be transported to the mitochondria, through a yet unspecified mechanism, for a role in mitochondrial DNA replication [1].

**The Zebrafish Model**

The zebrafish, *Danio rerio*, is an ideal model organism for phenotypic analysis of a variety of developmental conditions. Fast-developing and transparent, they possess a genome approximately 70% orthologous to that of humans [18], resulting in high conservation of key developmental signaling processes. Furthermore, zebrafish allow for recapitulation of phenotypes ranging from abnormal morphological characteristics to abnormal immunity or digestion [19-21]. *D. rerio* is a freshwater, tropical fish, established initially as a laboratory model organism in the late 1960s by George Streisinger [20]. Sexual maturity is reached within three to four months of development, however, early organ development makes *D. rerio* efficient for modeling prenatal and pediatric onset conditions observed in humans [19]. Adults can
generate large clutch sizes (200 or more embryos) [20], thereby providing ample sampling opportunity for F1 generations [20, 21], which would allow for investigations into heritability and germ line development of mutants. In addition, zebrafish can be maintained at high densities and at lower costs than other mammalian model systems [22]. Practical limits of a zebrafish model include, in some instances, dissimilarity from the human condition, with a notable example being lack of an orthologous structure such as the mammalian lung [19]. Taken together, these characteristics make zebrafish an ideal model organism for studying a disease such as Cartilage-Hair Hypoplasia focusing on cartilage, erythrocyte, and gut development.

The zebrafish has been used to model many relevant phenotypes that are associated with Cartilage-Hair Hypoplasia, particularly gastrointestinal deficiency, anemia, and cartilage dysplasia. First, certain markers of enteric (small intestine) neurons are conserved between humans and zebrafish which would be beneficial to visualize Hirschsprung-like symptoms. For example, a protein recognized initially as a Drosophila-specific neuron binding protein, ELAV, has been shown to belong to a family of other neuron specific ligands that include HuC and HuD in vertebrates [23]. This binding protein is expressed in all neurons after their generation, and the zebrafish elav/HuC homolog is 89% homologous to that of humans [23]. Zebrafish can be used to model Hirschsprung-like symptoms as shown by previous studies visualizing enteric neurons through an anti-HuC antibody with secondary antibody fluorescence [24]. Second, hematopoiesis is a highly-conserved process between humans and D. rerio. A study by Ransom et al. (1996) reported multiple genetic alterations that resulted in anemia phenotypes modeled in zebrafish; these included a bloodless condition (moonshine), decrease in blood cell count as shown in the cabernet (cab) mutant, and hypochromic blood with decreased cell count, observed in chianti (cia) [25]. These phenotypes, matching the expected human anemic phenotype, were visualized.
using a transgenic line with a fluorescent reporter gene such as DsRed under control of the *gata-1* promoter, an erythrocyte transcription factor [25]. Finally, cartilage assays have been utilized to observe the pharyngeal skeleton, often with Alcian Blue staining. This is a glycoprotein stain that allows visualization of spatial arrangement of pharyngeal elements, individual components, and differentiation of those elements [26]. A dysplasia such as the expected CHH phenotype could be seen with this method. Similar visualization techniques, such as GFP (green fluorescent protein) in *-1.4colla1:egfp* transgenic zebrafish [27], can enable assessment of the same structures. An original forward genetic screen by Neuhauss et al. (1996) showed that mutations expected to alter cartilaginous structures affected the pharyngeal elements of zebrafish, inducing changes in the ceratohyal angle; results from this study showed this angle to bend caudally, as more acute than controls, or more obtuse than controls, though this methodology utilized an Alcian Blue staining system [26]. More recent reverse genetic screens using the developed collagen transgenic reporter and VAST (Vertebrate Automated Screening Technology) screening have also been reported [28]. These assays were relevant to phenotyping in this project.

Zebras have been used in the laboratory setting to create models of human genetic disorders through injection of mutant mRNA and antisense morpholinos, to express genes ectopically or to create a loss of gene function, respectively [19, 21]. Morpholinos are common for this research purpose due to their specificity, stability, effect duration and non-toxicity; interference typically occurs at or near the translational start site or blocks the mRNA splicing process [19]. Once a successful model is created, relevant phenotypes can be assayed quantitatively or capped human mRNA can be introduced to quantify rescue of a phenotype of interest. Such wild-type (WT) human mRNA does not typically affect zebrafish anatomy and physiology, but introduced dominant mutations, for gain of function, can induce the analogous
phenotype and function to rescue the phenotype back to expected condition [19]. However, although morpholino (MO) modeling is widely used, one report has suggested a poor correlation between MO-induced phenotype and mutant phenotypes. Kok et al. (2015) found that mutants for ten separate genes failed to recapitulate published MO-induced phenotypes [21]. This research thus postulated that mutants should become the standard of phenotypic analysis as opposed to morphants. Similarly, a discrepancy in phenotype between mutants and morphants was noted in a study by Rossi et al (2015): in a study with egfl7 (encoding EGF-like-domain, multiple 7) a lack of phenotype was noted in mice mutants whereas severe vascular defects were published with MO knockdowns of the same gene other model organisms, such as zebrafish and frogs [29]. This study made use of CRISPR (clustered regularly interspaced palindromic repeats) technology to demonstrate that morpholino-induced knockdown does not result in the same phenotype as severe genetic lesions, and in many cases, exhibited no phenotype [29]. However, this study also showed that the discrepancy in phenotype may be due to genetic compensation; for example, upregulation of Emilins (extracellular matrix glycoproteins) when egfl7 was lost, which non-deleterious MO effects are not sufficient to trigger [29]. In addition, there are other limitations to using MOs, such as their potential to induce p53-dependent apoptosis, off-target effects, and toxicity effects unrelated to the desired gene [29, 30]. Thus, while WT human mRNA will eventually be used for phenotypic rescue, the model of interest in this study made use of the CRISPR-Cas9 genome editing system.

The recent development of genome editing approaches has revolutionized reverse genetics approaches in model organisms, including zebrafish. Whereas classical mutagenesis approaches using mutagens are laborious and introduced random mutations in the genome (forward genetics), genome editing now enables targeted mutagenesis of a gene of interest
(reverse genetics). Initial technologies for generating site-specific gene disruption include zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) ([31, 32] [21]). In these procedures, mRNA with the relevant ZFN or TALEN heterodimers are injected into single-cell embryos and induce double strand breaks, for which imprecise repair can introduce insertions or deletions, occasionally in the germ line. Though these methods provide valid genome editing techniques, this project made use of CRISPR and the CRISPR-associated protein Cas9, an endonuclease complex active in bacterial immunity. This complex can be targeted with site-specific guide RNAs (gRNAs) to cleave target DNA and introduce heritable modifications. This method has been shown to be effective in zebrafish, and more robust and efficient than comparable ZFNs or TALENs [33]. With the use of CRISPR-Cas9 and associated rmrp-specific gRNAs, we introduced heritable modifications of the RMRP ortholog in D. rerio to characterize the phenotypes of dysfunctional RMRP, and thus CHH, in human patients.

Though clinical symptoms of CHH have been well documented in existing literature, no viable animal model for the disease currently exists. A goal of this project was to create such an animal model. We used this model to characterize hallmark clinical manifestations of the disease such as anemia, gastrointestinal neuronal dysplasia, and chondrodysplasia, which will allow for further study of their mechanistic pathways in the future. Development of a zebrafish rmrp model will ultimately serve as a platform to provide insight into possible therapeutic avenues.

Materials & Methods
Zebrasfish & Embryo Maintenance

All embryos resulted from natural matings of zebrafish adults on either AB (1.4coll1a1:egfp [27]) or EK (gata1:dsRed [34]) backgrounds. All CRISPR gRNA and Cas9
protein injections were carried out within one hour post-fertilization, into the cell, at the one-cell stage. Embryos were maintained in egg water at 28.5°C according to standard procedures.

**CRISPR guide RNA Generation & Efficiency**

We queried the zebrafish genome (GRCz10) using Ensembl (http://www.ensembl.org) (EMBL-EBI) [35] to identify the zebrafish *rmrp* ortholog (ENSDARG00000080987). CRISPR guide RNA targets and flanking primers were designed using ChopChop [36, 37] (http://chopchop.cbu.uib.no/) algorithms using default parameters for gRNA selection (see Appendix A, Sequences). gRNA DNA templates were assembled through a modified PCR reaction and gRNAs were transcribed *in vitro* and purified, per GeneArt™ gRNA Precision Synthesis kit manufacturer’s instructions (Invitrogen by Thermo Fisher Scientific). gRNA integrity was verified by electrophoresis on a 1% agarose gel prior to microinjection into zebrafish embryos (see Appendix B, Gels).

We extracted DNA from zebrafish embryos at two days post-fertilization (dpf) using Proteinase K (20mg/ml; Eurofins Genomics) and Thermopol 10x PCR Buffer (New England Biolabs) in nuclease-free water for two hours at 55°C followed by 10 minutes at 95°C. *rmrp* regions flanking each gRNA target were amplified in a 50 μl reaction using primer set 1 (Appendix A) and Taq polymerase (New England Biolabs) at 95° for three minutes, followed 10 cycles of 30 seconds each of 95° and 64°, decreasing one degree per cycle, and 72° for one minute. Resulting PCR products were denatured at 95° then allowed to re-anneal. To visualize heteroduplexes (indicative of small insertion or deletions in DNA), we migrated samples on a 15% 1.0 mm precast PAGE (polyacrylamide gel electrophoresis, BioRad) gel at 120 V for five minutes, followed by 150 V for one hour; the gel was washed in 0.5% Ethidium Bromide then
imaged on a BioRad Chemidoc UV transilluminator. PCR product from individual embryos demonstrating heteroduplexing (Appendix B) were purified on a 1% agarose gel, and samples were then extracted using Qiagen Gel Extraction kit. A-overhangs were added to purified PCR product using Taq polymerase (New England Bio Labs), at 72° for 12 minutes.

Purified PCR products were cloned into TOPO4 vector (Invitrogen), transformed into TOP10 cells (Invitrogen), and incubated overnight on LB-agar plates containing 1 mg/ml (1x) ampicillin. Resulting clones were grown in liquid LB culture containing 1 mg/ml ampicillin, and plasmid was purified using the Qiagen DirectPrep® 96 Miniprep protocol according to manufacturer’s instructions. Plasmid DNA was sequenced bidirectionally using BigDye3.1 cycle sequencing reagent (Applied Biosystems) according to manufacturer’s instructions using rmrp specific primers, (Appendix A). Samples were purified using ethanol precipitation (3M Sodium Acetate (NaAc, pH 4.6) and 100% Ethanol (EtOH), followed by diluted EtOH washes and centrifugation), and purified reactions resuspended in HiDi formamide were sequenced on an ABI3730 capillary sequencer (Applied Biosystems).

Sequences were analyzed using Sequencher DNA Sequence Analysis Software (Gene Codes) by aligning experimental reads to reference sequence. CRISPR/Cas9 targeting efficiency was calculated as total number of clones per embryo with small insertions or deletions, and overall gRNA efficiency was calculated as the average of all embryo efficiencies associated with that gRNA. Efficiency is estimated as the percent of DNA molecules targeted.
Craniofacial Phenotyping

We collected embryos resulting from natural mating of heterozygous *-1.4col1a1:egfp* transgenic adults, and injected them at the one-cell stage with 100 pg gRNA +/- 200 pg Cas9 protein (PNA Bio Labs) for gRNA efficiency testing, and phenotyping. For phenotyping studies, gRNA concentration was adjusted (50pg), and injected in the presence or absence of Cas9 protein. These cocktails were made from 500 ng/μl stocks of gRNA, 1 μg/μl Cas9 protein, 10x phenol red, and water; 1 nl was injected into each embryo according to standard procedures with a Picospritzer microinjector (Parker). Larvae were maintained until 4 dpf and imaged live using the VAST Bioimager™ platform (Union Biometrica) on an Axio ScopeA.1 fluorescent microscope with an AxioCam 503 mono camera and Zen Pro (Zeiss.) software after sedation with Tricaine.

Facial cartilage patterning was assessed by measuring the ceratohyal angle of ventral images as described using the public source NIH image processing software, ImageJ [38, 39].

Enteric Neuron Staining & Imaging

Embryos collected from natural matings of heterozygous *-1.4col1a1:egfp* adults were injected at the one-cell stage with 1 nl of cocktails containing 50 pg gRNA 2 or gRNA 3 with or without Cas9 protein. Injection solutions were made from 500 ng/μl gRNA stock, 1 μg/μl Cas9 protein, 10x phenol red, and water. Embryos were maintained until 6 dpf, and larvae were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and washed with 1x phosphate-buffered saline with 0.1% Tween (PBST). Larvae were bleached (PBST, potassium hydroxide (KOH), hydrogen peroxide (H₂O₂)) essentially according to published protocols [40], and washed 5x in PBST.
before treatment with proteinase K (10 μg/mL diluted in PBST). Larvae were again treated with 4% PFA for 20 minutes before washing 3x with IF buffer (1% bovine serum albumin in PBST). They were then incubated for one hour in blocking solution (IF Buffer + 10% Fetal Calf Serum), followed by overnight incubation at 4°C in 1:500 HuC/D primary antibody (anti-mouse; Invitrogen Thermofisher) diluted in blocking solution. The following day larvae were incubated for two hours with fluorescent secondary antibody, AlexFluor™ anti-goat 488 (Invitrogen Thermofisher) and washed with IF buffer prior to imaging.

Image acquisition was performed using an AZ100 microscope and Digital Sight DS-U2 camera with NIS-Elements AR 4.20.01 64-bit software with automated Z-stacking (Nikon) followed by intensity quantification with ImageJ.

_Erythrocyte Imaging and Quantification_

Embryos collected from natural matings of _gata1:dsRed_ and _-1.4colla1:egfp_ heterozygous adults were injected at the one-cell stage with 1 nl of cocktails containing 50 pg gRNA 2 or gRNA 3 with or without Cas9 protein. Injection solutions were made from 500 ng/μl gRNA stock, 1 μg/μl Cas9 protein, 10x phenol red, and water. Embryos were maintained to 3 dpf and euthanized with 1x Tricaine prior to imaging on a Nikon AZ100 microscope with the same camera, software, and stacking as described above. This was followed by cell counting with the ImageJ plug-in, Image-based Tool for Counting Nuclei (ITCN) (Center for Bio-Image Informatics, UC Santa Barbara).
**Statistical Analysis**

A two-tailed t-test was used to test for significant difference against controls in all cases. We verified the validity of this test by confirming controls fit an approximately normal distribution. All significance is determined from $\alpha=0.05$ level. Statistical tests were run through the Microsoft Excel add-in, XLStat (Addinsoft) and Microsoft Excel. All data are represented in boxplot form to show the variability and data distribution.

**Results**

*Identification of efficient CRISPR gRNAs*

To target the zebrafish \textit{rmrp} locus, we first identified the single ortholog in the zebrafish genome, which is 53.9\% identical to the human sequence. Zebrafish \textit{rmrp} secondary structure is

\begin{center}
\textit{H. sapiens} RMRP (9p13.3) \hspace{0.5em} \textit{D. rerio} \textit{rmrp} (1p39.3)
\end{center}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Secondary structures of human \textit{RMRP} (from \cite{1}; left) and zebrafish \textit{rmrp} ortholog (Ensembl entry ENSDARG00000080987.1; right). Enzymatic sites are noted by red boxes, and the founder mutation 70A$\rightarrow$G in yellow.}
\end{figure}
also analogous to that of human *RMRP*, though not identical due to sequence differences. The overall stem-and-loop structure of the transcript is maintained between species, in addition to location and sequence of enzymatic sites (Figure 4). Next, we identified four gRNA target sites within the zebrafish *rmrp* locus. These were non-overlapping, relatively consecutive sequences towards the 5’ end of the transcript, located in between the two enzymatic sites. Guide 1 differs from the other guides in its target sequence on the forward (+) strand and its overlap with the 5’ enzymatic site, while the other three gRNAs do not overlap with the enzymatic sites, and target the reverse (–) strand (Figures 4 and 5). These guides were selected from 18 potential gRNA sequences due to efficiency, GC content, and complementarity characteristics.

**Figure 5:** 267 bp nucleotide sequence of zebrafish *rmrp* (black, bold italics); gRNA 1 is notated with pink highlight; gRNA 2 in blue highlight; gRNA 3 in orange highlight, and gRNA 4 in green highlight. Primer set 1 is in blue font, and primer set 2 in magenta font (See Appendix A). Enzymatic sites of the gene are indicated with red font (see: Figure 4).

To test the targeting efficiency of each of the four gRNAs, we determined a) the percentage of embryos with genome targeting events; and b) the estimated level of mosaicism induced by each gRNA. We injected 10 embryos with each CRISPR Guide 1-4 respectively (100 pg) with Cas9 protein (200pg). At 2 dpf, we collected embryos for DNA isolation and PCR amplification of regions flanking each gRNA target site. After denaturation and slow reannealing of PCR product, we migrated a sample corresponding to each of these 10 embryos on a
polyacrylamide gel (PAGE) (Figure 6). gRNAs 1, 2 and 3 showed heteroduplexes in all F0 embryos that migrated slower than homoduplexed wild type DNA amplified from controls, while

gRNA 4 showed minimal heteroduplexing for all 10 samples (Figure 6). Together, our data indicated that three of four gRNAs targeted consistently all embryos injected, while the fourth gRNA was inefficient and was not used in further studies.

To estimate the extent of mosaicism of F0 embryos injected with each of the three gRNAs that demonstrated heteroduplexes on the PAGE assay, we cloned and sequenced the PCR products that encompassed each gRNA. Sequencing of clones amplified from 5 embryos injected with gRNA 1 showed an estimated mosaicism of 50.24%, as indicated by the presence of insertions or deletions that differed from the wild-type reference sequence (Table 1, Figure 7). Each embryo had between 12-16 clones sequenced, with rmrp-specific targeting ranging from 31.25-66.66%. Mosaicism for each gRNA was calculated as the average percent of targeted
clones over all sequenced embryos. For gRNA 2, we sequenced clones from four different embryos (13-15 clones each), and observed 100% of clones with insertions or deletions, indicative of a high degree of mosaicism. We sequenced six embryos for gRNA 3 (12-22 clones each), and observed rmrp targeting that ranged from 81.81-100%, resulting in an overall gRNA efficiency of 95.99% (Table 1). Sequence alterations were variable between clones from the same injected embryo, and across embryos injected with the same gRNA with Cas9; these consisted of insertion events, deletions, or combinations thereof (Figure 7A-C). In conclusion, we deemed gRNAs 1-3 to be sufficiently effective for further phenotypic analysis.

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Table 1: Estimated mosaicism of CRISPR-Cas9 injected-embryos. Cloned PCR products amplified from embryos injected with 100 pg (top to bottom) guide 1, guide 2, or guide 3, + 200 pg Cas9. Embryo identifiers correspond to labels on PAGE gels (Figure 1). Numbers indicate the number of clones in each category. Overall guide 1 efficiency for 5 embryos (12-16 clones/embryo) was 50.24%; overall guide 2 efficiency for 4 embryos (13-15 clones/embryo) was 100%, and overall guide 3 efficiency was 95.99% for 6 embryos (12-22 clones/embryo).
Disruption of rmr7 results in a craniofacial phenotype

As a proxy for the cartilage defects that are a defining feature of CHH, we evaluated cartilage patterning in the zebrafish craniofacial skeleton. We generated F0 mutant embryos by injection with each of gRNAs 1, 2 and 3 with Cas9 protein, and acquired fluorescent ventral images at 4 dpf in a transgenic reporter line that labels collagen-expressing cells with GFP. The angle of the ceratohyal cartilage was measured as an indicator of altered patterning. We first assessed F0 mutants injected with 100 pg gRNA with or without Cas9. Guide 1 + Cas9 induced a significantly broader mean ceratohyal angle (n=65, 84.45°) versus controls (n=65, 80.43°; p=0.008, Figure 8A). We detected no significant differences between gRNA 1 alone and controls. Guide 2 + Cas9 also produced a significantly wider mean ceratohyal angle (n=40,
165.13°) than controls (n=47, 78.85°; p=3.04x10^{-19}; Figure 8B). Similar to guide 1, we observed no significant difference between guide 2 alone and controls (Figure 8B). Additionally, guide 3 + Cas9-injected F0 mutants showed a significant difference in mean ceratohyal angle (n=45, 130.47°) in comparison to controls (n=33, 74.56°; p=4.2x10^{-6} (Figure 8C). gRNAs 2 and 3 + Cas9 resulted in many inverted or incompletely developed ceratohyal angles at this concentration.

**Figure 8: F0 mutants display craniofacial defects.** A. Ceratohyal angle of larvae 4 days post-fertilization. Quantification of controls (uninjected), “G1 Alone” (100pg of guide 1), and “G1+Cas9” (100pg of guide 1 + 200pg Cas9), right. Sample size is indicated below each condition, and significant differences between conditions are indicated by a noted p-value (student’s t-test). No p-value indicates no significance between two conditions. Fluorescent ventral images (left) are representative of each condition. The top “Control” image shows the angle measured. Guide 1 + Cas9 shows a modest phenotype. B. Average ceratohyal angle of larvae 4 days post-fertilization. Quantification of controls (uninjected), “G2 Alone” (100pg of guide 2), and “G2+Cas9” (100pg of guide 2 + 200pg Cas9), right. Sample size is indicated below each condition, and significant differences between conditions are indicated by the p-value (student’s t-test). Fluorescent ventral images (left) are representative of each condition. Guide 2 + Cas9 shows a severe phenotype. C. Average ceratohyal angle of larvae 4 days post-fertilization. Quantification of controls (uninjected), “G3 Alone” (100pg of guide 3), and “G3+Cas9” (100pg of guide 3 + 200pg Cas9), right. Sample size is indicated below each bar, and significant differences between conditions are indicated by the p-value (student’s t-test). Fluorescent ventral images (right) are representative of each condition. Guide 3 + Cas9 shows a severe phenotype, but less so than Guide 2 + Cas9.
(30% of larvae for gRNA 2, 20% for gRNA 3, see: “G2+Cas9” Figure 8B, “G3+Cas9” Figure 8C). Notably, phenotypic severity was correlated with gRNA efficiency, as evidenced by a modest phenotype in guide 1 F0 embryos (50.24% mosaic) in comparison to gRNAs 2 and 3 which produced nearly 100% mosaicism. The experiment was repeated with a reduced amount of gRNA (50 pg) with the expectation that the phenotype would be less severe.

![Figure 9](image.png)

**Figure 9**: F0 mutants display craniofacial defects without toxicity. A. Representative fluorescent ventral images of control and experimental conditions for each data set, white angle indicating the measurement for each condition. B. Ceratohyal angle measurement spread of F0 larvae at 4 days post-fertilization with reduced guide RNA injection. Controls (uninjected), “G2 Alone” (50pg of guide 2), and “G2+Cas9” (50pg of guide 2 + 200pg Cas9). Significant differences between conditions is indicated by the p-value. Injection of 50pg of guide showed a less severe phenotype than from 100pg injected guide (Figure 8A-C) but a still significant phenotype. Replicate experiments for the same experimental conditions are shown.

To generate *rmrp* F0 mutants with a less severe craniofacial phenotype than that of embryos injected with 100 pg gRNA 2 or 3, we injected 50 pg of gRNA with the same concentration of Cas9 protein (200 pg). Injection of guide 2 with Cas9 resulted in a significantly wider mean ceratohyal angle in comparison to controls (replicate data generated by two
independent microinjections: 73.23° vs. 141.07°, \( p = 7.5 \times 10^{-11} \), and 79.23° vs. 253.37°, \( p = 9.5 \times 10^{-39} \), Figure 9A). Though still severe, a smaller percentage of larvae showed a fully inverted ceratohyal angle compared to the 100 pg condition (22% vs. 30%, respectively; Figure 8B, Figure 9B). Mean measurements of larvae injected with gRNA 2 alone were significantly wider than controls in the first experimental set, but the effect was marginal (\( p=0.02 \), Figure 9A, left); in the replicate batch, there were no significant differences between the batch injected with gRNA 2 alone versus controls (Figure 9A, right). F0 rmrp mutants produced by injection of gRNA 3 and Cas9 showed a significantly wider mean ceratohyal angle compared to controls in replicate batches (73.23° vs. 111.27°, \( p = 3.2 \times 10^{-5} \), and 71.42° vs. 89.56°, \( p = 1.9 \times 10^{-4} \), Figure 10A). Guide 3 alone showed a marginal effect (\( p=0.02 \)) compared to controls in replicate 1 (left)
and no significant differences compared to controls in replicate 2 (right). In summary, our evaluation of multiple independent reagents targeting *rmrp* demonstrate recapitulation of the CHH-relevant cartilage phenotype in zebrafish F0 mutants. Injection of embryos with gRNAs 2 or 3 with Cas9 both result in a consistent and significantly wider ceratohyal angle than that of controls, and the most severe effect is seen with gRNA 2.

*Lack of rmrp results in a gastrointestinal phenotype*

A hallmark phenotype of CHH cases is gastrointestinal deficiency. This clinical feature presents in the form of Hirschsprung-like symptoms, or a lack of enteric neurons resulting in inefficient peristalsis and digestion. We thus quantified the presence of enteric neurons in the zebrafish gut of *rmrp* F0 mutants. We generated F0 mutant embryos by injection with gRNAs 2 or 3 with Cas9 protein on the -1.4colla1:egfp background, followed by fixation at 6 dpf, bleaching, and immunostaining of the pan-neuronal marker, HuC/D. We acquired lateral

![Figure 11](image)

**Figure 11.** F0 mutants display enteric neuron deficits. **A.** Representative images of larvae fixed at 6 dpf for each condition, bleached and stained for the pan-neuronal marker, HuC/D. **B.** Number of enteric neurons counted on a consistent location of lateral images of the gut (rectangle in panel A), for each condition: 50 pg gRNA 2 +/- 200 pg Cas9, 50 pg G3 +/- 200 pg Cas9, and uninjected controls. Significant differences between conditions is indicated by the
fluorescent images and quantified neurons in a specified region of interest of the gut. First, we attempted to induce a phenotype by injecting 50 pg gRNA with 200 pg Cas9; we observed high enteric neuron count variability within each condition, ranging from 9 to 157 neurons in F0 mutants (Figure 11). We found both gRNAs 2 and 3 alone to be significantly different from controls (p = 0.05 for gRNA 2 alone, p = 0.0008 for gRNA 3 alone, Figure 11), though this may be due to small sample sizes. Both gRNA + Cas9 conditions demonstrated significantly reduced enteric neuronal counts compared to controls (p=1.3x10^-6, gRNA 2+Cas9; p = 4.9x10^-6, gRNA 3+Cas9; Figure 11B). We observed a significant decrease in neuronal count between gRNA 2 alone and gRNA 2 + Cas9 (p=0.0003), but no significant differences between gRNA 3 in the presence or absence of Cas9. Consistent with the cartilage patterning data, the greater most severe phenotypic effect was induced by gRNA 2 (Figure 11B).

**Figure 12.** F0 mutants display enteric neuron deficits. **A.** Representative images of larvae fixed at 6 dpf for each condition, bleached and stained for the pan-neuronal marker, HuC/D. **B.** Number of enteric neurons counted on a consistent location of lateral images of the gut (rectangle in panel A), for each condition: 100 pg G2 +/- 200pg Cas9, 50pg G3 +/- 200pg Cas9, and uninjected controls. Significant differences between conditions is indicated by the p-value. Sample sizes are shown below each bar.
Next, we repeated the assessment of *rmrp* F0 mutants with larger sample sizes and an increased amount of gRNA (100 pg vs. 50 pg; Figure 12). All conditions in which gRNA alone or gRNA and Cas9 was injected showed a significant reduction of enteric neurons compared to controls in as counted in the imaged region of interest. Of note, a significantly smaller number of enteric neurons is present in gRNA 2 + Cas9 vs. gRNA 2 alone larval batches (p=0.016), and no significant difference was detected for gRNA 3 + Cas9 versus gRNA 3 alone. In summary, gRNA 2, but not gRNA 3, is sufficient to produce an enteric neuron phenotype.

**rmrp F0 mutants do not display detectable differences in erythrocyte quantity**

To determine whether F0 mutants display an anemia phenotype consistent with CHH patients, we imaged and counted erythrocytes in a region of interest on F0 mutants. Visualization of erythrocytes was facilitated by generating *rmrp* mutant embryos on a transgenic reporter line in which the erythroid transcription factor, *gata1*, is tagged with the fluorescent protein, DsRed. We generated F0 mutant embryos by injection with gRNAs 2 or 3 with Cas9 protein and manually acquired lateral images of the resultant larvae. We first assessed F0 mutants injected with 50 pg gRNA with or without Cas9, and initially only imaged gRNA 2-injected larvae at 2 dpf (Figure 13). We observed a modest, but significant increase in number of erythrocytes present in the region of interest between gRNA 2 alone versus controls (p=0.019), as well as a significant but modest increase in erythrocytes in larvae injected with gRNA alone versus gRNA 2 + Cas9 (p=0.028). No significant differences were detected between gRNA 2 + Cas9 and controls. No conclusions could be drawn from these results alone, due to the unexpected significance of guide alone compared to both other conditions while the experimental condition (guide 2 + Cas9) showed no significant difference when compared to controls. We performed two replicate experiments using a higher concentration of gRNA (100 pg, Figure 14, 15).
Figure 13. F0 mutants do not display a deficit in erythrocytes. A. Representative images of larvae fixed at 2 dpf for each condition. B. Number of erythrocytes counted on a consistent location of lateral images of the tail (rectangle), for each condition: 50pg G2 +/- 200pg Cas9, and uninjected controls. Significant differences between conditions is indicated by the p-value. Sample sizes are shown below each bar.

Figure 14. F0 mutants do not display a deficit in erythrocytes. A. Representative images of larvae imaged at 2 dpf for each condition. B. Number of erythrocytes counted on a consistent location of lateral images of the tail (rectangle in panel A), for each condition: 100 pg G2 +/- 200 pg Cas9, and uninjected controls. Significant differences between conditions is indicated by the p-value. Sample sizes are shown below each bar.
We considered the possibility that assessment of a larger area of interest in our erythrocyte images would be required to detect a phenotype. Injection of 100 pg gRNA + Cas9 resulted in a significantly lower number of erythroctyes for both conditions with gRNA in the presence or absence of Cas9 in comparison to controls (p=1.3x10^{-6}, 7.8x10^{-4}, respectively). There was no significant difference between gRNA alone and gRNA plus Cas9 (Figure 14). A replicate experiment showed no significant difference in number of erythrocytes in the larger area of the tail between any conditions (Figure 15). To confirm these results, we evaluated larval batches injected with 100 pg gRNA 3 + 200 pg Cas9. We observed no significant differences in number of erythroctyes in the region of interest between any conditions, supporting our findings with gRNA 2 (Figure 16). Together, our data suggest that no red blood cell reduction is detectable in rmrp F0 models with these experimental parameters.

![Figure 15. F0 mutants do not display a deficit in erythrocytes. A. Representative images of larvae imaged at 2 dpf for each condition. B. Number of erythrocytes counted on a consistent location of lateral images of the tail (rectangle in panel A), for each condition: 100 pg G2 +/- 200 pg Cas9, and uninjected controls. Significant differences between conditions is indicated by the p-value, and no p-value indicates no significance. Sample sizes are shown below each bar.](image-url)
**Discussion**

Here, we have used CRISPR-Cas9 genome editing to generate a zebrafish model of CHH by targeting the *RMRP* ortholog in zebrafish, *rmrp*. We demonstrate that two of four gRNAs targeted the gene efficiently in F0 embryos to induce a cartilage dysplasia phenotype. Furthermore, we observed that genome-editing of *rmrp* with gRNA 2 plus Cas9 can also induce a reduction of enteric neurons. However, we were unable to detect any differences in erythrocyte numbers in *rmrp* F0 mutants in comparison to that of controls.

The CRISPR-Cas9 complex is a programmable genome-editing tool, which is a derivative of the bacterial immune system [41, 42]. It is a site-specific alternative to other genome editing technologies such as ZFNs (zinc finger nucleases), TALENS (TAL effector
nucleases), or knockdown effectors like morpholinos [41]. CRISPR-Cas9 disrupts genes by altering target sequences; a guide RNA (gRNA) is designed as a short, usually 24-26 base pair sequence around a protospacer adjacent motif (PAM, an NGG sequence), which is recognized by two amino acids in Cas9. The protein then uses this anchor to pull apart the complimentary strand and allow the guide segment to bind in its place. Cas9 excises the DNA, often with imperfect repair that results in small insertions or deletions [42]. We chose to use the CRISPR-Cas9 system to create an in vivo model of CHH in zebrafish, due to its reduced off-target effects and toxicity compared to other methods [29, 30].

We found that three of our designed gRNAs, when associated with the Cas9 protein, showed extensive heteroduplexing of PCR amplicons that flank target sites, indicative of introductions of small insertions or deletions near the PAM site associated with each guide target (Figure 6, Results). gRNA 4, in contrast, showed minimal heteroduplexing; the most prominent PCR product observed on the PAGE subsequent to denaturing and reannealing appeared to be a homoduplex consistent with the wild type sequence, ~300 bp. Thus, we concluded that gRNA 4 was inefficient and unsuitable for injection into zebrafish embryos. This finding is supported by the fact that the gRNA 4 target sequence had the lowest rank (6/18) of the four selected guides supplied by ChopChop algorithms (gRNAs 1, 2, and 3 were ranked 1, 2, and 5 respectively). These algorithms measure off-targeting effects in combination with sequence and location characteristics [36] (See: Appendix A, Sequences). Based on ranking information, we anticipated that gRNAs 1, 2, and 3 would result in phenotypes ranging in a similar ranking of severity: 1, 2, and then 3. However, after sequencing, we observed that guide 1 was least efficient, only showing rmrp disruptions in 50.24% of clones. This was an unexpected result, though the reason for this discrepancy is unknown. The guide could be causing off-target effects instead, or it could
potentially be caused by the guide being on the forward (+) strand; \textit{RMRP} and \textit{rmrp} are both found on the reverse (-) strand, as are guides 2, 3, and 4. gRNA 2 was the most efficient of the three gRNAs, as evidenced by 100\% of clones showing targeting effects; gRNA 3 was also efficient, although not to the same extent as gRNA 2 (95.99\% mosaicism).

We concluded that gRNAs 2 and 3, in combination with Cas9 protein, consistently disrupt the desired region of \textit{rmrp}. This creates approximately the same percentage of mutated molecules in humans with recessive disease (CHH is an autosomal recessive disorder, thus both copies of the gene are mutated), and will allow for effective study of the phenotypic manifestations in zebrafish as a vertebrate model. This conclusion is supported further by our phenotypic analyses, discussed below.

The first phenotype that we investigated was craniofacial patterning, as a surrogate readout for the skeletal and cartilage dysplasias of the human condition. Dwarfism, short, thickened long bones, and scalloped and widened metaphyses are all hallmarks of CHH in humans \[6, 7\], and we expected to see a dysplasia reflected in the organization of cartilage in the zebrafish. Previous studies have shown that genes that cause developmental chondrodysplasias in humans can result in a phenotype in the craniofacial region, such as Meckel’s cartilage or the ceratohyal arch \[43\]. For example, similar to \textit{RMRP}, biallelic mutations in \textit{NANS} (encoding the synthase for \textit{N}-acetylneuraminic acid, sialic acid) result in reduced \textit{NANS} activity, thus reduced sialic acid, and skeletal dysplasia \[43\]. This dysplasia results in a poorly developed ceratohyal angle, among other craniofacial defects. The published literature further supports that cartilage phenotypes can be modeled by CRISPR genome editing, and moreover, that ceratohyal angle is an indicator of the phenotype. Decreased doses of \textit{cep55}, active in cytokinesis, at 4 dpf have shown significant and dose-dependent increases in cartilage disorganization and angle of the
ceratohyal, both with morpholinos and CRISPR-Cas9 disruption [38]; CRISPR/Cas9-mediated alteration of smchd1, associated with an arhinia (absence of the nose) phenotype, similarly showed a significant increase in the ceratohyal angle in F0 mutants [39].

We hypothesized that CRISPR-Cas9 induced disruption of rmrp would result in an abnormal ceratohyal angle, and we observed the expected phenotype for all three gRNAs that were injected. Injection of gRNA 1 with Cas9 showed the smallest phenotypic effect, with many larvae showing no visible defect. Genome editing with gRNA 2 resulted in the most severe phenotype, although gRNA 3 also produced a significantly broader ceratohyal angle compared to controls. Our data reflect the guide efficiencies, guide 1 showing the smallest effect and guide 2 the most severe. We determined the effect of guide 1 was limited compared to those of guides 2 and 3, more analogous to a heterozygous condition at 50.24% mosaicism, and it was discontinued from phenotypic study.

We next aimed to investigate whether a gut phenotype was present in this model. A high prevalence of Hirschsprung’s disease, or a lack of enteric neurons resulting in limited peristalsis, exists in patients with severe forms of CHH [5, 44]. The CRISPR reagent most likely to induce Hirschsprung symptoms was gRNA 2, with 100% mosaicism, followed by guide 3, at 95.99% in F0 mutants. We observed that injection of both gRNA alone and gRNA + Cas9 resulted in a large phenotypic range of enteric neurons between conditions, though the overall effect was as expected: the majority of CRISPR gRNA 2 plus Cas9 injected larvae had significantly fewer enteric neurons as compared to controls.

However, all conditions were significantly different compared to controls; we thus compared the difference between gRNA alone and gRNA + Cas9 to draw conclusions about the
experimental condition (Cas9 presence). In both iterations, gRNA 2 + Cas9 showed a significantly lower number of enteric neurons in the region of interest compared to gRNA 2 injected alone. gRNA 3 alone and gRNA 3 + Cas9 were not significantly different. The fact that genome editing of rmrp with only the most efficient reagent (gRNA 2) was able to induce an enteric neuron phenotype was not surprising considering clinical observations from CHH cohorts. The prevalence of Hirschsprung’s Disease in CHH patients is only 9% (1:11)[44]. Additionally, Hirschsprung is more prevalent in males, at a male predominance of 4:1 [44], but we do not know if a relevant gender-specific effect is relevant in zebrafish. To better examine the effects of rmrp disruption on enteric neuron phenotypes, it will be necessary to repeat this assay with larger sample sizes in stable F2 mutants. A guide RNA alone phenotype was observed in these experiments, but we posit that this is due to potential off-target effects or mechanical disruption during injection.

Though no mouse model exists for CHH, mouse models have been shown to recapitulate a Hirschsprung phenotype, for example, the mouse mutant Dominant megacolon (Dom). The mutation in this model is a defect in the Sry-related transcription factor, Sox10 [45]. Sox10 is a factor in mouse neural crest development, thus this also lends to the hypothesis that Sox10 could be implicated in the development of CHH, as well as neural crest cells, and as such should be the focus of further study with rmrp zebrafish mutants.

The third phenotype studied in this project was reduced erythrocyte count, or anemia. In humans with CHH, childhood anemia is the most common, and it is often correlated with the presence of Hirschsprung symptoms [44]. Severe cases of CHH experience severe childhood anemia, as well as Hirschprung Disease, and have been correlated further with immunodeficiency, as with vaccine-related poliomyelitis [12]. Further, there is little that can
currently be done to help these anemia symptoms, as these patients have also been shown to tolerate iron chelation therapy poorly [13].

In a study by Mäkitie et al. (2001), 6/10 Finnish CHH patients were shown to have had severe childhood anemia. In a previous study with 88 patients, 73% were retrospectively evaluated as having childhood anemia as well; after puberty, hemoglobin typically reaches the normal range for these patients [46]. Based on these clinical data, it is possible that we did not evaluate the correct time point of hematopoietic development. It is also possible that we might observe anemia in this model in larval or juvenile stages, but our data reflects no significant differences in erythrocyte counts for gRNAs 2 or 3 + Cas9 compared to controls. Future work with this model could include screening at both later or earlier time points, and re-evaluation with a stable mutant. The lack of anemia could also be due to differences in human and zebrafish hematopoiesis. Interestingly, it has been shown that an anemia phenotype is observed in conjunction with Hirschspring phenotypes. We observed a loss of enteric neurons with gRNA 2 plus Cas9, though we did not observe anemia in multiple replications of gRNA 2 injections. The reason for this could be due to variation: though childhood anemia does appear to have a high prevalence in CHH patients, whether mild or severe, and a correlation does exist with Hirschspring symptoms, there is still a cohort of CHH patients who do not present with anemia. Overall, we conclude that a reduction in erythrocyte number is not detectable in this model, regardless of gRNA or efficiency.

**Future Directions**

In addition to documented enzymatic or mitochondrial roles, RMRP is the source of two micro (mi)RNAs, RMRP-S1 and RMRP-S2 [1] (Figure 17). Several of the pathogenic mutations
that cause CHH also map to these regions. A recent study showed that RMRP-S1 and RMRP-S2 quantities are reduced in fibroblast and B cell lines from CHH patients in comparison to that of controls [1]. These miRNAs have been linked to silencing activity of genes implicated functionally in tissue or cell types related directly to many CHH phenotypes, such as osteoblasts and erythrocytes [1]. In addition, Rogler et al. (2014) recorded differing stability of these small RNAs in healthy versus diseased cell lines: S1 and S2 were shown to be 60 and 70% reduced in mutant CHH fibroblasts respectively, while a B cell line showed 80% and 60% reduction [1]. In particular, RMRP-S1 regulates genes involved with connective tissues and osteoblasts, while RMRP-S2 regulates skeletal, muscular, and hematological abnormalities, as well as cancer [1]. Even so, a major caveat of this work is that conclusions were drawn from in vitro models; we propose that recapitulation of these findings with an animal model of RMRP dysfunction would bolster the validity of these findings in a spatio-temporally relevant context. A future direction of this project will be to correlate abnormal cartilage morphology in a vertebrate model with targets of these two miRNAs and subsequent integration of this information with the previously reported findings.

Figure 17. Schematic of RMRP secondary structure determined by SHAPE probing by Rogler et. al (2014) where orange denotes highest confidence; locations of miRNAs, RMRP-S1 & RMRP-S2, are shown by red bars in relation to known pathogenic mutations circled in grey [Figure 4A from [1]]
Further study with a zebrafish \textit{rmrp} model could be useful toward development of a gene therapy mechanism using human wild-type \textit{RMRP} RNA. Rescue attempts can target developmental pathways through genetic compensation. This is because genetic knockout, whether from lack of enzymatic activity or lack of proper transcription, is the cause of the observed phenotype. Such rescues have been published throughout the literature, particularly for craniofacial defects. Van Karnebeek \textit{et al.} (2016) showed partial skeletal rescue by adding sialic acid, the product of the NANS pathway previously discussed, to the egg water of growing embryos affected with their morpholino [43]. The delayed or absent development of branchial arches in other CRISPR and morpholino-based models have also been shown to be rescuable with human wild-type (hWT) mRNA of the affected genes [38, 39]. As the expected phenotype was observed for chondrodysplasia for \textit{rmrp} disruption, attempting rescue with hWT mRNA (\textit{RMRP}) is the next step, in accordance with published findings. This research can be translated into that of human clinical research in that a successful rescue of the zebrafish phenotype with hWT mRNA would support the idea that the human phenotype could show improvement with periodic embryological injection of WT mRNA, to compensate for lack of innately-produced \textit{RMRP} during development. Such a rescue attempt would require a safe method of neo-natal delivery, and stable \textit{RMRP} mRNA.

In conclusion, we have generated an animal model for Cartilage-Hair Hypoplasia that recapitulates chondrodysplasia and loss of enteric neuron phenotypes. This model is useful for studying the CHH disease mechanism and can be used for future therapeutic development. Study of this model can also elucidate principles that will be valuable for other ribosomopathies, ranging from mutations in genes encoding ribosomal proteins, such as \textit{RPS19} implicated in Diamond-Blackfan anemia, to genes required for ribosome synthesis, such as those implicated in
CHH or Treacher-Collins syndrome [47]. This model will be important in investigating other phenotypes associated with CHH, such as dwarfism, hypopigmentation, and tumor formation.
References


### Appendix A: Sequences

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<td>CTCAGTCACGTGGACGTCTTTAG</td>
<td>TCAGTAGCTGGTAAGTCCGTTAT</td>
</tr>
</tbody>
</table>
Appendix B: Gels

Figure B1. PCR amplicons to determine gRNA efficacy. A. (Left) Guides 1, 2, and 3; (right) primer 2F&1R followed by 2F & 2R run on zebrafish genomic DNA. Primer set 2F & 2R (see Appendix A: Sequences) produced the most specific PCR amplicon. B. PCR of guide template DNA across multiple samples. C. (left to right) Guides 1, 2, 3, and 4. Guide 4 did not show a band, and was re-run (see D). D. Re-run of Guide 4 on the same gel.