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The functional effect of pathogenic mutations in Rab escort protein 1

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Abstract

Choroideremia (CHM) is a chorioretinal degeneration with an X-linked pattern of inheritance. Affected males experience progressive atrophy of the choroid, retinal pigment epithelium and retina leading to eventual blindness. The *CHM* gene encodes Rab escort protein 1 (REP-1). REP-1 is involved in trafficking of Rab proteins in the cell. To date, the majority of reported mutations in the *CHM* gene cause a complete loss of REP-1 function. Here we report pathogenic mutations: a novel missense mutation, L550P; a truncation c.1542T>A, STOP; and two deletions (c.525_526delAG, c.1646delC) in the *CHM* gene and their phenotypic effect. To analyze the effect of mutations, the 3D structure of human REP-1 and the proteins associated with REP-1 function were modeled using sequence homology with rat proteins. *In silico* analysis of the missense mutation L550P suggests that the proline residue at position 550 destabilizes the β -structural elements, and the REP-1 tertiary structure. Truncation and deletion mutants are associated with a partial or total loss of the REP-1 essential activity and protein-protein interactions as predicted by the analysis of the structure and stability of these protein products. The presumptive loss of protein was confirmed by Western Blot analysis of protein from mononuclear cells and fibroblasts (FB) from CHM patients.

Keywords

Choroideremia; Rab escort protein 1; missense mutation; structural mechanism; protein destabilization

1. Introduction

CHM is a chorioretinal degeneration with an X-linked pattern of inheritance. Affected males experience progressive atrophy of the choroid, retinal pigment epithelium and retina leading to eventual blindness. The *CHM* gene encodes Rab escort protein 1 (REP-1). Molecular cloning of REP-1 revealed identity with the human *CHM* gene, an X-linked gene that when mutated results in a form of retinal degenerative disease [1–3]. To date, the majority of reported mutations in the *CHM* gene cause a complete loss of REP-1 protein function [4,5]. REP is

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Conflict of interest

The authors declare that there are no conflicts of interest.

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required ubiquitously and the absence of REP function could affect the role of Rab proteins in isoprenylation cycle. Rabs are *ras*-related GTPases that play a key role in the regulation of intracellular membrane transport by controlling the main steps of trafficking in secretory and endocytic pathways [6–8]. Like many other regulatory GTPases, Rabs are modified at their C-terminus in the cytoplasm by lipids, in many cases, two 20-carbon geranylgeranyl isoprenoids.

Rab geranylgeranyl transferase (RabGGTase) is a catalytic heterodimer primarily responsible for the post-translational modification of Rab proteins, and is composed of tightly associated α and β subunits of 60 and 38 kDa, respectively [1,9,10]. RabGGTase catalyzes the transfer of two isoprene lipids to two closely spaced cysteine residues at the Rab C-terminus using thioether bonds [10]. REP-1 acts as a component of the protein complex. There are two similar Rab escort proteins (75% sequence identity): REP-1 and REP-2. Mutational changes in REP-1 cause a functional deficiency of RabGGTase activity in extracts from lymphoblastoid cell lines from patients with CHM [2]. According to the current view, the function of geranylgeranylation is assured by homologous REP-2 in patients with CHM, and most cells will function properly with adequate REP-2.

Recently protein complexes of rat Rab7:rep-1, RabGGTase:rep-1, and yeast Rab:RabGDI were crystallized and an atomic structure of these proteins was determined using protein crystallography [11–13]. The structural mechanisms for recycling of Rabs between membrane compartments were proposed by Goody and colleagues [14]. These and other studies imply a complex mechanism when upon prenylation, rep-1 removes Rab proteins from the catalytic site of RabGGTase and delivers them to an acceptor protein, RabGDP dissociation inhibitor (RabGDI), mediating membrane association of prenylated Rab proteins [3]. When the prenylation reaction is reconstituted *in vitro* using purified components, rep-1 forms a complex with rab proteins, suggesting the following model: rep-1 binds newly synthesized Rab proteins and presents them to the catalytic site of RabGGTase.

Here we report novel pathogenic mutations in the *CHM* gene: a missense mutation L550P, a truncation c.1542T>A, STOP, and two deletions (c.525_526delAG, c.1646delC). To analyze the effect of mutations, the 3D structure of human REP-1 and proteins associated with REP-1 function were modeled using sequence homology with rat proteins. *In silico* analysis of the role of the missense mutation L550P suggests that the proline residue at position 550 destabilizes the β -structural elements and tertiary REP-1 structure. All mutants reported in this study are associated with a partial or total loss of REP-1 essential activity and protein-protein interactions as predicted by the analysis of structure and stability of these protein products. The loss of protein in all cases was confirmed by Western Blot analysis of protein from nonocular cells and fibroblasts from the CHM patients.

2. Materials and Methods

2.1. Patients

Clinical data from 4 male patients with CHM (aged 44–76) are shown in the Table 1. Pedigrees of the families with the clinical diagnosis of CHM showed X-linked transmission of the chorioretinal dystrophy in both affected males and carrier females. This study was approved by the CNS Institutional Review Board of the NIH (08-E1-#0017). Informed consent was obtained from each subject participating in this study. All males affected by the mutations experience concentric widespread chorioretinal atrophy and blindness as demonstrated in Fig. 1 (Panels A–D).

2.2. Isolation of mononuclear cells from the CHM patients and immunochemistry

Mononuclear cells from patient's peripheral blood (PBMCs) were collected in heparin sodium tubes (Vacutainer CPT; BD Labware, Bedford, MA). After density gradient centrifugation, PBMCs were collected, washed and homogenized in RIPA buffer containing 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM TrisHCl, pH 7.6, and a cocktail of protease inhibitors (Complete, Roche, Indianapolis, IN).

Protein concentration in extracts was determined using BCA reagent (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were loaded and separated by SDS-PAGE using 4–12% NuPAGE Novex Bis-Tris Gels and NuPAGE MOPS SDS Running Buffer (Invitrogen, Carlsbad, CA), electro-transferred to a 0.45- μ m nitrocellulose membrane (Invitrogen, Carlsbad, CA) and probed with mouse monoclonal anti-rep-1 antibody, clone 2F1 (Santa Cruz Biotech., Santa Cruz, CA) generated against the 415 C-terminal amino acids of rep-1 [4,8]. Immunoreactivity was detected with a corresponding second anti-IgG antibody conjugated with horseradish peroxidase (Zymed Lab. Inc., San Francisco, CA) and imaged on X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.3. Primary fibroblasts cell cultures

Primary dermal human fibroblast (FB) cultures were obtained as follows. Skin biopsies were used to establish dermal fibroblast primary cell lines from the CHM patients and age-matched healthy controls. The biopsy was immediately minced using sterilized forceps in a 60 mm dish containing 10% DMEM F12 media with 2.5 mg/l antibiotic/antimycotic (Invitrogen Life Sciences, Carlsbad, CA) and incubated for 10–14 days until fibroblasts started to attach to the plate. Media was changed every 2–3 days. Cells were spilt once they reached 80 % confluence. After passage 8, cells were grown to 100% confluence and left for one week in 10% DMEM F12 media. As skin biopsies contain multiple cell types, immunocytochemistry with anti-collagen I antibodies was performed on a subset of primary cultures to confirm the type of the cells used in the study.

2.4. Genotyping

Genomic DNA was isolated by standard methods from PBMCs. Briefly, genomic DNA from specimens was PCR-amplified using pairs as previously described [5] for analysis of all 15 coding exons of the *CHM* gene and their flanking splice sites. Bi-directional sequence was obtained and DNA sequence was analyzed and compared to the published gene sequence. The missense mutation L550P was not found in 89 chromosomes from a normal Caucasian population by using a TaqMan MGB allelic discrimination assay (Applied Biosystems). The TaqMan MGB genotyping custom assay was designed as follows. The DNA sequence of exon 14 containing the single nucleotide substitution (T>C) causing the L550P missense mutation was located and a small 162 bp subsequence containing the mutation site was selected as a context sequence for the TaqMan Minor Groove Binding (MGB) genotyping assay design by Applied Biosystems (Foster City, CA, USA). The context sequence was then screened for repeats and low complexity sequences via the repeat Masker website at <http://www.repeatmasker.org/>. Once it was determined that no repeats or low complexity sequences were found in the context sequence, it was passed to the Custom TaqMan Genomic Assays File Builder (version 3.1) software utility of Applied Biosystems. The location of the single nucleotide substitution was annotated in the sequence and an assay file was created and submitted to Applied Biosystems for design, synthesis, and functional testing. The designed forward and reverse primers and wild-type/mutant allele-specific probes for the custom genotyping assay are shown in Table 2. The primer sequences were also confirmed to align with the *CHM* gene in the specific location by using the *in-silico* PCR utility at the UCSC Genome Browser Website (<http://genome.ucsc.edu/>).

A set of 89 normal human male genomic DNA samples was subdivided into eight groups of 10 samples and a single group of nine samples in a pseudorandom fashion. DNA concentration and A260/A280 ratios were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). All DNA samples had concentrations greater than 1 ng/μL and a 260/280 of 1.5 or greater. All PCR reactions were setup using the Applied Biosystems TaqMan Genotyping Master Mix in 384-well optical reaction plates on the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis was performed on the computer controlling the 7900HT Sequence Detection System and the results were exported as a text file.

2.5. Homology modeling of human REP-1

Modeling of the human REP-1 was carried out by homology modeling based on 2.2 Å crystal coordinates for rat rep-1 protein in a complex with monophenylated Rab7 protein (PDB file: 1vg0) as the structural template [15]. REP-1 has a sequence identity of 66% to rat rep-1, covering a broader sequence range including sequence fragments which were not resolved in the atomic structure of rep-1 from electron density maps: residues 1–2, peptide 64–73, peptide fragment 107–210 and C-terminal fragment 607–650. Primary sequences were aligned by the method of Needleman and Wunsch [16], incorporated in the program Look, version 3.5.2 [17,18] for 3-dimensional structure prediction. Finally, full-length monomeric REP-1 and changes in protein structure corresponding to gene mutations: c.525_526delAG; c.1542T>A, STOP and c.1646delC, were built by the automatic segment matching method in the Look program [19] followed by 500 cycles of energy minimization. In the full-length structure of REP-1, the elements of structure corresponding to the unresolved fragments of rep-1 were generated in a conformation which has not been justified experimentally in order to show the possible location and structural role of these fragments in REP-1. The same program generated the conformation of protein with the L550P missense mutation and refined this structure by 500 cycles of self-consistent ensemble optimization [18].

The Impact module of the Maestro molecular modeling environment, version 8.0.308 (Schrodinger, LLC, NY) was used to evaluate the effect of the missense mutation. The predicted structures of the second domain of REP-1, in the absence and the presence of L550P mutation, were regularized by an energy minimization of 100 cycles of steepest descent and 200 cycles of conjugated gradient in the presence of water. Finally, REP-1 structure was equilibrated using 3ps molecular dynamics (2 fs step). The geometry of the predicted structures was tested with the Procheck program [20].

Structures of human REP-1, Rab7 and the Rab geranylgeranyl transferase (Rab GGTase) were generated as described above using the corresponding rat structures as structural templates (PDB files: 1vg0 and 1ltx) and were docked together to form a hypothetical oligomeric complex of human proteins.

3. Results

3.1. Genotyping

The individual mutations in the *CHM* gene found in the 4 patients presented in this work are shown in the Table 1. A total of 89 genomic DNA samples from normal subjects without CHM were screened along with the CHM positive patient DNA sample containing the missense single nucleotide substitution (T>C) located in exon 14 using a custom TaqMan minor groove binding (MGB) genotyping assay from Applied Biosystems (Foster City, CA, USA). This custom assay was developed to accurately detect either the wild-type allele (T) or the mutant allele (C) sequence in a genomic DNA sample. All of the 89 screened genomic DNA samples were determined to contain only the wild-type (T) allele. As an additional control, the CHM

patient DNA sample was included with each group of non-CHM patient DNA samples and was consistently called as the mutant allele (C).

3.2. Possible effects of mutations on the REP-1 structure

The human REP-1 structure based on homology modeling is shown in Figure 2. Structures of human (red) and rat (grey) rep-1 proteins are superimposed according to the sequence alignment (Fig. 2D). The globular domain of human REP-1 comprises 3 structural domains containing 21 helices and β -structural segments (β -strands), forming 3 major β -sheets in the protein similar to that of the rat rep-1 [11,12]. Two large fragments of rat rep-1 sequence of about 100 residues located in the domain 2 and last 50 residues, shown by the orange line in Fig. 2D are not visible in the electron density maps (PDB: 1vg0, 1ltx), suggesting that these residues do not have a defined position. In the predicted structure of human REP-1 however, the elements of structure corresponding to the unresolved fragments of rat rep-1 (residues 107–210 and 607–650) were generated in a conformation which had not been justified experimentally, in order to understand their possible location and structural role. Although residues 107–210 shown partially by the orange line in Fig. 2A on the right seem not to have a well defined conformation, the last 50 residues start from the poly-proline PPPPxP motif (Fig. 2B) and restrict the orientation of C-terminus to a limited number of conformations.

Possible effects of the REP-1 mutations based on the model of protein structure described above are shown in Table 3 and Figures 3. The most significant changes are those in which large parts of the molecule are absent. The first pathogenic mutation, c.1542T>A, STOP, is in exon 13 of the gene and would be expected to truncate the peptide just within the globular domain (Fig. 3B), removing about 150 amino acids from the C-terminus and changing a possible interaction with Rab-protein. The two other pathogenic mutations are a 2-bp deletion in exon 5 (c.525_526delAG) and a 1-bp deletion in exon 14 (c.1646delC). The first deletion eliminates 473 residues of the protein structure, thus disrupting essential rep-1 activity (Fig. 3A). The second deletion results in loss of almost 100 amino acids (Fig. 3C) and should also cause the loss of interaction with Rab protein. Overall, all 3 pathogenic changes remove large fragments of protein structure and significantly change conformational stability and an essential activity of REP-1.

A novel missense mutation c. 1679T>C (p. L550P) in the *CHM* gene was found in patients with CHM (Fig. 3 Panel D). The mother is heterozygous for the mutation (top tracing), an unaffected brother does not carry the mutation (middle tracing), and the proband has the mutation (bottom). In addition, the proband's affected brother carries the same mutation (data not shown). The mutation, L550P, although not as remarkable as the removal of large fragments of the polypeptide chain, seems highly prone to affect stability of REP-1. Indeed, the leucine residue L550 is conserved in at least 4 species (Fig. 3H) and the mutation L550P has a negative Blosum70 score (–3) suggesting that the leucine residue change to proline is relatively rare. The L550P mutation in the REP-1 structure is shown in Fig. 2A and C. In the wild type REP-1 structure, a side chain of leucine holds the ϕ backbone dihedral angle at approximately -141.3° . Due to the mutation L550P, the distinctive cyclic structure of proline's side chain locks its ϕ backbone dihedral angle at approximately -78.3° , significantly changing the conformation of the polypeptide backbone in the vicinity of the residue. This conformational change affects a hydrogen-bonding pattern. Fragments of wild type (light grey, Fig. 3E) and L550P mutant (magenta, Fig. 3F) REP-1 are shown by three β -strands formed by amino acid residues 98–101, 454–458 and 549–551, respectively. Residues of leucine and proline located in position 550 are shown in red. The hydrogen-bonds are shown by blue lines connecting the corresponding donor-acceptor pairs of atoms in the polypeptide chains. A conformation change due to the L550P mutation induces the loss of a single hydrogen bond stabilizing an interaction between two β -strands (Fig. 3F). Currently we are not able to do molecular dynamics

simulations for the whole REP-1 structure. However our molecular dynamics 3 ps simulations (data not shown), obtained for the REP-1 domain 2 in water, demonstrated more pronounced effect of the L550P mutation linked to the further loss of hydrogen bonds within domain 2.

In conclusion, our analysis of structural data suggests that the mutation L550P could affect the stability of the β -sheet and a whole protein molecule and, finally, disrupt its structural integrity. This is consistent with the literature on the ability of proline residues to disrupt regular secondary structural elements such as α -helices and β -sheets [21]. Overall, the destabilizing effect of the missense mutation L550P could change the native fold of REP-1 molecule and we might expect that the unfolded protein to be digested by the cell's proteolytic system.

3.3. Western Blot analysis of the rep-1 expression

PBMCs and FB cultures were used as cellular models of CHM. Western blot analysis of protein from PBMCs from normal controls and CHM patients is shown in Fig. 3G. Protein bands, demonstrating REP-1 protein expression in samples from normal patients, are labeled as Control 1 and Control 2. In contrast, proteins from CHM patients: deletions c.1646delC, c.525_526delAG (Panel H) and the truncation c.1542T>A, STOP (Panel G) show no REP-1 band. No detectable REP-1 expression (Panel G: lane2) in PBMCs or very low expression (Panel G: lane 5) in skin FB was also seen for the missense mutation L550P. Thus the absence of a detectable REP-1 band or a very low level of protein expression agrees with results of molecular modeling, suggesting a partial or complete loss of structural stability for the L550P mutant protein.

4. Discussion

Here we report four pathogenic mutations in the *CHM* gene: a novel missense mutation L550P, truncation c.1542T>A, STOP, deletions c.525_526delAG, c.1646delC and phenotypic data associated with CHM. To analyze the effect of mutations, the 3D structure of REP-1 and proteins associated with REP-1 function were modeled using the sequence homology with rat proteins. *In silico* analysis of the role of the missense mutation L550P demonstrated that the proline residue at position 550 destabilizes the β -structural elements, and hence REP-1 structure. This mutation results in a unfolded protein product that is rapidly degraded and thus relatively absent in the affected male in a way similar to that of truncation and deletion mutants [4,22]. Presented in this work, truncation and deletion mutants are associated with a partial or total loss of the REP-1 essential activity and protein-protein interactions as predicted by the analysis of structure and stability of these protein products. The partial or full loss of protein is confirmed by the Western blot analysis of protein from mononuclear cells and FB from the CHM patients. With the exception of the L550P mutation and that reported by Garcia-Hoyos et al. (2008), most currently known CHM mutations are linked to complete degradation of the REP-1 protein product and exclude REP-1 from isoprenylation cycle. In the latter example, alternate splicing resolved in a small amount of REP-1 of normal size.

For the missense mutation L550P, no REP-1 expression was detected in PBMCs or very low expression in skin FB as demonstrated by Western blots (Fig. 3, Panel G). In this work we employed mouse monoclonal anti-rep-1 antibody, clone 2F1, generated against the 415 C-terminal amino acids of rep-1, which includes the residue 550. Thus, although the absence of a detectable REP-1 band or a very low level of protein expression agrees with results of molecular modeling and suggests a partial or complete loss of structural stability for the L550P mutant protein, there is still the possibility that missense mutation at position 550 might affect the affinity for the monoclonal antibody. The precise location in the protein sequence of the epitope recognized by the antibody is not known (Santa Cruz, private communication). However it is very well known that epitopes recognized by antibodies are formed either by stretches hydrophilic residues (linear epitopes) or by hydrophilic residues located at the protein

surface (conformational epitopes). According to our structural model and to the structure of rat rep-1, the residue 550 is hydrophobic in both, wild type or mutant protein (Leu or Pro), and included in the consecutive patch of seven hydrophobic residues (546 ILWALYF 552). In addition, the amino acid 550 is buried in Rep-1 structure and therefore inaccessible for interaction with other molecules. Thus, for these reasons, the structural change due to mutation L550P should not affect the monoclonal antibody binding. We obtained similar results by using a polyclonal anti-REP-1 antibody raised against N-terminal amino acids 50–100 (clone N-17, Santa Cruz, CA). Although this antibody demonstrated less sensitivity, immunoblot analysis showed the complete absence of a REP-1 band in protein from a primary FB culture of the patient with the L550P mutation (data not shown). This suggests that the observed low level of protein expression might relate to a partial or complete loss of structural stability of REP-1 with the L550P mutation rather a result of changing the C-terminal antibody binding activity.

The results of our findings and literature on truncation mutants are summarized in Figure 4. Domains 1 and 2 (red and blue color) are involved in interactions with Rab-7 [11,12]. All mutations presented in our work and the majority of truncations of protein occurred in areas located in these two domains suggesting that domains 1 and 2 could be important for REP-1 function. In addition, all currently known truncations appear to be before the amino acid 600 leading to the loss of the poly-P motif and the rest of C-terminal sequence. This suggests that the REP-1 C-terminal fragment (last 50 residues) is significant functionally. This conclusion is in agreement with results on recombinant rat rep-1 protein that lacks the 70 C-terminal amino acids and is unable to assist in the geranylgeranylation of Rab proteins [23].

The loss of mutant REP-1 as demonstrated in Fig. 3G might be related to the loss of corresponding mRNA due to nonsense mediated decay, a cellular mechanism required to detect nonsense mutations and prevent the expression of truncated or erroneous protein [24]. In our preliminary experiments we used real-time PCR (TaqManMGB kit from ABI Biosystems) in order to evaluate levels of mRNA expression. The levels of mRNA expression (normalized to 18sRNA) as quantified by the ΔC_t threshold were 18.52, 20.35 and 16.99 for primary FB cultures of c.1542T>A, STOP; c.1646delC and p. L550P mutations, respectively. Comparable ΔC_t values were found for mRNA expression in primary FB cultures of 3 normal controls: 16.11, 17.55 and 17.32. These results suggest that levels of REP-1 mRNA expression in primary FB cultures from three of four genetically affected individuals (lanes 2–4, Table 3) are similar to 3 age-matched normal controls. Therefore, although the nonsense mediated decay could be a possible scenario for other mutations, for the mutations described herein we predict the major effect of mutation is on REP-1 function likely thorough protein destabilization or misfolding. This conclusion is consistent with the recent observation [25] that loss of REP-1 protein occurs rather than nonsense mediated decay.

There are several mechanisms leading to misfolded protein degradation. About 30% of cytosolic proteins carry KFERQ-targeting motifs in their sequences allowing a selective targeting of cytosolic proteins to the lysosome for degradation by the chaperone-mediated autophagy [26]. The absence of the KFERQ peptide in the REP sequence proposes a further option. Accordingly, misfolded or unassembled proteins are retained in the endoplasmic reticulum (ER) bound to chaperones or lectins until they are delivered to the cytosol for degradation in the ubiquitin-proteasome pathway [27]. Although the most significant structural changes affecting protein stability are expected for mutant proteins missing large fragments of structure (Table 3, Fig. 3), even for the mutation L550P, we predict the loss of stability. Indeed, in addition to the hydrogen-bonding pattern change due to the L550P mutation, we estimate a Gibbs free energy change due to the mutation of 23.22 kJ/mol by using the FoldX empirical force field [28]. This energy could be sufficient to unfold the single protein domain requiring >15 kJ/mol [29]. Thus, the mutant protein, L550P, is expected to have a markedly perturbed protein fold and together with other three mutants (Table 1), is predicted to be misfolded in

the ER and later digested in proteasome. This agrees with an observation, that mutant proteins with significant changes in protein fold (key residues or deletions) could generate proteins susceptible to hydrolysis similar to that of human haemoglobin in which about one-fifth of hundreds of missense proteins undergo rapid degradation [27].

Abbreviations

REP-1 and rep-1

Rab escort protein 1 from human and rat, respectively

FB

fibroblasts

PBMCs

peripheral blood mononuclear cells

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Figure 1.

Fundus photographs of CHM males showing extensive chorioretinal degeneration. Panels from left to right: Panel A: CHM c.1542T>A, STOP (76 year old male); Panel B: c.1646delC (49 year old male); Panel C: c.525_526delAG deletions (44 year old male) and Panel D: missense L550P mutation (44 year old male).

Figure 2.

The structure of human REP-1 is modeled by homology to rat protein. The superposition of rep-1 structures from rat (gray) and human (red) and the sequence alignment are shown on the Panel A and on Panel D, respectively. Panels B and C show the model of C-terminal extension connected through the 4 proline motif (Poly-P) to the rest of REP-1 and the location of the mutation L550P in the human protein, respectively. Rab-7 protein structure is shown in forest green. The side chain of residue L550 is shown by yellow (Panel C). Two fragments of protein sequence (part of loop 106–213 and C-terminal extension 557–653), which do not have a definitive structure in the rat (PDB file: 1vg0), are shown by orange (Panels A and D). Rat rep-1 ligands (geranylgeranyl and GDP) and metal ions are shown by cyan ball and stick models.

Figure 3.

Analysis of structural changes in REP-1 caused by genetic mutations in CHM patients is presented. The expected protein products and the parts of polypeptide chain removed from the protein due to mutation are shown by red and yellow, respectively. The last residues at the C-termini of expected protein products are represented by blue space-filling models. Mutations c.525_526delAG (Panel A), c.1512T>A, STOP (Panel B) and c.1646delC (Panel C) are shown respectively from left to right. Sequence tracings of partial fragments of the *REP-1* gene are shown on Panel D. The mother is a heterozygous for the missense mutation c. 1679T>C (p. L550P) in the *REP-1* gene (top), an unaffected brother does not carry the mutation (middle), and the proband has the mutation (bottom). Mutation sites are indicated by arrows. The β -sheet fragments of wild type (Panel E) and L550P mutant (Panel F) rep-1 formed by amino acid residues 98–101, 454–458 and 549–551 are shown by light grey and magenta, respectively. Leucine (Panel E) and proline (Panel F) residues located in position 550 are shown in red. The hydrogen-bonding pattern is shown by blue lines connecting the corresponding donor-acceptor pairs of atoms in polypeptide chains. Western blots of the monocyte fractions (CD14+) or dermal fibroblasts from the CHM patients are shown in Panel G. Lanes from c1 to c5 are controls. REP-1 protein bands for PMBCs in lanes from 1 to 4 correspond to Ex13 c.1542T>A, STOP; Ex14-L550P; Ex14 c.1646delC and Ex5 c525_526delAG mutations, respectively. Western blot for REP-1 from dermal FB for the Ex14-L550P mutation is shown in lane 5. The alignment of conserved parts of rep-1 sequences from different species is demonstrated in the Panel H. The position of the amino acid residue L550 is shown by an arrow.

Figure 4.

CHM-linked mutations affect the structure and function of REP-1. Three structural domains of REP-1 are shown in red (domain 1: residues 1–92, 370–446, 554–653), blue (domain 2: residues 93–270 and 447–553) and forest green (domain 3: residues 271–369), respectively. The missing crystal structure of rat protein fragments of loop 106–213 and the C-terminal extension 557–653 are indicated by dotted lines. The structures of Rab-7, RabGGTα and RabGGTβ are represented by forest green, purple and magenta, respectively. Color bars of corresponding colors located above the diagram show the domain structure and schematically represent areas involved in protein-protein interactions. Divided into fragments, the blue ribbon represents the CHM gene structure and its corresponding exon borders. CHM-linked mutations causing truncations of rep-1 from the Retina International Mutation Database (<http://www.retina-international.com/sci-news/repmut.htm>) and from the review [31] are shown in black letters. Mutations described in this work are shown in blue.

Table 1

Choroideremia phenotype

Mutation	Age	Refraction	Vision	Field	ERG
c.1542T>A, STOP	76	-	NLP*	-	-
c.1646delC ⁺	49	-	NLP	-	-
		OD -2.5+0.75 ×110	20/40-3	3 degrees, III4e, with 40 degree temporal island	2μV flicker [£]
c.525_526delAG [†]	44	OS -2.75 +1.00×90	20/63-1	3 degrees, III4e with inferior island	2μV flicker [£]
		OD -6.00+2.00×70	20/63	10 degrees, V4e	-
p. L550P	49	OS -7.25+3.00×95	20/25	5 degrees, V4e	-
		OD -10+1.25×165	20/50-2	5 degrees, III4e	NR
		OS -11+2.75×180	20/100-2	3 degrees, III4e	NR

* No light perception.

⁺ Reported in CNTF trial, NR: not recordable,

[£] photopic flicker response.

[†] IV-10 in Pedigree J of Sieving PA, Niffenegger BA and Berson EL [30].

Table 2

TaqMan genotyping PCR assay primer and probe sequences

Assay Component:	Sequence:
Forward Primer	TGAACAAGTAGAAAAGCCAAGAATTCTGT
Reverse Primer	CTGCTGATGTCTGACGAATCTCT
VIC Labeled Wild-Type (T) Probe Sequence:	ATATTGAAGTAAAGAGCCC
FAM Labeled Mutant (C) Probe Sequence:	TTGAAGTAAGGAGCCC

Table 3
Mutations observed in CHM patients and their expected effect on REP-1 structure

No	Exon	Gene mutation	Protein product	Expected molecular mass, kDa	Location in the structure and the predicted role of mutant protein
1	5	c.525_526delAG	G175KRKP	20	Rep-1 sequence ends at G175 followed by the tetra-peptide KRKP at the C-terminus (residues 176–79)
2	13	c.1542T>A,STOP	Y504X	57	Protein chain is truncated after Y504
3	14	p/leu550pro	L550P	73	Missense mutation (Blossum70 score is -3) affecting protein stability by breaking the hydrogen bonding pattern of β -sheet formed by residues 98–101, 454–458 and 549–551
4	14	c.1646delC	W548VFTSI	63	REP-1 sequence ends at the W548 followed by the penta-peptide VFTSI at the C-terminus (residues 549–553)