Single base substitutions in the CHM promoter as a cause of choroideremia

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Single base substitutions in the *CHM* promoter as a cause of choroideremia

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Abstract

Although over 150 unique mutations affecting the coding sequence of *CHM* have been identified in patients with the X-linked chorioretinal disease choroideremia (CHM), no regulatory mutations have been reported, and indeed the promoter has not been defined. Here we describe two independent families affected by CHM bearing a mutation outside the gene’s coding region at position c.-98: C>A and C>T, which segregated with the disease. The male proband of family 1 was found to lack *CHM* mRNA and its gene product Rab escort protein 1 (REP-1), while whole genome sequencing of an affected male in family 2 excluded the involvement of any other known retinal genes. Both mutations abrogated luciferase activity when inserted into a reporter construct, and by further employing the luciferase reporter system to assay sequences 5’ to the gene, we identified the *CHM* promoter as the region encompassing nucleotides c.-119 to c.-76. These findings suggest that the *CHM* promoter region should be examined in patients with choroideremia who lack coding-sequence mutations, and reveals, for the first time, features of the gene’s regulation.

Keywords

CHM, choroideremia, promoter, REP-1, ZNF143, THAP11

68 Introduction

69 Choroideremia (CHM; MIM# 303100) is an X-linked, recessively inherited chorioretinal
70 dystrophy with an incidence of 1/50,000. Progressive degeneration of photoreceptors, retinal
71 pigment epithelium (RPE), and the choroid causes affected hemizygous males to develop night
72 blindness in the first or second decade of life, followed by a decrease in peripheral visual fields
73 and an eventual loss of central visual acuity in advanced stages of the disease (Coussa and
74 Traboulsi, 2012). Carrier females, while usually asymptomatic, may exhibit signs of retinal
75 degeneration upon fundoscopic examination, and more rarely have reduced dark adaptation and
76 peripheral vision (Karna, 1986; Roberts et al., 2002).

77 To date, CHM has only been linked to mutations within the *CHM* gene, coding for REP-1
78 (Cremers et al., 1990). The protein serves as a molecular chaperone for small GTPases from the
79 Rab family, presenting them to Rab geranylgeranyl transferase which modifies them by the
80 covalent attachment of a lipid moiety. The lipid modification, known as prenylation, of target
81 Rab proteins is essential for intracellular vesicular transport (Seabra et al., 1992; Seabra et al.,
82 1993). A homologue, REP-2, encoded by *CHML* or choroideremia-like, functions similarly and
83 appears to compensate for the absence of REP-1 in all tissues except the eye (Cremers et al.,
84 1994). *CHM* spans over 150 kb of Xq21.2 and contains 15 exons (van Bokhoven et al., 1994). A
85 30 base pair 5'untranslated region, found on exon 1, precedes an open reading frame encoding
86 the 653 amino acid REP-1 protein. At least 163 unique pathogenic mutations have been reported
87 in the LOVD Retinal and Hearing Impairment Genetic Mutation Database
88 [https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php ; accessed Feb 2017] (Fokkema
89 et al., 2011). The mutation spectrum includes transitions and transversions leading to protein
90 truncation, splice defects, indels and large deletions ranging from a single exon to the full gene.

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91 Missense mutations predicted to alter protein structure or impair function (Sergeev et al., 2009;
92 Esposito et al., 2011), transposon insertions (Van Den Hurk et al., 2003), partial gene
93 duplications (Chi et al., 2013; Simunovic et al., 2016) and other variations are infrequently
94 found, but taken collectively, almost all known pathogenic variants in the *CHM* gene have been
95 loss of function mutations that abolish functional REP-1 (McTaggart et al., 2002; Simunovic et
96 al., 2016). Notably there is no apparent correlation between genotype and phenotype, with the
97 age at onset of symptoms, visual acuity and visual fields being unrelated to mutation type
98 (Freund et al., 2016; Simunovic et al., 2016).

99 The promoter driving expression of *CHM* has been heretofore unidentified. Analyses of
100 promoter mutations causing inherited diseases can be useful in identifying transcription factors
101 involved in the regulation and expression of a gene of interest, as the activity of RNA
102 polymerase II is mediated by the recruitment of general and sequence specific transcription
103 factors to *cis*-acting regulatory sequences. The core promoter directing basal level transcription
104 is generally found between nucleotides -40 and +50 of the transcription start site (TSS) (de
105 Vooght et al., 2009), while proximal promoter elements typically reside within 1 kilo bases (kb),
106 with additional enhancers, repressors, insulators acting even over distances of mega bases
107 (Maston et al., 2006). The investigation of regulatory elements in the context of human disease is
108 complicated by the fact that in contrast to loss of function mutations, regulatory defects may
109 produce small quantitative changes that are difficult to detect. Mutations in *cis*-acting regulatory
110 sequences are a significant cause of human disease, and according to statistics compiled by the
111 Human Gene Mutation Database [<http://www.hgmd.cf.ac.uk> ; accessed May 2016]
112 approximately 2% of disease causing point mutations are in non-coding regions of the genome
113 (Stenson et al., 2014). Though promoter defects can cause functionally important consequences

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for gene expression, their analysis is often not a regular part of DNA diagnostics as the investigation can be complex, laborious and difficult to perform.

In this study, we report the first known regulatory mutations causing choroideremia. The absence of a genetic defect in a CHM family prompted us to explore the upstream non-coding region, revealing the novel promoter variant c.-98C>A. Subsequently, whole genome sequencing in an unrelated family with CHM identified a second variant of the same residue, c.-98C>T. We demonstrate the mutations' effects on transcription using a luciferase assay, and employ the same system to further characterize the boundary of the *CHM* promoter that spans the location. The results suggest a disruption of a transcription factor binding site and impaired transactivation of the *CHM* promoter by the factor(s). Together, these studies further our understanding of regulation and expression of *CHM* and present a possible explanation for cases of unexplained choroideremia where no causative mutation is found within the gene.

Materials and Methods

Clinical Examination and Study Subjects

This study was approved by the institutions' respective ethics review boards. All procedures adhered to the tenets of the Declaration of Helsinki. Before participation, the purpose and risks of the study were explained, and informed consent was obtained. Blood samples were drawn, and a detailed pedigree and history was recorded.

We studied 4 males affected by CHM and 1 carrier female from two unrelated families. Individuals were examined by fundoscopy and functional ophthalmologic methods including some or all of: visual acuity testing, central visual fields, full field electroretinography, optical

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coherence tomography and fundus autofluorescence imaging. Choroideremia was diagnosed by senior clinicians with expertise in inherited retinal disease based on clinical findings alone. Affected males had a history of night vision loss and fundus appearance of peripheral retinopathy with broad areas of RPE and choroidal atrophy.

CHM Sanger Sequencing

DNA was extracted from lymphocytes using conventional methodologies. Proband 5116 from family 1 (C127) was initially screened for *CHM* mutations in the coding sequence and splice sites by direct Sanger sequencing from PCR amplicons (Furgoch et al., 2014). Additionally, 1kb of the 5'-flanking sequence was amplified with primers 5'-CAGGGAAGGCCCACTACTGC -3' and 5'-CTTGTGGAAATGAGATCAAGTTAGG-3' and sequenced with the same primers. With the exception of patient 111 and his parents, of family 2 who underwent whole genome sequencing, the remaining study individuals were genotyped only at position c.-98 to confirm the presence or absence of the respective variant. Annotation is in accordance with GenBank: NM_000390.3, where +1 represents the start of translation.

Cell Culture

Leukocytes from patient 5116 were separated with Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA) from 10mL of whole human blood collected in acid citrate dextrose (ACD-A) tubes (BD, Franklin Lakes, NJ, USA). A lymphoblastoid cell line was established by Epstein-Barr virus transformation within 48 hours of collection (Anderson and Gusella, 1984). Cells were maintained in RPMI-1640 supplemented with 15% fetal calf serum and penicillin-streptomycin.

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mRNA and Protein Analysis

Total RNA was extracted from 5×10^6 lymphoblastoid cells from patient 5116 and a healthy control, with the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. cDNA was transcribed from $5 \mu\text{g}$ of total RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA) using a random hexamer primer mix. PCR amplification from cDNA with forward primer 5'-TAATAGTCACATGACACGTTTCCCG-3', paired with 5'-TGGATTTCAGGCAAACCCGT-3', or 5'-TTTAAAATGAGCAAGTCAATGTGC-3' as a reverse primer was used to detect partial transcript (spanning the junction of exon 1 and 2), or the entire coding sequence of *CHM* encompassed by 15 exons, respectively. GAPDH was also amplified as a positive control with intron-spanning primers.

Protein was extracted from 2×10^6 lymphoblastoid cells and immunoblot analysis of REP-1 was performed as previously described (Furgoch et al., 2014).

Whole Genome Sequencing

UK proband 111 (GC406) and his unaffected parents 190 and 192 underwent whole genome sequencing (WGS) as part of the 100,000 Genomes Project. Briefly, genomic DNA was processed using the Illumina TruSeq DNA PCR-Free Sample Preparation kit (Illumina Inc) and sequenced using an Illumina HiSeq X Ten, generating minimum coverage of 15X for >97% of the callable autosomal genome. Reads were aligned to build GRCh37 of the human genome using the Isaac aligner (Illumina Inc). SNVs and indels were identified using Platypus v0.8.1 and annotated using Cellbase (<https://github.com/open.cb/cellbase>). Variant filtering was performed using minor allele frequency (MAF) in publicly available and in-house datasets, predicted

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183 protein impact and familial segregation. Surviving variants were prioritized using a pre-specified
184 virtual gene panel from PanelApp (<https://panelapp.extge.co.uk/crowdsourcing/PanelApp/>)
185 Posterior segment abnormalities v1.7. Allelic state was required to match the curated mode of
186 inheritance for variants in panel genes.

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188 **Promoter Analysis**

189 *Construction of luciferase reporter plasmids*

190 Primers were initially designed to amplify a 1063 bp fragment from upstream of the *CHM*
191 transcription start site (c.-1093_c.-31) from control and patient 5116's DNA. The c.-98C>T
192 mutation present in family 2 was generated by site directed mutagenesis from the control
193 plasmid. All sequences cloned contained only untranscribed DNA and did not include the 30
194 base pair 5'UTR directly upstream of the start codon. The fragment was cloned according to
195 standard protocols, with SacI and HindIII restriction sites added to primers allowing digest by
196 the enzymes (Thermo Scientific, Rockford, IL, USA). Ligation into the promoterless firefly
197 luciferase reporter vector, pGL3-Basic (Promega, Madison, WI, USA) generated constructs
198 pGL3-1063_-31 and pGL3-1063_-31 c.-98 C>A. Subsequently, a 4kb fragment, and a series of
199 nested deletions ranging from 2kb to 44bp were cloned into pGL3-Basic with the same
200 restriction sites added and used for subsequent digest, to identify the minimal promoter necessary
201 and sufficient for transcription. pGL3-Basic lacks a eukaryotic promoter sequence upstream of
202 the reporter luciferase gene, and expression of luciferase in transfected cells depends on a
203 functional promoter sequence to be inserted upstream of the *luc+* gene. pGL3-Basic itself served
204 as a negative control. A schematic of all inserts assayed is provided in Figure 1A. All constructs

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generated were Sanger sequenced to ensure fidelity. Primer sequences for the generation of plasmid inserts are available upon request.

Luciferase Assay

HEK293T cells cultured with standard reagents and conditions were seeded in 24 well plates and transfected at 70% confluency with polyethylenimine (PEI). Each well was transfected with a total of 650ng of plasmid DNA, including 600ng of pGL3 construct and 50ng of the internal control pRL-CMV, a cytomegalovirus promoter driven *Renilla* luciferase reporter vector. Briefly, plasmid DNA was diluted into 25 μ L of PBS, and mixed by vortexing with 1 μ L of 1mg/mL branched PEI (Sigma-Aldrich, St. Louis, MO, USA) in 25 μ L PBS. After a 15 minute incubation, 50 μ L of the reaction was added dropwise to each well. Forty-eight hours after transfection, luciferase activity was assayed using the Dual-Luciferase Reporter Assay kit and measured with the Glomax Explorer (both Promega, Madison, WI, USA). Relative luciferase activity was obtained by dividing the relative light units (RLU) produced by the firefly luciferase pGL3 construct by the RLUs produced by *Renilla* luciferase control reaction. Light generated by the reaction can be correlated with the amount of luciferase protein produced which in turn is proportional to promoter activity driving the gene's expression. Values from an n=6 were averaged and normalized to that of the reference construct, pGL3-1093_-31 to obtain a relative measure of activity.

Statistical analysis

All data were expressed as mean $\pm$ SD. Data from the luciferase assay represents two independent experiments with triplicate measurements. Differences between groups were examined for statistical significance using Student's *t*-test. A *P*-value <0.01 denoted the presence of a statistically significant difference.

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229 **Results**

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231 *Clinical Characterization of CHM*

232 *Family 1*

233 We investigated a progressive retinal degeneration in a Caucasian family of American origin
234 (C127). Remarkably, the proband 5116 was the offspring of a consanguineous union between
235 second cousins, with an affected father and carrier mother. To our knowledge, this has never
236 been reported with choroideremia, and it necessitated a thorough and accurate diagnosis of X-
237 linked CHM and exclusion of an autosomal recessive, or even an unusual male-male
238 transmission as cause for the disease. An investigation of the extended family for which the
239 pedigree is reported in Figure 2A however, clearly demonstrated the X-linked inheritance of the
240 disease.

241 Patient 5116 was initially seen at age 56 years, when he was referred by a retinal specialist with a
242 diagnosis of choroideremia. At his most recent examination, age 76, best corrected visual acuity
243 (BCVA) was measured as 6/19 OD and 6/15 OS. Visual fields were reduced to less than 5
244 degrees. The full-field ERG showed non-detectable dark adapted rod-driven responses as well as
245 non-detectable cone responses to a 30 Hz flicker stimulus. OCT imaging indicated loss of the
246 photoreceptor layer across the periphery of the fundus with only a small island of RPE remaining
247 in the macula. Posterior segment examination showed a hypo-pigmented fundus with significant
248 atrophy of the RPE and choroid with areas of bare sclera (Figure 3). These findings are
249 consistent with an advanced state of choroideremia.

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The proband's daughter 5113 was examined at 33 years of age and did not report any vision difficulties. BCVA when examined was 6/6, both eyes. Full-field ERG testing of the left eye showed dark adapted rod-driven responses were reduced by 50% in amplitude (42.5 μ V) and a normal b-wave implicit time (81.6 msec). Light-adapted cone-driven responses to a 30 Hz flicker stimulus were reduced by 50% in amplitude (27.6 μ V) and borderline reduced in implicit time (25.6 msec). These findings were consistent with a classic carrier state of choroideremia.

The proband's father and one brother 5149 were reported to have been previously diagnosed with choroideremia, while a second brother 5147 was reported to be unaffected. Findings are summarized in Table 1.

Family 2:

Family 2 (GC406) was a Caucasian family of British origin with a history of choroideremia.

Proband 111 was first examined at age 13, displaying a reduced but not delayed full field ERG with flicker and bright flash responses both within normal limits. He remained asymptomatic until age 32. Confrontational visual field testing at age 35 showed bilateral infratemporal scotoma, and BCVA was 6/5 in both eyes. He is considered symptomatically mild with retained central macular structure.

Of his two maternal uncles, 151 was diagnosed at age 12. Fundus abnormalities typical of choroideremia were noted. 161 was diagnosed at age 8, and upon examination at age 37 exhibited moderately constricted visual fields particularly in the superior field bilaterally. BCVA was reduced to count fingers vision OD and 6/36 OS. Fundus examination was consistent with a clinical diagnosis of choroideremia. The pedigree is reported in Figure 2B, and clinical findings summarized in Table 1.

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Genetic Analysis

Family 1 (C127)

Genetic analysis of proband 5116 did not reveal any pathogenic mutation in the coding sequence of the *CHM* gene or splice site boundaries. Yet at position c.-98 relative to the translation start site, a hemizygous C>A transversion was detected (hg38, chrX:g.86047629G>T NM_000390.3, c.-98C>A). The variant was not listed in the latest release of dbSNP [<http://www.ncbi.nlm.nih.gov/SNP/>; accessed Feb 2017] (Sherry et al., 2001). The proband's affected brother (5149) was also found to harbour the variant while it was absent in the unaffected brother (5147) available for testing. The obligate carrier status was confirmed in the proband's daughter (5113). The location of the variant strongly suggested a regulatory mutation, as evaluation of entries in the HGMD reveals that most promoter mutations are located between +50 and -500 from the TSS of a gene (Stenson et al., 2014).

Family 2 (GC406):

Prior genetic analysis of the coding exons of the *CHM* gene did not reveal a pathogenic mutation. Whole genome sequencing was performed on proband 111 and his parents 190 and 192, as part of the 100,000 Genomes Project. After variant filtering, no causative rare coding variants were identified in any retinal disease gene.

In light of the clinical diagnosis of choroideremia in the family, the complete *CHM* gene was interrogated for rare variants (≤ 0.001 MAF in 1Kgenome project and internal cohort of over 2000 whole genome sequencing samples) hemizygous in the proband and carried by his mother. One such variant was identified, the transition, c.-98C>T (hg38, chrX:g.86047629G>A NM_000390.3, c.-98C>T). The variant was confirmed in the two affected maternal uncles 151 and 161 by direct Sanger sequencing.

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297 ***Molecular diagnosis of choroideremia in 5116***

298 Immunoblot analysis of protein harvested from a cell line derived from patient 5116 failed to

299 detect the *CHM* gene product REP-1, providing conclusive confirmation of the clinical diagnosis

300 of choroideremia (Figure 4A). To provide evidence for a regulatory mutation and subsequently

301 reduced transcription, we intended to compare the level of expression between normal and

302 patient samples through qPCR. Endpoint PCR from a cDNA template; however, failed to

303 amplify the 15 exon, 2200 base pair transcript, indicating it was absent or present at a level

304 below the detection threshold, and thus the quantitative assay was not performed. To largely rule

305 out the possibility of a splice defect, we also attempted to amplify a minimal portion of the

306 transcript, a 93 base pair fragment from the 5'-UTR to a region spanning the boundary of exon 1

307 and 2, and found the patient's cells lacked even this short fragment (Figure 4B). A control

308 housekeeping gene was nevertheless readily detected and both partial and full length transcripts

309 were amplified from normal cDNA.

311 ***Effect of the c.-98C>A and c.-98C>T mutations on transcription of CHM***

312 As a starting point, an approximately 1kb fragment upstream of the TSS was assayed for ability

313 to drive gene expression, since the majority of elements necessary for transcription are expected

314 to be found within this region (Rockman and Wray, 2002). Comparing the robust luciferase

315 activity produced by cells transfected with this wild type construct pGL3-1093_-31, to that of

316 pGL3-1093_-31 c.-98C>A and c.-98C>T, we observed complete abrogation of promoter activity

317 (Figure 1B) in the mutants. The drop from 100 ± 9.5 to 2.0 ± 0.3 and 1.3 ± 0.4 respectively, as

318 measured in normalized relative light units (RLU) ± 1 standard deviation is even significantly

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319 lower than that of the negative control pGL3-basic which does not contain a promoter sequence,
320 reading at 5.8 ± 0.9 . This startling observation strongly suggested that the mutation spans an
321 element essential to promoter activity.

322 Based on unpublished reports suggesting other activating elements may exist up to 2.8kb
323 upstream of the TSS (Kaiser NW, *et al.* IOVS 2004;45:ARVO E-Abstract 2451), we additionally
324 compared the activity of two larger constructs approximately 4kb and 2kb long. Typically,
325 regulatory regions upstream of core promoter sequences contain multiple TF specific binding
326 motifs, where several copies of the same factor or cooperation between different factors serve to
327 synergistically stimulate transcriptional activity of a given gene (Maston et al., 2006). Yet in the
328 case of *CHM*, the longer sequences did not stimulate additional luciferase expression. pGL3-
329 3983_-31 and pGL3-2027_-31 did not significantly differ from pGL3-1093_-31, producing 83.7
330 ± 4.9 and 96.2 ± 7.6 RLU respectively. The somewhat diminished expression as compared to
331 the reference 1kb construct may be attributed to a lower molar amount of the larger plasmids
332 being delivered during transfection.

334 ***Characterization of CHM promoter***

335 To further characterize the promoter, we proceeded to assay progressively shorter constructs,
336 deleting sequences from the -5' and -3' of the 1kb construct to define its boundaries (Figure 1A).
337 Working from the 5' end, the approximately 400, 300, and 100 base pair constructs pGL3-437_-
338 31, pGL3-346_-31, pGL3-119_-31 did not significantly differ from the 1 kb containing reference
339 plasmid pGL3-1093_-31, reading at 100.2 ± 11.4 , 96.1 ± 10.6 , and 97.7 ± 8.1 RLU respectively.
340 Yet upon deleting a further 11 base pairs, we observed a significant drop to 39.8 ± 7.6 RLU with
341 pGL3-108_-31. Having delineated the -5' boundary of the promoter as extending to no further

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than nucleotide c.-119, we subsequently tested pGL3-119_-76 bearing a deletion from the -3' end, and found no significant difference at 93.4 ± 14 RLU. It was the final construct, pGL3-119_-82, which yielded significantly lower activity producing only 27.4 ± 4 RLU. Though further characterization of the promoter region down to the single nucleotide level could be performed, we have defined the borders of the promoter to within the region of c.-119 to c.-76. This short 44 base pair DNA region upstream of the *CHM* coding sequence is able to stimulate transcription in an *in-vitro* assay at a level that is not significantly different from that of a 1kb, or even a 4kb fragment and can be understood to contain essential *cis*-acting elements that positively regulate *CHM* expression.

Discussion

To date, all genetic defects causing choroideremia, which include all major mutation classes, have been observed in exons, introns or intron/exon boundaries of the *CHM* gene (Fokkema et al., 2011). No mutations have been reported in the promoter region of *CHM*, and the regulation of the gene has remained essentially unexplored. In this study, we present the first report of promoter mutations, c.-98C>A and c.-98C>T, causing choroideremia. The critical role of this residue for gene expression is highlighted by the complete abrogation of reporter activity in a promoter assay when the nucleotide is mutated. Though we demonstrate the first examples in a novel mutation class, it is worthwhile noting that the phenotypes observed in affected individuals were typical of choroideremia. In family 1, the genotype c.-98C>A in proband 5116 resulted in severe retinopathy, while characteristic milder signs were found in the carrier female 5113. Males from family 2 bearing the c.-98C>T mutation manifested with chorioretinal disease, but with varying degrees of severity; proband 111 remained symptomatically mild while in his

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maternal uncles 151 and 161, disease progression was more typical. CHM is known to exhibit a wide interfamilial, and also intrafamilial variability (Moosajee et al., 2014). As the mutations c.-98C>A and c.-98C>T appear to completely abolish transcription, producing no detectable levels of *CHM* mRNA and REP-1 protein in patient cells, it is not surprising for the disease to present in a classical manner consistent with phenotypes observed for the loss of function mutations. Whole genome sequencing in the parent-offspring trio of family 2 allowed the exclusion of a deep intronic *CHM* mutation, as well as mono or biallelic mutations in any other retinal dystrophy gene as a cause of the observed symptoms. We therefore conclude that the two mutations at residue c.-98 impair *CHM* transcription enough to result in choroideremia. For the several gene therapy trials currently underway employing a “gene replacement” strategy (Dimopoulos et al., 2015), individuals bearing these genotypes would be suitable candidates. The two variants have been submitted and published in the LOVD Retinal and Hearing Impairment Genetic Mutation Database (Fokkema et al., 2011).

Having identified mutations that evidently abolished transcription, we interpreted their location to span a region crucial for gene expression, and set out to define the boundaries of the gene’s promoter. Surprisingly, after analyzing fragments as long as 4kb, we found a short 44 base pair DNA fragment to be wholly responsible for driving expression. The region c.-119 to c.-76 comprises the entirety of the *CHM* proximal promoter, and is able to drive robust transcription in an *in-vitro* luciferase assay. The region implicated amounts to less than 3% of the length of the *CHM* coding sequence where the large majority of reported causative mutations have been found (Fokkema et al., 2011). A recent investigation of a large disease cohort (74), found causative mutations in the gene in 94% of cases previously diagnosed with CHM (Simunovic et al., 2016).

Promoter mutation causing choroideremia

388 The remaining 6% can be understood to be comprised of regulatory mutations, incorrect
389 diagnoses or deep intronic variant causing cryptic splicing (Carss et al., 2017). Promoter defects
390 are therefore expected to be responsible for a small minority of choroideremia cases. The
391 identified region; however, becomes an obvious area for examination in patients in whom no
392 coding sequence mutation is found.

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394 A promoter or regulatory mutation can be expected to either increase or decrease transcriptional
395 activity mediated by the altered binding capacity of trans-acting protein factors specific to a
396 DNA sequence in the promoter region. In this case, the interaction appears to be entirely
397 disrupted based on the null expression of a reporter driven by mutated sequence in our luciferase
398 assay. Impaired transcription due to a 1-bp mutation in a promoter region is unusual, but has
399 been reported previously as in the case of single base mutations stimulating additional
400 transcriptional activity at the *OVOL2* promoter causing autosomal-dominant corneal endothelial
401 dystrophies (Davidson et al., 2016). On the other hand, decreased transactivation of *NMNAT1*
402 due to a single nucleotide change in the promoter was found to cause Leber congenital amaurosis
403 (Coppieters et al., 2015).

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405 While we delineate the *CHM* promoter boundary to this small area upstream of the gene, we
406 cannot exclude the possibility of distant enhancer, repressor, or intronic elements also
407 contributing to regulation; our findings suggest the sequence between c.-119 and c.-76 is
408 essential, but not necessarily sufficient for transcription. The wider upstream sequence of *CHM*
409 lacks the consensus sequences often found in RNA polymerase II promoters, such as CAAT and
410 TATA boxes; as well it is neither GC rich nor associated with any CpG islands. Bioinformatic

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analysis with MotifMap, a dataset of computationally predicted transcription factor binding sites based on binding motifs [<http://motifmap.ics.uci.edu/>] (Daily et al., 2011) identifies a putative binding motif for the transcription factor zinc finger protein 143 (ZNF143) contained within the region of the now revealed *CHM* promoter. ZNF143 participates in the regulation of RNA pol II and III mediated transcription of protein coding, non-coding, and small nuclear RNA (Schaub et al., 1997; Myslinski et al., 1998) and was initially connected with the binding motif TTCCCATTATGCACCGCG (SBS1) (Myslinski et al., 2006). Genome-wide studies revealed the binding site for the factor to be frequently found with an adjacent 5' accessory sequence, forming the ACTACAATTCCCATTATGCACCGCG (SBS2) motif. SBS2 is comprised of both a THAP domain-containing protein 11 (THAP11) and ZNF143 binding site, with the factors believed to act in a competitive manner (Ngondo-Mbongo et al., 2013). The recruitment of THAP11 to its canonical binding site ACTAYRNNNCCCR is most frequently associated with up-regulation genes essential to protein biosynthesis and energy production (Dejosez et al., 2010). More recently, ZNF143 was suggested to cooperatively occupy SBS2 sites with THAP11 and a third factor, the scaffold protein host cell factor 1 (HCFC1) *in-vivo* (Vinckevious et al., 2015). SBS2 is closely matched by the sequence found at position c.-108 to c.-84 upstream of *CHM*, ACTACAACACCCAGAATGCACTGTT. Notably, ZNF143's binding at promoters was recently implicated in chromatin looping with distal regulatory elements (Bailey et al., 2015), suggesting the involvement of yet other factors in the total regulation of expression of *CHM*.

Of the three transcription factors implicated above, the binding of at least ZNF143 is supported by publicly available chromatin immunoprecipitation sequencing data released as part of the ENCODE project [<https://genome.ucsc.edu/ENCODE/>] (ENCODE Project Consortium, 2012).

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434 In all cell types assayed: lymphoblastoid, HeLa and K562 cell lines, as well as embryonic stem
435 cells, the promoter region of *CHM* interacted with ZNF143, for which a consensus binding
436 sequence GAACTACAATTCCCAGAAGGC, again is closely matched by
437 GAACTACAACACCCAGAATGC found between position c.-110 to c.-91 relative to the A of
438 the *CHM* start codon. The position-weight matrix for ZNF143 (Figure 5) establishes the relative
439 frequency of the base C at position c.-98 to be 100%, supporting the importance of the residue
440 and the resulting pathogenesis when mutated. Furthermore, surveying the UCSC genome
441 browser (<http://genome.ucsc.edu/>) multiz alignment of 100 vertebrate genomes tract, the region
442 c.-119 to c.-76 shows a high degree of conservation among mammals, and absolute conservation
443 of residue C at positions corresponding to c.-98 pointing to an important biological role for the
444 sequence (Kuhn et al., 2007; Blanchette et al., 2004). Representatives of birds and amphibian
445 classes, however, lack homology in the region, in while in fish a corresponding region is absent
446 altogether. The promoter, therefore, cannot be considered an ultra-conserved non-coding element
447 (Dimitrieva and Bucher, 2013). Several alignments are listed in Table 2.

448
449 Studies of *CHM* mRNA and protein localization have found a broad expression profile for both.
450 In mice, evidence of transcription was found in multiple cell types and in every major layer of
451 retina (Keiser et al., 2005), while immunolabeling of primate retina showed REP-1 localized to
452 both rod and cones (Dimopoulos et al., 2015). Studies in human and primate retina found that
453 mRNA levels did not correspond to the pattern of disease expression; little *CHM* was detected in
454 the RPE and choroid, and there were no marked regional differences in the concentration of
455 *CHM* mRNA apparent with foveal versus mid-peripheral total RNA despite affected males
456 typically exhibiting a preservation of central vision until late in the disease (Bernstein and Wong,

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3 457 1998). Additionally, REP-1 can be readily detected in human fibroblasts or peripheral blood
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6 458 mononuclear cells (Furgoch et al., 2014; MacDonald et al., 1998). Taken together with
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8 459 ZNF143's characterization as one of the most common and ubiquitously expressed TFs
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10 460 (Myslinski et al., 1998) a picture emerges of widespread and non-specific transcription of *CHM*,
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12 461 despite choroideremia's manifestation as an ocular disease. Indeed, patients' apparent lack of
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14 462 systemic symptoms can be understood to result not from tissue specific expression of REP-1, but
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16 463 from the differing affinities of REP-1 and REP-2 for target Rabs, which may themselves be
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18 464 differentially expressed or possess tissue or cell specific activity. Investigators have implicated
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20 465 Rab27 (Seabra et al., 1995) and Rab38 (Kohnke et al., 2013) as possible contributors.
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27 467 The study presented here also poses interesting questions, such as whether mutations of other
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29 468 residues less critical to transactivator binding in the *CHM* promoter, that diminish, but not
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31 469 completely abolish mRNA expression, can result in a milder phenotype, or a different rate of
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33 470 progression of choroideremia. Currently, the dbSNP database lists no known SNPs between c.-
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35 471 119 to c.-76 (Sherry et al., 2001). Having shown the *CHM* region responsible for regulation of its
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37 472 expression, described for the first time the features of its promoter, and extended the inventory of
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39 473 molecular changes causing choroideremia, the findings are of clinical and diagnostic interest and
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41 474 present an obvious area of examination for patients with CHM in whom no coding sequence
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43 475 mutation has been found. Further elucidating the roles of ZNF143, THAP11, HCFC1 or other
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45 476 distal factors will prove an important step toward understanding the complete picture of *CHM*'s
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47 477 regulation.
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55 479 **Acknowledgements**
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Promoter mutation causing choroideremia

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492
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499 **Figure Legends**

500 **Figure 1.** Functional Analysis of the *CHM* Promoter.

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(A) The constructs portrayed on the left were inserted upstream of the luciferase gene in pGL3-basic. Nucleotide +1 represents the translation start site. The promoterless pGL3-basic served as a negative control. (B) The mutations c.-98C>A, and c.-98C>T abolish transcription, while the minimal construct c.-119_-76 is sufficient for robust expression of the reporter gene not significantly different from even that of the nearly 4kb construct. All constructs were transiently transfected into HEK293T cells. A dual-luciferase reporter assay was used to assess the potential promoter activity of various sized inserts and the c.-98 mutants. Promoter activity is shown as a ratio of firefly luciferase over *Renilla* luciferase present on the transfection control plasmid pRL-CMV to account for inter-well variation. Activity is normalized to that of the reference construct pGL3-1093_-31 which is artificially set to equal 100. Activity significantly different (P<0.01) from the reference construct is denoted by an asterisk. Error bars represent ± 1 SD.

Figure 2. Pedigree Structure of Affected Families with *CHM* Promoter Mutations

(A) Family1. The parents of proband 5116 were second cousins, sharing a set of great grandparents. The inheritance pattern mimics male-male transmission, but is nevertheless consistent with X-linked inheritance upon examination of the wider family pedigree. (B) Family 2. The pedigree showing three generations affected by choroideremia examined in this study. Inheritance follows an X-linked pattern.

Figure 3. Retinal features in patient 5116.

Legend: Left column (OD), right column (OS). (A) Fundus photographs of the proband taken at age 76 showing typical choroideremia changes, with atrophy of the choroid and RPE. A small island of preserved RPE remains in the central macula, surrounded by atrophic peripheral areas of apparent bare sclera. (B)(C) Fundus autofluorescence image demonstrating areas of residual RPE (L) and the corresponding OCT image (R). Preserved retinal areas with normal

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524 autofluorescence exhibit thicker choroid and preserved retinal lamination. An outer retinal
 525 tubulation is seen in the right fundus.

526 **Figure 4.** Molecular confirmation of choroideremia in patient 5116.

527 **(A)** Patient 5116 lacks REP-1. Western blot results show the absence of a ~100 kDa band
 528 corresponding to REP-1 in a lymphoblastoid cell line derived from the patient (lane 2), which is
 529 present in a normal control (lane 1). A β -actin antibody was used as a loading control to ensure
 530 an adequate protein sample in each lane, with the 42 kDa band present in both samples.

531 **(B)** Patient 5116 lacks *CHM* mRNA. cDNA synthesized from the mRNA harvested from a
 532 patient generated lymphoblastoid cell line was used as template for PCR. Lanes 2 and 3 show a
 533 475 bp band resulting from the amplification of the *GAPDH* control housekeeping gene from
 534 5116 and a normal control, indicating cDNA of adequate quality. Lanes 6 and 9 demonstrate an
 535 absence of amplification from the patient's cDNA of both partial and full length coding
 536 sequence, respectively, as compared to PCR products sized 93 and 2200 base pairs amplified
 537 from normal cDNA observed in lanes 5 and 10.

538 **Figure 5.** Consensus binding sequences for transcription factor ZNF143.

539 **(A)** Partial map of the human *CHM* gene; arrow indicates transcription start site. **(B)** Expanded
 540 sequence of the minimal *CHM* promoter from c.-119 to c.-76, as identified through the analysis
 541 of progressive deletion constructs. Position c.-98 is marked with an asterisk. **(C)** Sequence logo
 542 derived from publically available ChIP-seq data released as part of the ENCODE project, with
 543 the position weighted matrix below (ENCODE Project Consortium, 2012). An invariant C is
 544 found at position corresponding to c.-98 of *CHM*.

546 **Table 1.** Clinical data of genotyped individuals

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Table 2. Multiz alignment of the promoter region of 15 *CHM* orthologs

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For Peer Review

Promoter mutation causing choroideremia

1 Single base substitutions in the *CHM* promoter as a cause of choroideremia

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Abstract

Although over 150 unique mutations affecting the coding sequence of *CHM* have been identified in patients with the X-linked chorioretinal disease choroideremia (CHM), no regulatory mutations have been reported, and indeed the promoter has not been defined. Here we describe two independent families affected by CHM bearing a mutation outside the gene's coding region at position c.-98: C>A and C>T, which segregated with the disease. The male proband of family 1 was found to lack *CHM* mRNA and its gene product Rab escort protein 1 (REP-1), while whole genome sequencing of an affected male in family 2 excluded the involvement of any other known retinal genes. Both mutations abrogated luciferase activity when inserted into a reporter construct, and by further employing the luciferase reporter system to assay sequences 5' to the gene, we identified the *CHM* promoter as the region encompassing nucleotides c.-119 to c.-76. These findings suggest that the *CHM* promoter region should be examined in patients with choroideremia who lack coding-sequence mutations, and reveals, for the first time, features of the gene's regulation.

Keywords

CHM, choroideremia, promoter, REP-1, ZNF143, THAP11

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Introduction

Choroideremia (CHM; MIM# 303100) is an X-linked, recessively inherited chorioretinal dystrophy with an incidence of 1/50,000. Progressive degeneration of photoreceptors, retinal pigment epithelium (RPE), and the choroid causes affected hemizygous males to develop night blindness in the first or second decade of life, followed by a decrease in peripheral visual fields and an eventual loss of central visual acuity in advanced stages of the disease (Coussa and Traboulsi, 2012). Carrier females, while usually asymptomatic, may exhibit signs of retinal degeneration upon fundoscopic examination, and more rarely have reduced dark adaptation and peripheral vision (Karna, 1986; Roberts et al., 2002).

To date, CHM has only been linked to mutations within the *CHM* gene, coding for REP-1 (Cremers et al., 1990). The protein serves as a molecular chaperone for small GTPases from the Rab family, presenting them to Rab geranylgeranyl transferase which modifies them by the covalent attachment of a lipid moiety. The lipid modification, known as prenylation, of target Rab proteins is essential for intracellular vesicular transport (Seabra et al., 1992; Seabra et al., 1993). A homologue, REP-2, encoded by *CHML* or choroideremia-like, functions similarly and appears to compensate for the absence of REP-1 in all tissues except the eye (Cremers et al., 1994). *CHM* spans over 150 kb of Xq21.2 and contains 15 exons (van Bokhoven et al., 1994). A 30 base pair 5'untranslated region, found on exon 1, precedes an open reading frame encoding the 653 amino acid REP-1 protein. At least 163 unique pathogenic mutations have been reported in the LOVD Retinal and Hearing Impairment Genetic Mutation Database [https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php ; accessed Feb 2017] (Fokkema et al., 2011). The mutation spectrum includes transitions and transversions leading to protein truncation, splice defects, indels and large deletions ranging from a single exon to the full gene.

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3 91 Missense mutations predicted to alter protein structure or impair function (Sergeev et al., 2009;
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5 92 Esposito et al., 2011), transposon insertions (Van Den Hurk et al., 2003), partial gene
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7 93 duplications (Chi et al., 2013; Simunovic et al., 2016) and other variations are infrequently
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9 94 found, but taken collectively, almost all known pathogenic variants in the *CHM* gene have been
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11 95 loss of function mutations that abolish functional REP-1 (McTaggart et al., 2002; Simunovic et
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13 96 al., 2016). Notably there is no apparent correlation between genotype and phenotype, with the
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15 97 age at onset of symptoms, visual acuity and visual fields being unrelated to mutation type
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17 98 (Freund et al., 2016; Simunovic et al., 2016).
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20 99 The promoter driving expression of *CHM* has been heretofore unidentified. Analyses of
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22 100 promoter mutations causing inherited diseases can be useful in identifying transcription factors
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24 101 involved in the regulation and expression of a gene of interest, as the activity of RNA
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26 102 polymerase II is mediated by the recruitment of general and sequence specific transcription
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28 103 factors to *cis*-acting regulatory sequences. The core promoter directing basal level transcription
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30 104 is generally found between nucleotides -40 and +50 of the transcription start site (TSS) (de
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32 105 Vooght et al., 2009), while proximal promoter elements typically reside within 1 kilo bases (kb),
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34 106 with additional enhancers, repressors, insulators acting even over distances of mega bases
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36 107 (Maston et al., 2006). The investigation of regulatory elements in the context of human disease is
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38 108 complicated by the fact that in contrast to loss of function mutations, regulatory defects may
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40 109 produce small quantitative changes that are difficult to detect. Mutations in *cis*-acting regulatory
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42 110 sequences are a significant cause of human disease, and according to statistics compiled by the
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44 111 Human Gene Mutation Database [<http://www.hgmd.cf.ac.uk> ; accessed May 2016]
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46 112 approximately 2% of disease causing point mutations are in non-coding regions of the genome
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48 113 (Stenson et al., 2014). Though promoter defects can cause functionally important consequences
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for gene expression, their analysis is often not a regular part of DNA diagnostics as the investigation can be complex, laborious and difficult to perform.

In this study, we report the first known regulatory mutations causing choroideremia. The absence of a genetic defect in a CHM family prompted us to explore the upstream non-coding region, revealing the novel promoter variant c.-98C>A. Subsequently, whole genome sequencing in an unrelated family with CHM identified a second variant of the same residue, c.-98C>T. We demonstrate the mutations' effects on transcription using a luciferase assay, and employ the same system to further characterize the boundary of the *CHM* promoter that spans the location. The results suggest a disruption of a transcription factor binding site and impaired transactivation of the *CHM* promoter by the factor(s). Together, these studies further our understanding of regulation and expression of *CHM* and present a possible explanation for cases of unexplained choroideremia where no causative mutation is found within the gene.

Materials and Methods

Clinical Examination and Study Subjects

This study was approved by the institutions' respective ethics review boards. All procedures adhered to the tenets of the Declaration of Helsinki. Before participation, the purpose and risks of the study were explained, and informed consent was obtained. Blood samples were drawn, and a detailed pedigree and history was recorded.

We studied 4 males affected by CHM and 1 carrier female from two unrelated families. Individuals were examined by fundoscopy and functional ophthalmologic methods including some or all of: visual acuity testing, central visual fields, full field electroretinography, optical

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coherence tomography and fundus autofluorescence imaging. Choroideremia was diagnosed by senior clinicians with expertise in inherited retinal disease based on clinical findings alone. Affected males had a history of night vision loss and fundus appearance of peripheral retinopathy with broad areas of RPE and choroidal atrophy.

CHM Sanger Sequencing

DNA was extracted from lymphocytes using conventional methodologies. Proband 5116 from family 1 (C127) was initially screened for *CHM* mutations in the coding sequence and splice sites by direct Sanger sequencing from PCR amplicons (Furgoch et al., 2014). Additionally, 1kb of the 5'-flanking sequence was amplified with primers 5'-CAGGGAAGGCCCACTACTGC -3' and 5'-CTTGTGGAAATGAGATCAAGTTAGG-3' and sequenced with the same primers. With the exception of patient 111 and his parents, of family 2 who underwent whole genome sequencing, the remaining study individuals were genotyped only at position c.-98 to confirm the presence or absence of the respective variant. Annotation is in accordance with GenBank: NM_000390.3, where +1 represents the start of translation.

Cell Culture

Leukocytes from patient 5116 were separated with Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA) from 10mL of whole human blood collected in acid citrate dextrose (ACD-A) tubes (BD, Franklin Lakes, NJ, USA). A lymphoblastoid cell line was established by Epstein-Barr virus transformation within 48 hours of collection (Anderson and Gusella, 1984). Cells were maintained in RPMI-1640 supplemented with 15% fetal calf serum and penicillin-streptomycin.

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mRNA and Protein Analysis

Total RNA was extracted from 5x10⁶ lymphoblastoid cells from patient 5116 and a healthy control, with the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. cDNA was transcribed from 5µg of total RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA) using a random hexamer primer mix. PCR amplification from cDNA with forward primer 5’-TAATAGTCACATGACACGTTTCCCG-3’, paired with 5’-TGGATTTCAGGCAAACCCGT-3’, or 5’-TTTAAAATGAGCAAGTCAATGTGC-3’ as a reverse primer was used to detect partial transcript (spanning the junction of exon 1 and 2), or the entire coding sequence of *CHM* encompassed by 15 exons, respectively. GAPDH was also amplified as a positive control with intron-spanning primers.

Protein was extracted from 2x10⁶ lymphoblastoid cells and immunoblot analysis of REP-1 was performed as previously described (Furgoch et al., 2014).

Whole Genome Sequencing

UK proband 111 (GC406) and his unaffected parents 190 and 192 underwent whole genome sequencing (WGS) as part of the 100,000 Genomes Project. Briefly, genomic DNA was processed using the Illumina TruSeq DNA PCR-Free Sample Preparation kit (Illumina Inc) and sequenced using an Illumina HiSeq X Ten, generating minimum coverage of 15X for >97% of the callable autosomal genome. Reads were aligned to build GRCh37 of the human genome using the Isaac aligner (Illumina Inc). SNVs and indels were identified using Platypus v0.8.1 and annotated using Cellbase (<https://github.com/open.cb/cellbase>). Variant filtering was performed using minor allele frequency (MAF) in publicly available and in-house datasets, predicted

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183 protein impact and familial segregation. Surviving variants were prioritized using a pre-specified
184 virtual gene panel from PanelApp (<https://panelapp.extge.co.uk/crowdsourcing/PanelApp/>)
185 Posterior segment abnormalities v1.7. Allelic state was required to match the curated mode of
186 inheritance for variants in panel genes.

Promoter Analysis*Construction of luciferase reporter plasmids*

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190 Primers were initially designed to amplify a 1063 bp fragment from upstream of the *CHM*
191 transcription start site (c.-1093_c.-31) from control and patient 5116's DNA. The c.-98C>T
192 mutation present in family 2 was generated by site directed mutagenesis from the control
193 plasmid. All sequences cloned contained only untranscribed DNA and did not include the 30
194 base pair 5'UTR directly upstream of the start codon. The fragment was cloned according to
195 standard protocols, with SacI and HindIII restriction sites added to primers allowing digest by
196 the enzymes (Thermo Scientific, Rockford, IL, USA). Ligation into the promoterless firefly
197 luciferase reporter vector, pGL3-Basic (Promega, Madison, WI, USA) generated constructs
198 pGL3-1063_-31 and pGL3-1063_-31 c.-98 C>A. Subsequently, a 4kb fragment, and a series of
199 nested deletions ranging from 2kb to 44bp were cloned into pGL3-Basic with the same
200 restriction sites added and used for subsequent digest, to identify the minimal promoter necessary
201 and sufficient for transcription. pGL3-Basic lacks a eukaryotic promoter sequence upstream of
202 the reporter luciferase gene, and expression of luciferase in transfected cells depends on a
203 functional promoter sequence to be inserted upstream of the *luc+* gene. pGL3-Basic itself served
204 as a negative control. A schematic of all inserts assayed is provided in Figure 1A. All constructs

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3 205 generated were Sanger sequenced to ensure fidelity. Primer sequences for the generation of
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8 207 *Luciferase Assay*
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10 208 HEK293T cells cultured with standard reagents and conditions were seeded in 24 well plates and
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12 209 transfected at 70% confluency with polyethylenimine (PEI). Each well was transfected with a
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14 210 total of 650ng of plasmid DNA, including 600ng of pGL3 construct and 50ng of the internal
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16 211 control pRL-CMV, a cytomegalovirus promoter driven *Renilla* luciferase reporter vector.
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18 212 Briefly, plasmid DNA was diluted into 25μL of PBS, and mixed by vortexing with 1μL of
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20 213 1mg/mL branched PEI (Sigma-Aldrich, St. Louis, MO, USA) in 25μL PBS. After a 15 minute
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22 214 incubation, 50μL of the reaction was added dropwise to each well. Forty-eight hours after
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24 215 transfection, luciferase activity was assayed using the Dual-Luciferase Reporter Assay kit and
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26 216 measured with the Glomax Explorer (both Promega, Madison, WI, USA). Relative luciferase
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28 217 activity was obtained by dividing the relative light units (RLU) produced by the firefly luciferase
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30 218 pGL3 construct by the RLUs produced by *Renilla* luciferase control reaction. Light generated by
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32 219 the reaction can be correlated with the amount of luciferase protein produced which in turn is
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34 220 proportional to promoter activity driving the gene's expression. Values from an n=6 were
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36 221 averaged and normalized to that of the reference construct, pGL3-1093_-31 to obtain a relative
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38 222 measure of activity.
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41 223 *Statistical analysis*
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44 224 All data were expressed as mean ± SD. Data from the luciferase assay represents two
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46 225 independent experiments with triplicate measurements. Differences between groups were
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48 226 examined for statistical significance using Student's *t*-test. A *P*-value <0.01 denoted the presence
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50 227 of a statistically significant difference.
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Results

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Clinical Characterization of CHM***Family 1***

We investigated a progressive retinal degeneration in a Caucasian family of American origin (C127). Remarkably, the proband 5116 was the offspring of a consanguineous union between second cousins, with an affected father and carrier mother. To our knowledge, this has never been reported with choroideremia, and it necessitated a thorough and accurate diagnosis of X-linked CHM and exclusion of an autosomal recessive, or even an unusual male-male transmission as cause for the disease. An investigation of the extended family for which the pedigree is reported in Figure 2A however, clearly demonstrated the X-linked inheritance of the disease.

Patient 5116 was initially seen at age 56 years, when he was referred by a retinal specialist with a diagnosis of choroideremia. At his most recent examination, age 76, best corrected visual acuity (BCVA) was measured as 6/19 OD and 6/15 OS. Visual fields were reduced to less than 5 degrees. The full-field ERG showed non-detectable dark adapted rod-driven responses as well as non-detectable cone responses to a 30 Hz flicker stimulus. OCT imaging indicated loss of the photoreceptor layer across the periphery of the fundus with only a small island of RPE remaining in the macula. Posterior segment examination showed a hypo-pigmented fundus with significant atrophy of the RPE and choroid with areas of bare sclera (Figure 3). These findings are consistent with an advanced state of choroideremia.

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250 The proband's daughter 5113 was examined at 33 years of age and did not report any vision
251 difficulties. BCVA when examined was 6/6, both eyes. Full-field ERG testing of the left eye
252 showed dark adapted rod-driven responses were reduced by 50% in amplitude (42.5 μ V) and a
253 normal b-wave implicit time (81.6 msec). Light-adapted cone-driven responses to a 30 Hz flicker
254 stimulus were reduced by 50% in amplitude (27.6 μ V) and borderline reduced in implicit time
255 (25.6 msec). These findings were consistent with a classic carrier state of choroideremia.

256 The proband's father and one brother 5149 were reported to have been previously diagnosed
257 with choroideremia, while a second brother 5147 was reported to be unaffected. Findings are
258 summarized in Table 1.

259 *Family 2:*

260 Family 2 (GC406) was a Caucasian family of British origin with a history of choroideremia.

261 Proband 111 was first examined at age 13, displaying a reduced but not delayed full field ERG
262 with flicker and bright flash responses both within normal limits. He remained asymptomatic
263 until age 32. Confrontational visual field testing at age 35 showed bilateral infratemporal
264 scotoma, and BCVA was 6/5 in both eyes. He is considered symptomatically mild with retained
265 central macular structure.

266 Of his two maternal uncles, 151 was diagnosed at age 12. Fundus abnormalities typical of
267 choroideremia were noted. 161 was diagnosed at age 8, and upon examination at age 37
268 exhibited moderately constricted visual fields particularly in the superior field bilaterally. BCVA
269 was reduced to count fingers vision OD and 6/36 OS. Fundus examination was consistent with a
270 clinical diagnosis of choroideremia. The pedigree is reported in Figure 2B, and clinical findings
271 summarized in Table 1.

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Genetic Analysis*Family 1 (C127)*

Genetic analysis of proband 5116 did not reveal any pathogenic mutation in the coding sequence of the *CHM* gene or splice site boundaries. Yet at position c.-98 relative to the translation start site, a hemizygous C>A transversion was detected (hg38, chrX:g.86047629G>T NM_000390.3, c.-98C>A). The variant was not listed in the latest release of dbSNP [<http://www.ncbi.nlm.nih.gov/SNP/>; accessed Feb 2017] (Sherry et al., 2001). The proband's affected brother (5149) was also found to harbour the variant while it was absent in the unaffected brother (5147) available for testing. The obligate carrier status was confirmed in the proband's daughter (5113). The location of the variant strongly suggested a regulatory mutation, as evaluation of entries in the HGMD reveals that most promoter mutations are located between +50 and -500 from the TSS of a gene (Stenson et al., 2014).

Family 2 (GC406):

Prior genetic analysis of the coding exons of the *CHM* gene did not reveal a pathogenic mutation. Whole genome sequencing was performed on proband 111 and his parents 190 and 192, as part of the 100,000 Genomes Project. After variant filtering, no causative rare coding variants were identified in any retinal disease gene.

In light of the clinical diagnosis of choroideremia in the family, the complete *CHM* gene was interrogated for rare variants (≤ 0.001 MAF in 1Kgenome project and internal cohort of over 2000 whole genome sequencing samples) hemizygous in the proband and carried by his mother. One such variant was identified, the transition, c.-98C>T (hg38, chrX:g.86047629G>A NM_000390.3, c.-98C>T). The variant was confirmed in the two affected maternal uncles 151 and 161 by direct Sanger sequencing.

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297 ***Molecular diagnosis of choroideremia in 5116***

298 Immunoblot analysis of protein harvested from a cell line derived from patient 5116 failed to

299 detect the *CHM* gene product REP-1, providing conclusive confirmation of the clinical diagnosis

300 of choroideremia (Figure 4A). To provide evidence for a regulatory mutation and subsequently

301 reduced transcription, we intended to compare the level of expression between normal and

302 patient samples through qPCR. Endpoint PCR from a cDNA template; however, failed to

303 amplify the 15 exon, 2200 base pair transcript, indicating it was absent or present at a level

304 below the detection threshold, and thus the quantitative assay was not performed. To largely rule

305 out the possibility of a splice defect, we also attempted to amplify a minimal portion of the

306 transcript, a 93 base pair fragment from the 5'-UTR to a region spanning the boundary of exon 1

307 and 2, and found the patient's cells lacked even this short fragment (Figure 4B). A control

308 housekeeping gene was nevertheless readily detected and both partial and full length transcripts

309 were amplified from normal cDNA.

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311 ***Effect of the c.-98C>A and c.-98C>T mutations on transcription of CHM***

312 As a starting point, an approximately 1kb fragment upstream of the TSS was assayed for ability

313 to drive gene expression, since the majority of elements necessary for transcription are expected

314 to be found within this region (Rockman and Wray, 2002). Comparing the robust luciferase

315 activity produced by cells transfected with this wild type construct pGL3-1093_-31, to that of

316 pGL3-1093_-31 c.-98C>A and c.-98C>T, we observed complete abrogation of promoter activity

317 (Figure 1B) in the mutants. The drop from 100 ± 9.5 to 2.0 ± 0.3 and 1.3 ± 0.4 respectively, as

318 measured in normalized relative light units (RLU) ± 1 standard deviation is even significantly

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319 lower than that of the negative control pGL3-basic which does not contain a promoter sequence,
320 reading at 5.8 ± 0.9 . This startling observation strongly suggested that the mutation spans an
321 element essential to promoter activity.

322 Based on unpublished reports suggesting other activating elements may exist up to 2.8kb
323 upstream of the TSS (Kaiser NW, *et al.* IOVS 2004;45:ARVO E-Abstract 2451), we additionally
324 compared the activity of two larger constructs approximately 4kb and 2kb long. Typically,
325 regulatory regions upstream of core promoter sequences contain multiple TF specific binding
326 motifs, where several copies of the same factor or cooperation between different factors serve to
327 synergistically stimulate transcriptional activity of a given gene (Maston et al., 2006). Yet in the
328 case of *CHM*, the longer sequences did not stimulate additional luciferase expression. pGL3-
329 3983_-31 and pGL3-2027_-31 did not significantly differ from pGL3-1093_-31, producing 83.7
330 ± 4.9 and 96.2 ± 7.6 RLU respectively. The somewhat diminished expression as compared to
331 the reference 1kb construct may be attributed to a lower molar amount of the larger plasmids
332 being delivered during transfection.

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334 ***Characterization of CHM promoter***

335 To further characterize the promoter, we proceeded to assay progressively shorter constructs,
336 deleting sequences from the -5' and -3' of the 1kb construct to define its boundaries (Figure 1A).
337 Working from the 5' end, the approximately 400, 300, and 100 base pair constructs pGL3-437_-
338 31, pGL3-346_-31, pGL3-119_-31 did not significantly differ from the 1 kb containing reference
339 plasmid pGL3-1093_-31, reading at 100.2 ± 11.4 , 96.1 ± 10.6 , and 97.7 ± 8.1 RLU respectively.
340 Yet upon deleting a further 11 base pairs, we observed a significant drop to 39.8 ± 7.6 RLU with
341 pGL3-108_-31. Having delineated the -5' boundary of the promoter as extending to no further

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342 than nucleotide c.-119, we subsequently tested pGL3-119_-76 bearing a deletion from the -3'

343 end, and found no significant difference at 93.4 ± 14 RLU. It was the final construct, pGL3-

344 119_-82, which yielded significantly lower activity producing only 27.4 ± 4 RLU. Though

345 further characterization of the promoter region down to the single nucleotide level could be

346 performed, we have defined the borders of the promoter to within the region of c.-119 to c.-76.

347 This short 44 base pair DNA region upstream of the *CHM* coding sequence is able to stimulate

348 transcription in an *in-vitro* assay at a level that is not significantly different from that of a 1kb, or

349 even a 4kb fragment and can be understood to contain essential *cis*-acting elements that

350 positively regulate *CHM* expression.

351

352 **Discussion**

353 To date, all genetic defects causing choroideremia, which include all major mutation classes,

354 have been observed in exons, introns or intron/exon boundaries of the *CHM* gene (Fokkema et

355 al., 2011). No mutations have been reported in the promoter region of *CHM*, and the regulation

356 of the gene has remained essentially unexplored. In this study, we present the first report of

357 promoter mutations, c.-98C>A and c.-98C>T, causing choroideremia. The critical role of this

358 residue for gene expression is highlighted by the complete abrogation of reporter activity in a

359 promoter assay when the nucleotide is mutated. Though we demonstrate the first examples in a

360 novel mutation class, it is worthwhile noting that the phenotypes observed in affected individuals

361 were typical of choroideremia. In family 1, the genotype c.-98C>A in proband 5116 resulted in

362 severe retinopathy, while characteristic milder signs were found in the carrier female 5113.

363 Males from family 2 bearing the c.-98C>T mutation manifested with chorioretinal disease, but

364 with varying degrees of severity; proband 111 remained symptomatically mild while in his

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maternal uncles 151 and 161, disease progression was more typical. CHM is known to exhibit a wide interfamilial, and also intrafamilial variability (Moosajee et al., 2014). As the mutations c.-98C>A and c.-98C>T appear to completely abolish transcription, producing no detectable levels of *CHM* mRNA and REP-1 protein in patient cells, it is not surprising for the disease to present in a classical manner consistent with phenotypes observed for the loss of function mutations. Whole genome sequencing in the parent-offspring trio of family 2 allowed the exclusion of a deep intronic *CHM* mutation, as well as mono or biallelic mutations in any other retinal dystrophy gene as a cause of the observed symptoms. We therefore conclude that the two mutations at residue c.-98 impair *CHM* transcription enough to result in choroideremia. For the several gene therapy trials currently underway employing a “gene replacement” strategy (Dimopoulos et al., 2015), individuals bearing these genotypes would be suitable candidates. The two variants have been submitted and published in the LOVD Retinal and Hearing Impairment Genetic Mutation Database (Fokkema et al., 2011).

Having identified mutations that evidently abolished transcription, we interpreted their location to span a region crucial for gene expression, and set out to define the boundaries of the gene’s promoter. Surprisingly, after analyzing fragments as long as 4kb, we found a short 44 base pair DNA fragment to be wholly responsible for driving expression. The region c.-119 to c.-76 comprises the entirety of the *CHM* proximal promoter, and is able to drive robust transcription in an *in-vitro* luciferase assay. The region implicated amounts to less than 3% of the length of the *CHM* coding sequence where the large majority of reported causative mutations have been found (Fokkema et al., 2011). A recent investigation of a large disease cohort (74), found causative mutations in the gene in 94% of cases previously diagnosed with CHM (Simunovic et al., 2016).

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388 The remaining 6% can be understood to be comprised of regulatory mutations, incorrect
389 diagnoses or deep intronic variant causing cryptic splicing (Carss et al., 2017). Promoter defects
390 are therefore expected to be responsible for a small minority of choroideremia cases. The
391 identified region; however, becomes an obvious area for examination in patients in whom no
392 coding sequence mutation is found.

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394 A promoter or regulatory mutation can be expected to either increase or decrease transcriptional
395 activity mediated by the altered binding capacity of trans-acting protein factors specific to a
396 DNA sequence in the promoter region. In this case, the interaction appears to be entirely
397 disrupted based on the null expression of a reporter driven by mutated sequence in our luciferase
398 assay. Impaired transcription due to a 1-bp mutation in a promoter region is unusual, but has
399 been reported previously as in the case of single base mutations stimulating additional
400 transcriptional activity at the *OVOL2* promoter causing autosomal-dominant corneal endothelial
401 dystrophies (Davidson et al., 2016). On the other hand, decreased transactivation of *NMNAT1*
402 due to a single nucleotide change in the promoter was found to cause Leber congenital amaurosis
403 (Coppieters et al., 2015).

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405 While we delineate the *CHM* promoter boundary to this small area upstream of the gene, we
406 cannot exclude the possibility of distant enhancer, repressor, or intronic elements also
407 contributing to regulation; our findings suggest the sequence between c.-119 and c.-76 is
408 essential, but not necessarily sufficient for transcription. The wider upstream sequence of *CHM*
409 lacks the consensus sequences often found in RNA polymerase II promoters, such as CAAT and
410 TATA boxes; as well it is neither GC rich nor associated with any CpG islands. Bioinformatic

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analysis with MotifMap, a dataset of computationally predicted transcription factor binding sites based on binding motifs [<http://motifmap.ics.uci.edu/>] (Daily et al., 2011) identifies a putative binding motif for the transcription factor zinc finger protein 143 (ZNF143) contained within the region of the now revealed *CHM* promoter. ZNF143 participates in the regulation of RNA pol II and III mediated transcription of protein coding, non-coding, and small nuclear RNA (Schaub et al., 1997; Myslinski et al., 1998) and was initially connected with the binding motif TTCCCATTATGCACCGCG (SBS1) (Myslinski et al., 2006). Genome-wide studies revealed the binding site for the factor to be frequently found with an adjacent 5' accessory sequence, forming the ACTACAATTCCCATTATGCACCGCG (SBS2) motif. SBS2 is comprised of both a THAP domain-containing protein 11 (THAP11) and ZNF143 binding site, with the factors believed to act in a competitive manner (Ngondo-Mbongo et al., 2013). The recruitment of THAP11 to its canonical binding site ACTAYRNNNCCCR is most frequently associated with up-regulation genes essential to protein biosynthesis and energy production (Dejosez et al., 2010). More recently, ZNF143 was suggested to cooperatively occupy SBS2 sites with THAP11 and a third factor, the scaffold protein host cell factor 1 (HCFC1) *in-vivo* (Vinckevicius et al., 2015). SBS2 is closely matched by the sequence found at position c.-108 to c.-84 upstream of *CHM*, ACTACAACACCCAGAATGCACTGTT. Notably, ZNF143's binding at promoters was recently implicated in chromatin looping with distal regulatory elements (Bailey et al., 2015), suggesting the involvement of yet other factors in the total regulation of expression of *CHM*.

Of the three transcription factors implicated above, the binding of at least ZNF143 is supported by publicly available chromatin immunoprecipitation sequencing data released as part of the ENCODE project [<https://genome.ucsc.edu/ENCODE/>] (ENCODE Project Consortium, 2012).

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3 434 In all cell types assayed: lymphoblastoid, HeLa and K562 cell lines, as well as embryonic stem
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6 435 cells, the promoter region of *CHM* interacted with ZNF143, for which a consensus binding
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8 436 sequence GAACTACAATTCCCAGAAGGC, again is closely matched by
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10 437 GAACTACAACACCCAGAATGC found between position c.-110 to c.-91 relative to the A of
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12 438 the *CHM* start codon. The position-weight matrix for ZNF143 (Figure 5) establishes the relative
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14 439 frequency of the base C at position c.-98 to be 100%, supporting the importance of the residue
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16 440 and the resulting pathogenesis when mutated. Furthermore, surveying the UCSC genome
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18 441 browser (<http://genome.ucsc.edu/>) multiz alignment of 100 vertebrate genomes tract, the region
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20 442 c.-119 to c.-76 shows a high degree of conservation among mammals, and absolute conservation
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22 443 of residue C at positions corresponding to c.-98 pointing to an important biological role for the
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24 444 sequence (Kuhn et al., 2007; Blanchette et al., 2004). Representatives of birds and amphibian
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26 445 classes, however, lack homology in the region, in while in fish a corresponding region is absent
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28 446 altogether. The promoter, therefore, cannot be considered an ultra-conserved non-coding element
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30 447 (Dimitrieva and Bucher, 2013). Several alignments are listed in Table 2.
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39 449 Studies of *CHM* mRNA and protein localization have found a broad expression profile for both.
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41 450 In mice, evidence of transcription was found in multiple cell types and in every major layer of
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43 451 retina (Keiser et al., 2005), while immunolabeling of primate retina showed REP-1 localized to
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45 452 both rod and cones (Dimopoulos et al., 2015). Studies in human and primate retina found that
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47 453 mRNA levels did not correspond to the pattern of disease expression; little *CHM* was detected in
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49 454 the RPE and choroid, and there were no marked regional differences in the concentration of
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51 455 *CHM* mRNA apparent with foveal versus mid-peripheral total RNA despite affected males
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55 456 typically exhibiting a preservation of central vision until late in the disease (Bernstein and Wong,
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1998). Additionally, REP-1 can be readily detected in human fibroblasts or peripheral blood mononuclear cells (Furgoch et al., 2014; MacDonald et al., 1998). Taken together with ZNF143's characterization as one of the most common and ubiquitously expressed TFs (Myslinski et al., 1998) a picture emerges of widespread and non-specific transcription of *CHM*, despite choroideremia's manifestation as an ocular disease. Indeed, patients' apparent lack of systemic symptoms can be understood to result not from tissue specific expression of REP-1, but from the differing affinities of REP-1 and REP-2 for target Rabs, which may themselves be differentially expressed or possess tissue or cell specific activity. Investigators have implicated Rab27 (Seabra et al., 1995) and Rab38 (Kohnke et al., 2013) as possible contributors.

The study presented here also poses interesting questions, such as whether mutations of other residues less critical to transactivator binding in the *CHM* promoter, that diminish, but not completely abolish mRNA expression, can result in a milder phenotype, or a different rate of progression of choroideremia. Currently, the dbSNP database lists no known SNPs between c.-119 to c.-76 (Sherry et al., 2001). Having shown the *CHM* region responsible for regulation of its expression, described for the first time the features of its promoter, and extended the inventory of molecular changes causing choroideremia, the findings are of clinical and diagnostic interest and present an obvious area of examination for patients with CHM in whom no coding sequence mutation has been found. Further elucidating the roles of ZNF143, THAP11, HCFC1 or other distal factors will prove an important step toward understanding the complete picture of *CHM*'s regulation.

Acknowledgements

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Figure Legends

Figure 1. Functional Analysis of the *CHM* Promoter.

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(A) The constructs portrayed on the left were inserted upstream of the luciferase gene in pGL3-basic. Nucleotide +1 represents the translation start site. The promoterless pGL3-basic served as a negative control. (B) The mutations c.-98C>A, and c.-98C>T abolish transcription, while the minimal construct c.-119_-76 is sufficient for robust expression of the reporter gene not significantly different from even that of the nearly 4kb construct. All constructs were transiently transfected into HEK293T cells. A dual-luciferase reporter assay was used to assess the potential promoter activity of various sized inserts and the c.-98 mutants. Promoter activity is shown as a ratio of firefly luciferase over *Renilla* luciferase present on the transfection control plasmid pRL-CMV to account for inter-well variation. Activity is normalized to that of the reference construct pGL3-1093_-31 which is artificially set to equal 100. Activity significantly different ($P < 0.01$) from the reference construct is denoted by an asterisk. Error bars represent ± 1 SD.

Figure 2. Pedigree Structure of Affected Families with *CHM* Promoter Mutations

(A) Family1. The parents of proband 5116 were second cousins, sharing a set of great grandparents. The inheritance pattern mimics male-male transmission, but is nevertheless consistent with X-linked inheritance upon examination of the wider family pedigree. (B) Family 2. The pedigree showing three generations affected by choroideremia examined in this study. Inheritance follows an X-linked pattern.

Figure 3. Retinal features in patient 5116.

Legend: Left column (OD), right column (OS). (A) Fundus photographs of the proband taken at age 76 showing typical choroideremia changes, with atrophy of the choroid and RPE. A small island of preserved RPE remains in the central macula, surrounded by atrophic peripheral areas of apparent bare sclera. (B)(C) Fundus autofluorescence image demonstrating areas of residual RPE (L) and the corresponding OCT image (R). Preserved retinal areas with normal

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3 524 autofluorescence exhibit thicker choroid and preserved retinal lamination. An outer retinal
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5 525 tubulation is seen in the right fundus.
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8 526 **Figure 4.** Molecular confirmation of choroideremia in patient 5116.
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10 527 **(A)** Patient 5116 lacks REP-1. Western blot results show the absence of a ~100 kDa band
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12 528 corresponding to REP-1 in a lymphoblastoid cell line derived from the patient (lane 2), which is
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14 529 present in a normal control (lane 1). A β -actin antibody was used as a loading control to ensure
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16 530 an adequate protein sample in each lane, with the 42 kDa band present in both samples.
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18 531 **(B)** Patient 5116 lacks *CHM* mRNA. cDNA synthesized from the mRNA harvested from a
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20 532 patient generated lymphoblastoid cell line was used as template for PCR. Lanes 2 and 3 show a
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22 533 475 bp band resulting from the amplification of the *GAPDH* control housekeeping gene from
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24 534 5116 and a normal control, indicating cDNA of adequate quality. Lanes 6 and 9 demonstrate an
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26 535 absence of amplification from the patient's cDNA of both partial and full length coding
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28 536 sequence, respectively, as compared to PCR products sized 93 and 2200 base pairs amplified
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30 537 from normal cDNA observed in lanes 5 and 10.
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32 538 **Figure 5.** Consensus binding sequences for transcription factor ZNF143.
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34 539 **(A)** Partial map of the human *CHM* gene; arrow indicates transcription start site. **(B)** Expanded
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36 540 sequence of the minimal *CHM* promoter from c.-119 to c.-76, as identified through the analysis
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38 541 of progressive deletion constructs. Position c.-98 is marked with an asterisk. **(C)** Sequence logo
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40 542 derived from publically available ChIP-seq data released as part of the ENCODE project, with
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42 543 the position weighted matrix below (ENCODE Project Consortium, 2012). An invariant C is
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44 544 found at position corresponding to c.-98 of *CHM*.
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55 546 **Table 1.** Clinical data of genotyped individuals
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547 **Table 2.** Multiz alignment of the promoter region of 15 *CHM* orthologs

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For Peer Review

Figure 1. Functional Analysis of the CHM Promoter. (A) The constructs portrayed on the left were inserted upstream of the luciferase gene in pGL3-basic. Nucleotide +1 represents the translation start site. The promoterless pGL3-basic served as a negative control. (B) The mutations c.-98C>A, and c.-98C>T abolish transcription, while the minimal construct c.-119_-76 is sufficient for robust expression of the reporter gene not significantly different from even that of the nearly 4kb construct. All constructs were transiently transfected into HEK293T cells. A dual-luciferase reporter assay was used to assess the potential promoter activity of various sized inserts and the c.-98 mutants. Promoter activity is shown as a ratio of firefly luciferase over Renilla luciferase present on the transfection control plasmid pRL-CMV to account for inter-well variation. Activity is normalized to that of the reference construct pGL3-1093_-31 which is artificially set to equal 100. Activity significantly different (P<0.01) from the reference construct is denoted by an asterisk. Error bars represent $\pm$ 1 SD.

Figure 1
609x406mm (96 x 96 DPI)

Figure 2. Pedigree Structure of Affected Families with CHM Promoter Mutations
(A) Family1. The parents of proband 5116 were second cousins, sharing a set of great grand-parents. The inheritance pattern mimics male-male transmission, but is nevertheless consistent with X-linked inheritance upon examination of the wider family pedigree. (B) Family 2. The pedigree showing three generations affected by choroideremia examined in this study. Inheritance follows an X-linked pattern.

Figure 2
478x349mm (96 x 96 DPI)

Figure 3. Retinal features in patient 5116.

Legend: Left column (OD), right column (OS). (A) Fundus photographs of the proband taken at age 76 showing typical choroideremia changes, with atrophy of the choroid and RPE. A small island of preserved RPE remains in the central macula, surrounded by atrophic peripheral areas of apparent bare sclera. (B)(C) Fundus autofluorescence image demonstrating areas of residual RPE (L) and the corresponding OCT image (R). Preserved retinal areas with normal autofluorescence exhibit thicker choroid and preserved retinal lamination. An outer retinal tubulation is seen in the right fundus.

Figure 3

Figure 4. Molecular confirmation of choroideremia in patient 5116. (A) Patient 5116 lacks REP-1. Western blot results show the absence of a ~100 kDa band corresponding to REP-1 in a lymphoblastoid cell line derived from the patient (lane 2), which is present in a normal control (lane 1). A β -actin antibody was used as a loading control to ensure an adequate protein sample in each lane, with the 42 kDa band present in both samples. (B) Patient 5116 lacks CHM mRNA. cDNA synthesized from the mRNA harvested from a patient generated lymphoblastoid cell line was used as template for PCR. Lanes 2 and 3 show a 475 bp band resulting from the amplification of the GAPDH control housekeeping gene from 5116 and a normal control, indicating cDNA of adequate quality. Lanes 6 and 9 demonstrate an absence of amplification from the patient's cDNA of both partial and full length coding sequence, respectively, as compared to PCR products sized 93 and 2200 base pairs amplified from normal cDNA observed in lanes 5 and 10.

Figure 4
32x16mm (600 x 600 DPI)

Figure 5. Consensus binding sequences for transcription factor ZNF143. (A) Partial map of the human CHM gene; arrow indicates transcription start site. (B) Expanded sequence of the minimal CHM promoter from c.-119 to c.-76, as identified through the analysis of progressive deletion constructs. Position c.-98 is marked with an asterisk. (C) Sequence logo derived from publically available ChIP-seq data released as part of the ENCODE project, with the position weighted matrix below (ENCODE Project Consortium, 2012). An invariant C is found at position corresponding to c.-98 of CHM.

Figure 5

439x257mm (96 x 96 DPI)

Patient	Age at exam	Sex	Genotype	OD/OS	BCVA	ERG Scotopic b-wave, μ V	ERG Photopic b-wave, μ V	ERG 30-Hz flicker, μ V	Visual Field	Fundus	
5116	76	M	c.-98C>A	OD	6/19	0	0	0.2	< 5 degrees	Widespread chorioretinal atrophy	
				OS	6/15	0	0	0.2	< 5 degrees	Widespread chorioretinal atrophy	
5149	-	M	c.-98C>A	Affected; not examined							
5113	33	F	c.-98C/A heterozygous	OD	6/6	Not tested				Carrier signs	
				OS	6/6	↓50%	Normal	↓50%	Not tested	Carrier signs	
5147		M	c.-98C	Unaffected; not examined							
111	35	M	c.-98C>T	OD	6/5	Not tested				Focal areas of chorioretinal atrophy in periphery	
				OS	6/5	Not tested				Focal areas of chorioretinal atrophy in periphery	
151	-	M	c.-98C>T	Data unavailable							Chorioretinal atrophy
161	37	M	c.-98C>T	OD	Count fingers vision	Not tested			Moderately constricted	Small central areas of functional retina	
				OS	6/36	Not tested			Moderately constricted	Small central areas of functional retina	
190	-	M	c.-98C	Unaffected; not examined							
192	-	F	c.-98C/T heterozygous	Not examined							

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<i>Homo sapiens</i> (human)	tcca--cccaa-gaactacaa-caccc	cagaatgcact-g-----tttt-ctt--ttc
<i>Pan troglodytes</i> (chimp)	tcca--cccaa-gaactacaa-caccc	cagaatgcact-g-----tttt-cct--ttc
<i>Pongo abelii</i> (orangutan)	ttccatcccaa-gaactacaa-caccc	cagaatgcatt-g-----tttttcct--ttc
<i>Callithrix jacchus</i> (marmoset)	tcca--cccaa-gaactacaa-caccc	cagaatgcatt-g-----tttt-cct--ttc
<i>Mus musculus</i> (mouse)	gcgt--ctcta-gaactacaa-caccc	cagaatgcact-g-----tttt-tcc--ttc
<i>Rattus norvegicus</i> (rat)	gcta--cccta-aaactacaa-caccc	cagaatgcact-g-----tttt-tcc--ttc
<i>Equus caballus</i> (horse)	gcca--cccaa-gaactacaa-taccc	cagaatgcact-g-----tttt-ccc--ttc
<i>Canis familiaris</i> (dog)	gcca--cccac-gaactacaa-tatcc	cagaatgcatt-g-----tttt-tcc--ttc
<i>Pteropus vampyrus</i> (megabat)	gcca--cccaa-ggactacaa-taccc	cagaatgcact-----tt-tcc---c
<i>Dasypus novemcinctus</i> (armadillo)	tcaa--cca-atgaactacaa-taccc	cagaatgcact-a-----tttt-cct--ttc
<i>Monodelphis domestica</i> (opossum)	tcct--gccac-gaactacaa-atccc	cagaatgcatt-g-----cgct-ccc---g
<i>Anolis carolinensis</i> (lizard)	-----a-gaactacag-caacc	cacaatgcact-gcaatatggt-cac--atc
<i>Gallus gallus</i> (chicken)	=====	=====
<i>Xenopus tropicalis</i> (X. tropicalis)	=====	=====
<i>Dani rerio</i> (zebrafish)		

Base c.-98 and its corresponding location in other species is highlighted