Consequences of mutations in the non-coding *RMRP* RNA in cartilage-hair hypoplasia

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Cartilage-hair hypoplasia (CHH), also known as metaphyseal chondrodysplasia McKusick type (OMIM no. 250250), is an autosomal recessive, multi-systemic disease characterized by disproportionate short stature, fine and sparse hair, deficient cellular immunity and a predisposition to malignancy. It is caused by mutations in *RMRP*, the RNA component of the ribonucleoprotein complex RNase MRP, and, thus, CHH represents one of few Mendelian disorders caused by mutations in a nuclear encoded, non-coding RNA. While studies in yeast indicate that *RMRP* contributes to diverse cellular functions, the pathogenesis of the human condition is unknown. Studies of our CHH patient cohort revealed mutations in both the promoter and the transcribed region of *RMRP*. While mutations in the promoter abolished transcription *in vitro*, *RMRP* RNA levels in patients with transcribed mutations were also decreased suggesting an unstable RNA. *RMRP* mutations introduced into the yeast ortholog, *NME1*, exhibited normal mitochondrial function, chromosomal segregation and cell cycle progression, while a CHH fibroblast cell line exhibited normal mitochondrial content. However, the most commonly found mutation in CHH patients, 70A>G, caused an alteration in ribosomal processing by altering the ratio of the short versus the long form of the 5.8S rRNA in yeast. Transcriptional profiling of CHH patient RNAs showed upregulation of several cytokines and cell cycle regulatory genes, one of which has been implicated in chondrocyte hypertrophy. These data suggest that alteration of ribosomal processing in CHH is associated with altered cytokine signalling and cell cycle progression in terminally differentiating cells in the lymphocytic and chondrocytic cell lineages.

INTRODUCTION

Cartilage-hair hypoplasia (CHH), also known as metaphyseal chondrodysplasia McKusick type (OMIM no. 250250), was first described in the Amish by Victor McKusick (1). It is an autosomal recessive disorder characterized by skeletal involvement with short stature, together with variable features like blond fine sparse hair, and defective cellular immunity affecting T-cell mediated responses (2). Patients may have severe combined immunodeficiency requiring bone marrow transplantation or they may be asymptomatic (3,4). Gastrointestinal dysfunction (5) such as malabsorption or

Hirschsprung's disease is frequently observed (6). A predisposition to certain cancers, primarily lymphomas, has been reported as well (2,7). Shortening of tubular bones is evident at birth, with the metaphyses widened, scalloped and irregularly sclerotic. These metaphyseal changes are usually more severe in the knee region than in the proximal femora helping to distinguish CHH from other metaphyseal chondrodysplasias (8). The incidence of CHH in the Amish is 1.5 in 1000 births; and in Finland, it is 1 in 18 000–23 000 live births. CHH was mapped to 9p13 by linkage analysis (9) and Ridanpaa *et al*. (10) found causative mutations in the *RMRP* gene (OMIM no. 157660) in most of the CHH cases

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studied. The worldwide emerging mutation spectrum in CHH includes the major 70A>G transition mutation with an ancient founder origin established in Finland (11–14).

RMRP is the RNA component of the RNase MRP (ribonuclease mitochondrial RNA processing) complex. It is an untranslated, intronless gene transcribed by the DNA dependent RNA polymerase III (RNA PolIII). The human *RMRP* transcript is 267 bases long and the promoter region contains several putative promoter elements, a TATA box, a proximal sequence element (PSE), a SP1 and oct-1 binding elements. At the 3′ end is a RNA PolIII stop signal with a run of five deoxythymidines. It is encoded in the nucleus (15) but the complex is localized primarily in the nucleolus and to a lesser extent in the mitochondria (16,17). The sequence of the *RMRP* transcript is highly conserved among a variety of different species, including human, mouse, rat, cow, *Xenopus*, yeast, *Arabidopsis* and tobacco (18). The length of the transcript varies among different species. Secondary structure models for RMRP reveal a complex structure, the core of which is required for assembly and function of the ribonucleoprotein complex (19,20). So far, in humans, ten proteins have been identified as part of this ribonucleoprotein complex (21).

In *Saccharomyces cerevisiae*, the *RMRP* ortholog *NME1* (for *nuclear mitochondrial endonuclease 1*) is an essential gene required for viability (22). Extensive characterization of the gene has revealed that deletion of the least conserved portion (nt 186–211 forming the P8 hairpin) has no phenotypic effect (23). Studies in yeast have attributed multiple functions to this ribonucleoprotein complex. It is involved in mitochondrial DNA replication by cleaving the RNA that primes mitochondrial DNA replication (15,24). Some *nme1* mutants exhibit a delay in the progression of the cell cycle at the end of mitosis in association with morphological changes. These mutants arrest in the late cycle of mitosis as large budded cells with dumbbell-shaped nuclei and extended spindles (25). One reason for the cell cycle delay in these mutants might be the increased level of *CLB2* (B-type cyclin) mRNA. Normally, the RNase MRP complex cleaves the 5′ UTR of *CLB2* mRNA. That in turn causes a rapid degradation of *CLB2* mRNA and efficient cell cycle progression (26). RNase MRP also plays a role in processing of ribosomal RNAs (27,28). In yeast, it cleaves pre-ribosomal RNA at the A_3-site and promotes the production of the short form of the 5.8S rRNA. In addition to these multiple cellular roles, the functional analysis of the RNase MRP endoribonuclease is further complicated by the fact that eight proteins of the complex are shared with a related ribonucleoprotein, called RNase P. RNase P is also a endoribonuclease but it is mainly involved in tRNA precursors maturation (29). In yeast, two RNase MRP specific proteins have been identified, Snm1 (30) and most recently Rmp1 (31).

While mutations in RNAs can cause disease, this has been primarily restricted to mitochondrial disorders. An exception is the *human telomerase RNA* (*hTR*). Mutations affecting this transcript are responsible for the rare autosomal dominant form of dyskeratosis congenita (CD) (32). CD is a progressive bone marrow failure syndrome that is characterized by abnormal skin pigmentation, nail dystrophy and mucosal leukoplakia.

As *RMRP* is not translated into a protein, it is not obvious how mutations may affect its putative catalytic action as part of a larger ribonucleoprotein complex or in as yet uncharacterized functions. To answer this question, we have analyzed the expression pattern of *RMRP* during development and the effect of *RMRP* promoter mutations on RNA PolIII transcription. Furthermore, we analyzed the effects of base pair substitutions found in CHH patients that were localized in conserved regions of the RNA using *S. cerevisiae* as a model organism. Finally, we have correlated the findings of these experiments in humans by transcriptional profiling of CHH patient RNAs.

RESULTS

RMRP is ubiquitously expressed during development

RMRP is ubiquitously expressed in *Xenopus laevis* throughout development. It is more highly expressed in those tissues containing large numbers of mitochondria (33). To determine whether the expression pattern of *RMRP* correlated with the distribution of affected tissues in CHH patients, we determined the temporal spatial pattern of *RMRP* expression during mouse development. We found that *Rmrp* is ubiquitously expressed at E10.5, E11.5, E12.5, E13.5, E14.5 and E17.5 during mouse embryogenesis by RNA *in situ* hybridization. Interestingly, *Rmrp* is more strongly expressed in the hypertrophic chondrocytes and pericondrium than in the zone of proliferating chondrocytes, correlating with the primary feature in human CHH as a metaphyseal dysplasia (Fig. 1A) (data not shown). *RMRP* is also ubiquitously expressed in all human adult tissues tested, though less so in skin and pancreas when compared with the *GAPDH* expression pattern that served as a RNA loading control (Fig. 1B).

RMRP promoter duplications result in reduced expression

In vitro transcription studies have shown that an 84 bp 5′ flanking region of the human *RMRP* promoter sequence is sufficient for *RMRP* transcription. When this 84 bp fragment and a 737 bp upstream sequence were injected into frog oocytes, the 737 bp promoter showed a stronger transcription efficiency than the 84 bp promoter *in vivo*, whereas no difference was observed *in vitro* (34).

To elucidate the effect of the *RMRP* promoter duplication found in CHH patients on *RMRP* transcription, the human *RMRP* promoter was further characterized in transfection studies *in vitro*. In RNA PolIII promoters, the TATA box is usually located at a fixed distance downstream of the PSE element (35–38). Because, promoters of RNA PolIII transcribed genes are usually very short, e.g. the human U6 promoter is 265 bp long (39) and the mouse U6 promoter 355 bp long (40), we studied two putative *RMRP* promoter sequences 352 and 841 bp in length (Fig. 2B). The putative 352 bp minimal promoter contains the TATA signal, PSE, oct-1 and the SP1 binding elements (Fig. 2A). To evaluate the strength of *RMRP* promoter variants, we inserted a short hairpin RNA (shRNA) directed against luciferase (shRNAluc) under the control of the *RMRP* promoter (RMRPshRNAluc). Hence, promoter strength can be correlated with the degree of downregulation
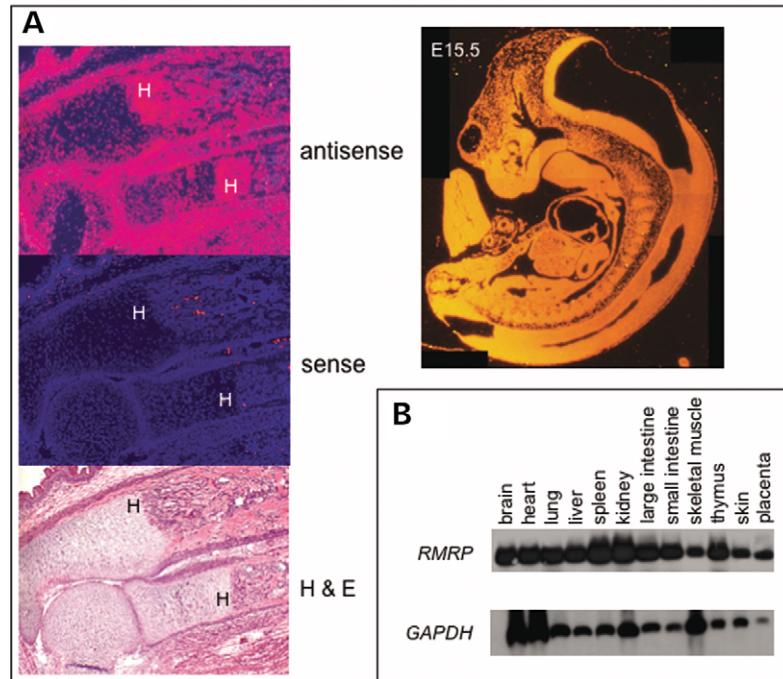
Figure 1. Expression pattern of the *Rmrp* gene. (**A**) *In situ* hybridization of an E15.5 mouse embryo. (**B**) Adult human multiple tissue northern blot. *Rmrp* is ubiquitously expressed in human and mouse. H, hypertrophic chondrocytes.

of luciferase expression in cells co-transfected with RMRPshRNAluc and a luciferase expression plasmid. The U6 promoter driving the shRNAluc serves as a positive control for this assay (U6luc), whereas the U6 promoter (U6) or shLuc (luc) alone serve as negative controls for this assay.

As shown in Figure 2B, both *RMRP* promoters constructs tested resulted in the down-regulation of luciferase gene expression when compared with U6 promoter (U6) or the shRNAluc (luc) alone. The degree of down-regulation for each was comparable to that resulting from U6 promoter-driven shRNAluc expression. The 352 bp promoter seems to be stronger than the 841 bp promoter ($P < 0.002$). This result suggests that the 352 bp sequence upstream of the *RMRP* transcription start site is sufficient to drive RNA expression and also might be stronger than the previously described 737 bp promoter.

We next investigated the impact of the promoter duplications identified in CHH patients on *RMRP* expression in this assay. The mutant promoters of CHH patient nos 4, 12 and 16 were amplified with the same primer pair as the wild-type (wt) 352 bp *RMRP* promoter. CHH no. 4 had a -11_-25dup, CHH no.12 a -6_-25dup, and CHH no. 16 a -15_-24dup, respectively. As shown in Figure 2C, the activities of the mutant *RMRP* promoters were reduced relative to the wt (−352 bp) promoter and were associated with lower shRNA expression and higher luciferase activities. Interestingly, the promoter activity was not completely abolished, as the measured luciferase activity was still diminished relative to the U6 promoter and shRNAluc alone controls. This suggests that the promoter duplications found in CHH patients are hypomorphic alleles that lead to decreased but not abolished *RMRP* transcription *in vitro* at least as defined in this assay.

Lobo and Hernandez (37) reported that the specificity of the RNA PolIII promoter can be converted to a RNA polymerase II promoter (RNA PolII) and vice versa by alterations in the distance between the TATA box and the transcription start site or by generating a TATA box (37). To test whether the promoter duplications found in the CHH cohort might convert the RNA PolIII-specific promoter to a RNA PolII-specific promoter, the same promoters as mentioned earlier were cloned upstream of the RNA PolII transcribed luciferase reporter gene. Transfection of these constructs into cos7 and HeLa cells showed that they had no activity, suggesting that CHH promoter duplications do not convert the RNA PolIII-specific promoter into a RNA PolII-specific promoter (data not shown).

RMRP expression level in CHH patients

To determine the effects of promoter duplications as well as single base pair substitution mutations on *RMRP* transcripts *in vivo*, we performed real-time RT–PCR to analyze the expression level of *RMRP* in three CHH patients with previously described mutations and six unaffected controls. Leukocytes from CHH patient nos 2 (compound heterozygous for 89C>G; 124C>T), 8 (homozygous for 70A>G) and 16 (with a promoter duplication -23_-14dup; and a 180G>A transition) as well as from sex and ethnicity matched controls were obtained. In addition, we collected samples from CHH no. 2 almost a year later to perform a longitudinal comparison. The RNA expression levels were normalized using the constitutively expressed gene *HPRT* as a reference (Fig. 3). Interestingly, *RMRP* RNA level is decreased in all three patients tested irrespective of the nature of mutation detected
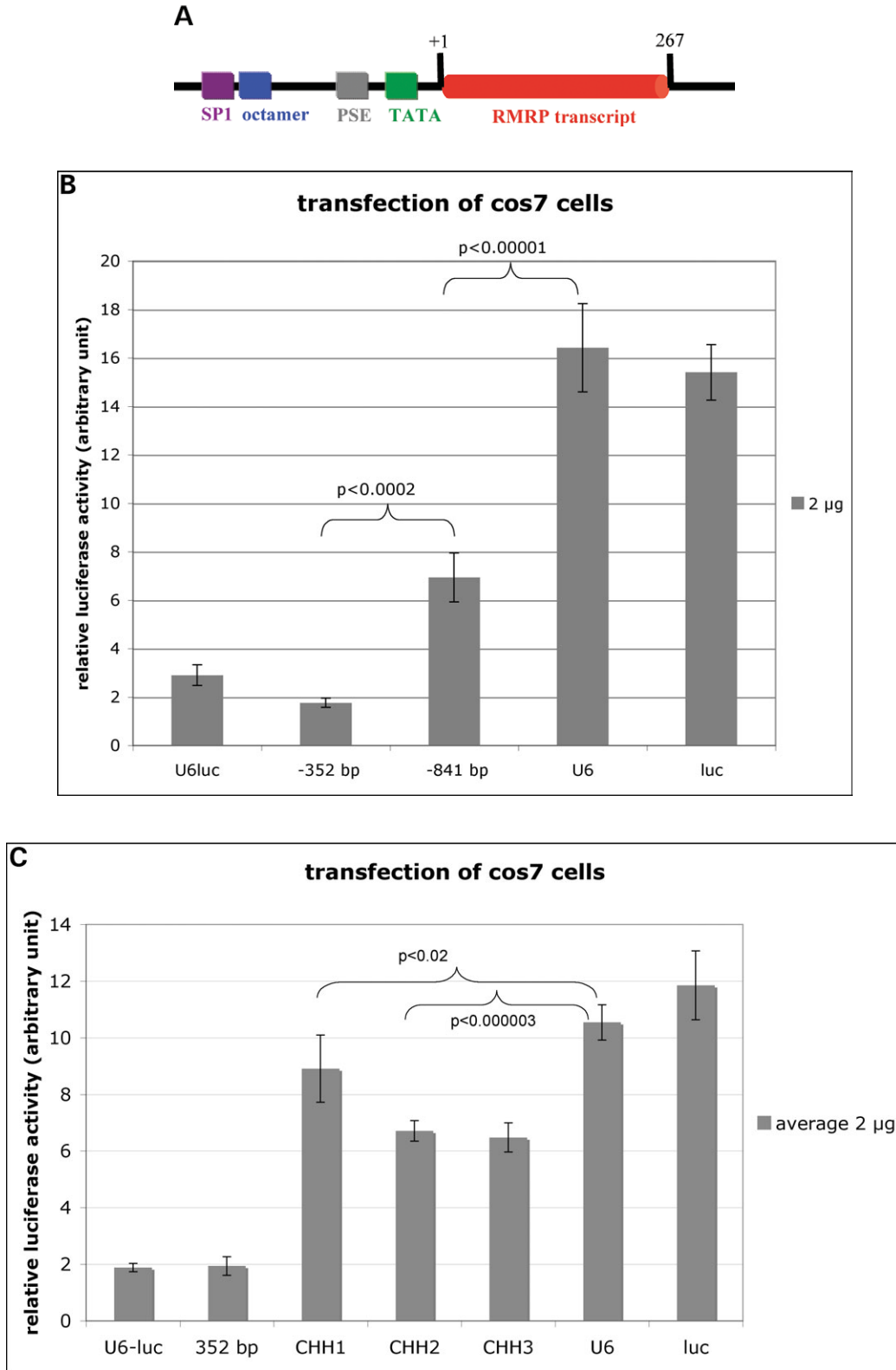
Figure 2. Human *RMRP* gene and promoter structure. (**A**) The *RMRP* transcript consists of 267 bp. The putative promoter region contains a TATA box, proximal sequence element (PSE), an Oct-1 binding site and a SP1 binding site. (**B**) The U6 promoter expressing the shRNA targeted against the luciferase gene serves as positive control. Two different lengths of the *RMRP* promoter were amplified: 352 and 841 bp. The U6 promoter without the shRNA and the shRNA without any promoter serve as negative controls and indicate the baseline luciferase activity without inhibition. The 352 bp *RMRP* promoter showed greater down-regulation of the luciferase activity than both the U6 and the 841 bp *RMRP* promoter. (**C**) The promoter activity of the mutant *RMRP* promoter is significantly decreased when compared with the *RMRP* wt promoter. The decrease in promoter activity results in an increase of luciferase activity.
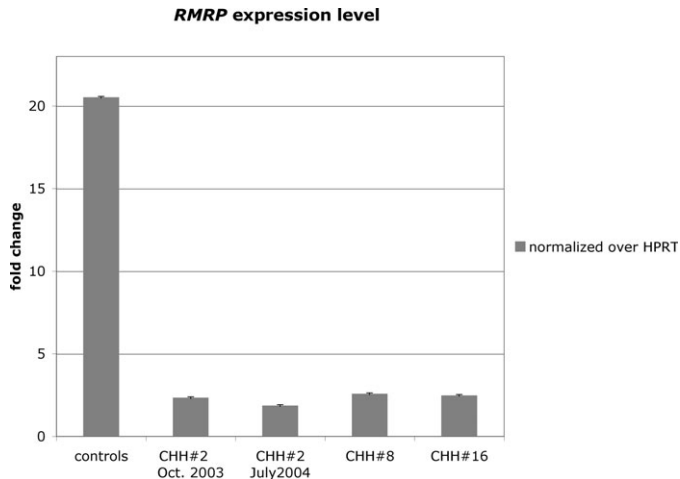
RMRP expression level

Figure 3. *RMRP* RNA expression level in two CHH patients. Quantitative real-time RT–PCR was performed on total RNA isolated from CHH patient leukocytes. From patient CHH no. 2, two samples were obtained (October 2003 and July 2004). CHH no. 2 is compound heterozygous for 89C>G and 124C>T. CHH no. 8 is homozygous for the 70A>G base pair substitution. CHH no. 16 is compound heterozygous for the promoter duplication -14_-23dup and the base pair substitution 180G>A. The *RMRP* RNA level in leukocytes is approximately 6- to 7-fold down-regulated in both patients when compared with six control samples.

in these patients. These data suggest that mutations even in the transcribed region may affect transcription efficiency and/or RNA stability *in vivo*.

The cognate 70A>G *RMRP* mutation in the yeast ortholog *NME1* alters rRNA processing

To study the functional consequences of *RMRP* mutations, we introduced mutations we found in CHH patients that were located at evolutionarily conserved positions from yeast to human into the yeast ortholog *NME1* (Fig. 4A). This included the most frequently found mutation 70A>G (yeast84A>G), as well as 124C>T (yeast168C>T), 180G>A (yeast216G>-A) and 262G>C (yeast331G>T) into the *NME1* gene (Fig. 3A and B). In addition, we introduced a 2 bp deletion mutant (yeast267_268ΔAT) as a positive control, as previous random mutagenesis studies showed that many deletion mutants exhibited altered phenotypes (23). As the yeast *NME1* gene is 72 bp longer than *RMRP*, the human mutation nomenclature was used to designate mutant strains. Figure 5 shows that the result of viability testing of haploid yeast strains bearing the mutant alleles. Each of the *nme1* point mutant alleles was able to sustain growth in the absence of wild-type *NME1*, and, therefore, these mutations did not abolish the essential function of *NME1* in yeast. In contrast, a strain bearing the 2 bp deletion had severely diminished growth in the absence of wild-type *NME1*, indicating its essential function was impaired. This supports the hypothesis that *RMRP* might be essential in humans and null alleles might be incompatible with life. Until now, no deletions of the entire *RMRP* gene have been reported in humans.

A well-described function of *NME1* is the processing of the pre-rRNA (27,28). To determine whether the human mutations

could affect this function, we examined the steady state levels of the 5.8S rRNAs in yeast strains bearing the mutant *nme1* allele (Fig. 6). The 5S rRNA and the tRNAs served as loading controls. As previously reported, there was a higher concentration of the $5.8S_S$ rRNA (short form of the 5.8S rRNA) than the $5.8 S_L$ rRNA (long form of the 5.8S rRNA) in wild-type cells: this ratio is usually 10:1. However, two of the five *nme1* mutant strains exhibit an alteration in this ratio. The $nme1^{70A>G}$ mutant strain had a ratio of approximately 2:3 of the short versus the long form of the 5.8S rRNA, whereas the 2 bp deletion mutant lacked the short form of the 5.8S rRNA completely. This latter mutant showed only minimal growth on non-fermentable carbon sources, indicating that most of its function is abolished. These data suggest that the human mutations including the common 70A>G variant can alter ribosomal RNA processing and that this is a highly conserved function for *RMRP* from yeast to humans. This is also the first example of a yeast mutant completely lacking the $5.8S_S$ rRNA that still remains viable.

Because *RMRP* has been shown to play a role in mitochondrial DNA replication (15), cell cycle progression at the end of mitosis (25), and chromosomal segregation during mitosis (41), we examined mitochondrial function, cell cycle progression and chromosome segregation in the *nme1* mutant strains. However, each of these was indistinguishable from the wild-type control (data not shown).

Transcriptional profiling of human CHH leukocytes

To gain insight into the pathogenesis of human CHH caused by mutations in the *RMRP* gene, we performed transcriptional profiling of human leukocytes using the Affymterix Human Genome U133 chip set. For this study, two CHH patients were available; the first patient was compound heterozygous for an 89C>G transversion and a 124C>T transition, and the second patient was homozygous for the most frequently found 70A>G transition. The transcription profile was compared to two controls matched for ethnicity and sex. The data were normalized using GCRMA from the statistical analysis software, Bioconductor. The genes that were differentially expressed in the two patients relative to the normal controls are listed in Tables 1 and 2, respectively.

Ninety-nine genes are commonly 2-fold or more up-regulated in both patients when compared with both controls. Forty-seven (47.5%) of these genes play a role in the immune system. Sixteen (16.2%) play a role in cell cycle regulation either via cell growth or apoptosis. Sixteen of the ninety-nine genes are involved in signal transduction, five of the sixteen genes belong to the family of G-coupled receptors. Five of them are transcription factors (Table 1).

Thirty-eight genes were 2-fold or more down-regulated in both patients when compared with unaffected controls. Seventeen genes (44.7%) play a role in the immune system. Seven genes are involved in cell cycle regulation and apoptosis. Nine genes (23.7%) are involved in signal transduction. Six genes (15.8%) have enzymatic activities. Four genes (10.5%) have an unknown function. Interestingly, no transcription factors were 2-fold or more down-regulated in this experiment.
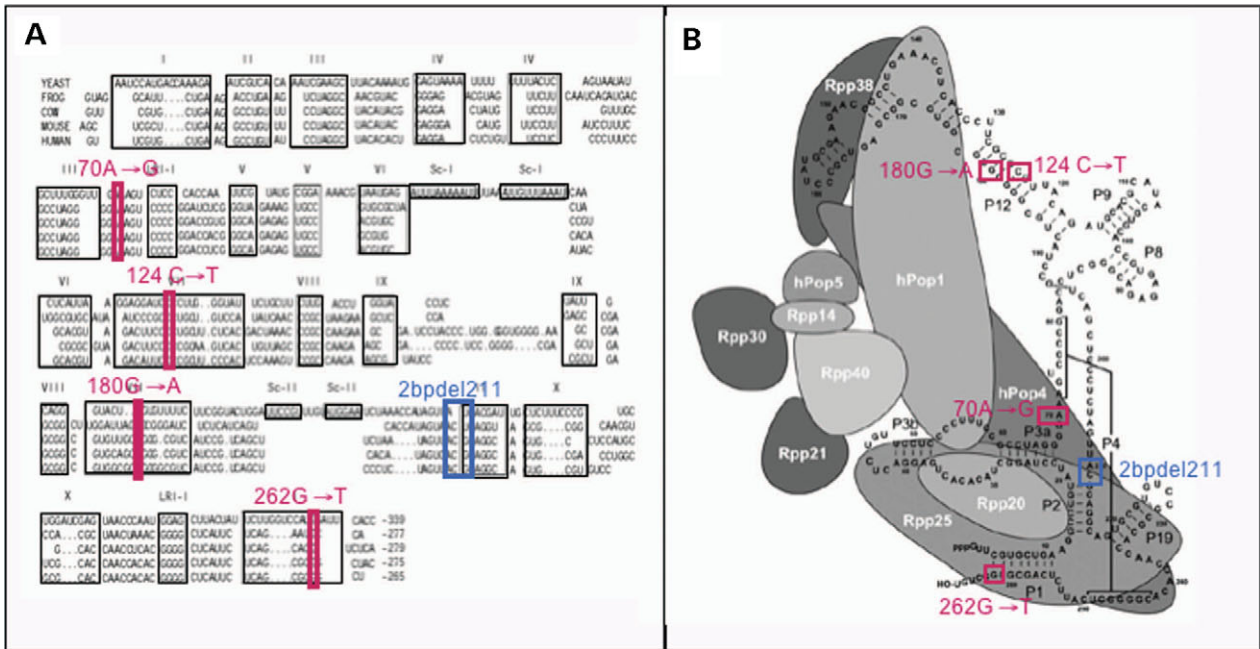
Figure 4. *RMRP* sequence and gene structure. (**A**) *RMRP* is highly conserved among a variety of different species. Mutations that have been introduced into the yeast ortholog *NME1* are highlighted [modified from Schmitt *et al.* (1992)]. (**B**) The positions of the human mutations in this complex are highlighted in a putative structure of the RNase MRP complex [modified from Welting *et al.* (24)].
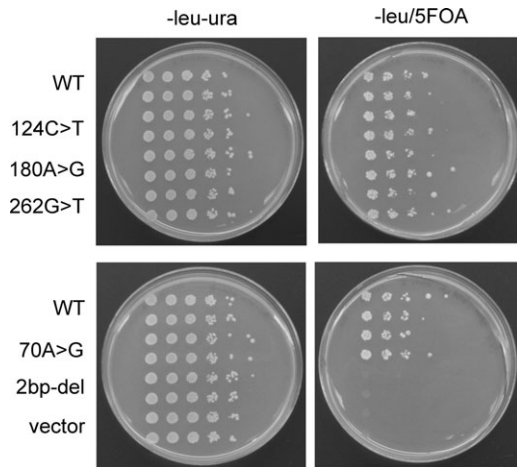

Figure 5. Viability tests of point mutations introduced into the *NME1* gene. The experiment was performed in duplicate. To control for cell numbers the yeast strains were grown on −leu-ura medium to maintain the wt *NME1* plasmid (left). The wt *NME1* plasmid is shuffled out by growth on −leu/5FOA (right). The introduced *nme1* point mutations grew equally well when compared with the wt control. The 2 bp-del mutant was lethal, which is indicated by a lack of growth on −leu 5-FOA.

PF4V1, *IL8*, *CCR3* and *STAT1* up-regulation was confirmed by real-time quantitative RT–PCR amplification (Fig. 7). There was not unexpectedly inter-patient variability, especially in *PF4V1*. The up-regulation of *G0S2*, *AMF4*, *LRAP* and *HLA-DRB1* as well as the down-regulation of *INDO* has also been confirmed via quantitative RT–PCR of the patients (data not shown). Moreover, a longitudinal comparison of the same patient that was compound heterozygous

for 89C>G and 124C>T showed up-regulation of seventy-two of the ninety-nine genes again when using the Affymetrix Human Genome 2.0 plus chip a year later in an independent assay. Also 24 genes previously found to be down-regulated were confirmed in this experiment.

DISCUSSION

To link the CHH pathogenesis with *RMRP* gene structure and transcript function in cellular and subcellular processes, we have generated genomic and functional data from *in vitro* and *in vivo* studies.

In eukaryotes, the RNA PolIII transcribes structural or catalytic RNAs that are usually shorter than 400 bp. There are three different types of RNA PolIII promoters. The type 1 promoter contains an intragenic internal control region consisting of an A box, an intermediate element and a C box. This is exemplified by the 5S rRNA gene (42). The type 2 promoter is also intragenic and consists of an A box and a B box and is exemplified by typical tRNAs (43–46). The type 3 RNA PolIII promoter is external. The core promoter consists of a PSE, element with a TATA box that is located at a fixed distance downstream of the PSE element (35,38). The PSE element on its own is sufficient for snRNA transcription by RNA PolIII (37,47).

The *RMRP* gene is transcribed by RNA PolIII and sequence elements of a type 3 promoter are present (48). The core sequence elements such as the PSE element and a TATA box can be found upstream of the transcription initiation site of the *RMRP* gene. In addition, transcription factor binding sites like a SP1 binding element and an octamer (recruits the transcription factor Oct-1) sequence could serve as distal

Table 1. List of genes that are up regulated in CHH patients

Name	Fold change	Adhesion	Apoptosis	Cell cycle	Cell growth	Immune response	Metabolic pathways	Transport	Transcription factor	Others	Signal transduction	Comments
G0S2	12.82			■								Putative lymphocyte cell cycle G0/G1 switch gene 2
INDO	9.32					■	Indoleamine 2,3-dioxygenase					Degrades L-tryptophan
P2RY14	8.06					■					■	G-coupled receptor (GPR105)
RSAD2	7.46					■						Radical S-adenosyl methionine domain containing 2
HLA-DQA1	6.92					■			■			Major histocompatibility complex, class II, DQ alpha 1
IFIT1	6.68					■						Cell migration, extracellular matrix stability
LR8	6.41									■		Organogeneis
SERPING1	6.32					■						Hereditary angineurotic edema (HANE)
TNFAIP6	6.15	■				■						Tumor necrosis factor, alpha-induced protein 6
SORD	5.82						Glucose pathway					Sorbitol dehydrogenase
ORM1	5.58					■		■				Orosomucoid 1
C4BPA	5.54					■						Complement component 4 binding protein, alpha
GPR44	5.24					■					■	G-coupled receptor
APOBEC3B	5.21			■	■					■		C to U RNA editing
PF4V1	5.10					■						Heparin binding, chemokine activity, cellular chemotaxis
CCR3	5.06	■				■					■	G-coupled receptor
RNASE3	4.96						Ribonuclease			■		Ribonuclease, RNase A family, 3 (eosinophil cationic protein)
HLA-DPA1	4.79					■						Major histocompatibility complex, class II, DP alpha 1
SMPD3	4.63				■		Glycosphingolipid metabolism				■	Sphingomyelin phosphodiesterase 3 (=NA)
EMR1	4.50	■										G-coupled receptor
CD9	4.47	■			■						■	Trigger platelet activation and aggregation, lit appears to promote muscle cell fusion and support myotube maintenance.
IL8	4.41			■		■						CXCL8 (interleukin 8)
PSPHL	3.81			■			Phospho serine phosphotase			■		Phosphoserine phosphatase-like (CO9)
IFI44L	3.78					■?						Interferon-induced protein 44-like, expresssed in osteoblasts, Histocombatibility 28
IER3	3.76		■Protects from			■						Immediate early response 3
HCA112	3.71											Hepatocellular carcinoma-associated antigen 112, function unknown
CEACAM1	3.71	■	■			■						Differentiation and arrangement of tissue three-dimensional structure, angiogenesis, tumor suppression, metastasis

Continued

Table 1. *Continued*

Name	Fold change	Adhesion	Apoptosis	Cell cycle	Cell growth	Immune response	Metabolic pathways	Transport	Transcription factor	Others	Signal transduction	Comments
G1P3	3.51					■						IFN-alpha inducible, belongs to FAM14 family
LAIR2	3.43					■						Leukocyte-associated Ig-like receptor 2; secreted and may help modulate mucosal tolerance
MLH3	3.41						DNA mismatch repair					MutL homolog 3,
CEBPE	3.39								■			CCAAT/enhancer binding protein (C/EBP), epsilon
STAT1	3.29								■		■	IFN-alpha and gamma inducible
ALOX15	3.25					■	Lipid metabolism	Electron transport		■		Arachidonate 15-lipoxygenase
INPP1	3.18						Inositol phosphate metabolism					Inositol polyphosphate-1-phosphatase
MX1	3.16		■			■						Dynamin family; production of microtubule bundles
CLC	3.14					■	Lysophospholipase			■		Charot-Leyden crystal protein; belongs to Galaptin (S-Lectin) family
SLC29A1	3.14							■				Nucleoside transport
TBC1D8	3.14			■								TBC1 domain family, member 8; GTPase activating protein for Rab family protein(s)
HLA-DQB1	3.10					■						Major histocompatibility complex, class II, DQ beta 1
IL5RA	3.05					■					■	Interleukin 5 receptor, alpha; Cytokine-cytokine receptor interaction; Jak-STAT signaling pathway
SIGLEC8	3.03	■									■	Sialic acid binding Ig-like lectin 8
BTNL8	3.01					■?						Butyrophilin-like 8; Ig-like
CD59	3.01					■					T-cell activation complexed to a protein tyrosine kinase.	CD59 antigen p18−20; Potent inhibitor of the complement membrane attack complex (MAC) action
FOLR3	2.93						Mediate delivery of 5-methyltetrahydrofolate to the interior of cells					Folate receptor 3; protein is constitutively secreted in hematopoietic tissues and is a potential serum marker for certain hematopoietic malignancies
v-FOS	2.85		■ In some cases	■	■	■			■		■	FBJ murine osteosarcoma viral oncogene homolog
HERC5	2.71						Ubiquitin ligases					Hect domain and RLD 5; The 350-amino acid HECT domain is predicted to catalyze the formation of a thioester with ubiquitin before transferring it to a substrate, and the RLD is predicted to act as a guanine nucleotide exchange factor for small G proteins

Gene	Value			Function		Description
STAT2	2.71		■		■	Signal transducer and activator of transcription 2
RNASE2	2.66		■?	Ribonuclease	■	Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)
RAB20	2.64				Small GTPase mediated signal transduction	RAB20, member RAS oncogene family; Plays a role in apical endocytosis/recycling.
PASK	2.60				■	AS domain containing serine/threonine kinase; intracellular signaling pathways
OAS3	2.58		■			2′-5′-oligoadenylate synthetase 3: This enzyme is induced by interferons and catalyzes the 2′, 5′ oligomers of adenosine in order to bind and activate RNase L. This enzyme family plays a significant role in the inhibition of cellular protein synthesis and viral infection resistance.
MYO15B	2.57					Myosin XVB, pseudogene; transcribed and unprocessed
FBXL11	2.57			Ubiquitin dependent protein degradation		F-box and leucine-rich repeat protein 11
FBP1	2.57			Catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate		Fructose-1,6-bisphosphatase 1
OASL	2.57			May interact with Rnase L in RNA degradation		2′-5′-oligoadenylate synthetase-like
IFIT3	2.57		■?			Interferon-induced protein with tetratricopeptide repeats 3
PAPSS1	2.55			Nucleic acid metabolism	■	3′-phosphoadenosine 5′-phosphosulfate synthase 1
IGSF2	2.55	■	■			Immunoglobulin superfamily, member 2
G1P2	2.55		■		■	IFN-alpha inducible; transacting-binding factor
TM6SF1	2.53					Transmembrane 6 superfamily member 1; function unknwon
CCR1	2.51		■		■	Chemokine (C-C motif) receptor 1
PAM	2.46			Peptidylglycine alpha-hydroxylating monooxygenase (PHM) and peptidyl-alpha-hydroxyglycine alpha-amidating lyase (PAL)		Peptidylglycine alpha-amidating monooxygenase: catalytic domains work sequentially to catalyze neuroendocrine peptides to active alpha-amidated products

Continued

Table 1. *Continued*

Name	Fold change	Adhesion	Apoptosis	Cell cycle	Cell growth	Immune response	Metabolic pathways	Transport	Transcription factor	Others	Signal transduction	Comments
KIR2DS2	2.45					■				Role in RA?		Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2
P2RX1	2.45										Functions as a ligand-gated ion channel with relatively high calcium permeability	Purinergic receptor P2X, ligand-gated ion channel, 1; Binding to ATP mediates synaptic transmission between neurons and from neurons to smooth muscle, being responsible, for example, for sympathetic vasoconstriction in small arteries, arterioles and vas deferens
SCO2	2.45						Catalyzes transfer of reducing equivalents from cytochorme c to oxygen					SCO cytochrome oxidase deficient homolog 2; pumps protons across the inner mitochondrial membrane
CAT	2.43						Catalase activity					Oxidative stress; cat deficiency causes acatalasia
BTN3A2	2.39			■		■				development		Butyrophilin, subfamily 3, member A2
F5	2.39						Essential factor of the blood coagulation cascade					Coagulation factor V
IFIT5	2.39					■						Interferon-induced protein with tetratricopeptide repeats 5
IFI44	2.31					■						Interferon-induced protein 44
MXD1	2.31								■			MAX dimerization protein 1; repressor of transcription
C1orf16	2.31											Chromosome 1 open reading frame 16; function unknown
C1RL	2.25					■	Proteolysis, peptidolysis					Complement component 1, r subcomponent-like
ADM	2.25						May function as a hormone in circulation control					Adrenomedullin; absence of Adm may be one cause of nonimmune hydrops fetalis
LOC44060	2.23					■						Fc-gamma receptor I B2
GK	2.17						Catalyzes the phosphorylation of glycerol by ATP					Glycerol kinase
CCL3	2.17					■						Chemokine (C-C motif) ligand 3
KCNJ15	2.16						Allows potassium to flow into a cell rather than out of the cell					Potassium inwardly-rectifying channel, subfamily J, member 15
FES	2.16					■	Tyrosine-specific protein kinase activity and that activity is required for maintenance of cellular transformation					Feline sarcoma oncogene; FES has a role in regulating the innate immune response

Gene	Ratio					Function		Description
PLAUR	2.14					Ole in localizing and promoting plasmin formation		Plasminogen activator, urokinase receptor
ZC3HAV1	2.13				■			Zinc finger CCCH-type, antiviral 1
EIF4A1	2.11							Eukaryotic translation initiation factor 4A, isoform 1; subunit of a high molecular weight protein complex involved in cap recognition and is required as a single polypeptide chain for mRNA binding to ribosome
CCL23	2.11				■			Chemokine (C-C motif) ligand 23
PRO0611	2.08							PRO0611 protein; function unknown
HAL	2.08					Ytosolic enzyme catalyzing the first reaction in histidine catabolism		Histidine ammonia-lyase
DAPK1	2.08		■					Death-associated protein kinase 1
CASP4	2.07		■					Caspase 4, apoptosis-related cysteine protease
SUI1	2.07							Putative translation initiation factor; Promotes the assembly of 48S ribosomal complexes at the authentic initiation codon of a conventional capped mRNA
HK2	2.07					Committing glucose to the glycolytic pathway		Hexokinase 2; phosphorylate glucose to produce glucose-6-phosphate
TRIB1	2.07			■		Protein kinase activity		Tribbles homolog 1
SFI1	2.06			■				Sfi1 homolog, spindle assembly associated
IFIT2	2.06				■			Interferon-induced protein with tetratricopeptide repeats 2
KLRC3	2.06				■			Killer cell lectin-like receptor subfamily C, member 3
ABCA7	2.03				■		Transport molecules acrosscellular membranes	ATP-binding cassette, sub-family A (ABC1), member 7; transport various molecules across extra- and intra-cellular membranes
TPM1	2.03						■	Tropomyosin 1; actin-binding protein
BLM	2.01					Helicase activity		Bloom syndrome
PLSCR1	2.01					Mediates transbilayer migration of phospholipids upon binding calcium ions		Phospholipid scramblase 1
AU158442	2.00							PLACE2 Homo sapiens cDNA clone PLACE2000394 3′, mRNA
DSC2	2.00	■						Desmocollin 2

Table 2. Genes that are down regulated in CHH patients

Name	Fold change	Adhesion	Apoptosis	Cell cycle	Cell growth	Immune response	Metabolic pathways	Transport	Transcription factor	Others	Signal transduction	Comments
LRAP	−16.00					■?						Leukocyte-derived arginine aminopeptidase
HLA-DRB1	−15.03					■						Major histocompatibility complex, class II, DR beta 1
AMFR	−4.03						Ubiquitin ligase activity					Autocrine motility factor receptor; tumor motility-stimulating protein secreted by tumor cells
APOBEC3A	−3.76			■	■		RNA editing enzyme					Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A; maybe an expressed pseudogene
IGL@	−3.39					■						Immunoglobulin lambda locus
CD36	−3.36	■				■						Glycoprotein of the platelet surface, serves as receptor for thrombospondin in platelets
DEFA1	−3.05					■						Defensin, alpha 1, myeloid-related sequence; Defensins are a family of microbicidal and cytotoxic peptides thought to be involved in host defense
CDKN1C	−2.95			■								Cyclin-dependent kinase inhibitor 1C
SERPINB9	−2.93	■				■						Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9
ADRB2	−2.85										■	G-coupled receptor family; beta-2-adrenergic receptor
MGC27165	−2.81											Hypothetical protein MGC27165; unknown function
IGKC	−2.79					■						Immunoglobulin kappa constant
TXNDC5	−2.60		■?				Protein-disulfide isomerase					Thioredoxin domain containing 5
TNFRSF17	−2.58			■							■	Tumor necrosis factor receptor superfamily, member 17
FCER1A	−2.58					■						C fragment of IgE, high affinity I, receptor for; alpha polypeptide
MS4A3	−2.48			■							■	Membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific); Hematopoietic modulator for the G1-S cell cycle transition
SESN1	−2.48				■?							Sestrin 1; Defects in SESN1 may be involved in heterotaxia
RPS26	−2.46						Ribosome					Protein component of 40S ribosomal subunit; autoregulates it's own expression
KLRG1	−2.38					■						Killer cell lectin-like receptor subfamily G, member 1
SLPI	−2.35					■						Secretory leukocyte protease inhibitor (antileukoproteinase); Increased leukocyte elastase activity in mice lacking Slpi leads to impaired wound healing due to enhanced activity of transforming growth factor-beta.
GZMH	−2.33		■?				Granzyme H					Also known as cytotoxic T-lymp;hocyte-associated serine esterase
HOP	−2.33											Homeodomain-only protein; Atypical homeodomain protein which does not bind DNA and is required to modulate cardiac growth and development

Gene	Value				Description	Note
SLC1A4	−2.31				Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Amino acid exchanger
CD8A	−2.28	■			CD8 antigen, alpha polypeptide (p32)	
XCL1	−2.27	■			Chemokine (C motif) ligand 1	
HBD	−2.27			■	Hemoglobin, delta; he delta (HBD) and beta (HBB) genes are normally expressed in the adult; Mutations in the delta-globin gene are associated with beta-thalassemia	
PRSS23	−2.23		■		Protease, serine, 23; member of the trypsin family of serine proteases; function is unknown	
TGFBR3	−2.23	■	■		Transforming growth factor, beta receptor III	
XCL2	−2.22	■	■		Chemokine (C motif) ligand 2	
IGHG1	−2.17				Immunoglobulin heavy constant gamma 1	
RYBP	−2.16				RING1 and YY1 binding protein	
ITGB1BP1	−2.08		■		Integrin beta 1 binding protein 1	
CX3CR1	−2.04	■	■		Hemokine (C-X3-C motif) receptor 1; Belongs to the G-protein coupled receptor 1 family	RA?
CCR2	−2.04	■			Chemokine (C-C motif) receptor 2	
RRAS2	−2.04				Related RAS viral (r-ras) oncogene homolog 2	
PTCH	−2.01				Patched homolog; protein is the receptor for sonic hedgehog	
KIAA0882	−2.01				Function unknown	
IL6R	−2.00	■		■	Interleukin 6 receptor	

sequence elements (DSE) to enhance the transcription of *RMRP* similar to the DSE element of the human *U6 snRNA* gene (49). Our *in vitro* studies show that 352 bp upstream of the transcription start site of the *RMRP* gene represents a region sufficient for activating transcription. The promoter duplications identified in CHH patients decrease the transcription activity *in vitro*, but they do not abolish transcription completely. This is consistent with our real-time RT–PCR data of patient leukocytes and correlates well with the yeast studies in that the transcribed human mutations cannot completely abolish the essential function required for viability. Hence, CHH mutant alleles are likely hypomorphic, as null alleles are likely incompatible with life.

Because *RMRP* is highly conserved among a variety of species, we studied the functional consequences of CHH mutations occurring at conserved positions between humans and yeast. Because *RMRP* may play a role in priming of mitochondrial DNA replication, loss of RNase MRP function might result in mitochondrial depletion.

However, our yeast studies and studies on human CHH fibroblasts do not support a significant defect in mitochondrial function or DNA content. It may be that other RNases compensate for RNase MRP in mitochondria (50) or mutations that affect the mitochondrial function of RNase MRP may be lethal.

Mutations in protein components of RNase MRP result in cell cycle delay in yeast. These mutants have an exit-from-mitosis defect (25). This defect is caused by an increase in Clb2 (B-type cyclin) levels caused by an increase in *CLB2* mRNA stability. Normally, *CLB2* mRNA levels decrease rapidly when the cell completes mitosis. RNase MRP cleaves the 5′-UTR of *CLB2* causing rapid degradation of the mRNA by the *Xrn*1 nuclease (5′ → 3′ exoribonuclease), but not by exosomes that are usually responsible for mRNA degradation (26). In this study, no cell cycle phenotype could be observed in the *nme1*[mutant] strains. There was no detectable change in the cell cycle progression under normal growth conditions or after stress induction through γ-irradiation. Interestingly, we also did not see significant differences in human *CLB2* mRNA in patient versus control white blood cells.

In yeast, RNase MRP plays a role in pre-ribosomal RNA processing. It cleaves at the A_3-site that in turn leads to the production of the $5.8S_S$ rRNA. Two of the five *nme1*[mutant] strains show an alteration in the normal ratio of the $5.8S_L$ rRNA versus $5.8S_S$ rRNA. Interestingly, the CHH 70A>G variant is just outside of the P4 domain. This position is highly conserved among a variety of species. In yeast, it can form a 9 bp duplex with the 5.8S rRNA (51). This mutation might, therefore, affect the maturation of the 5.8S rRNA that is reflected by the change of the ratio of the long versus short form of the 5.8S rRNA. The 70A>G might cause a subtle alteration in the local structure of the active site, and hence, lead to less efficient cleavage of the 5.8S rRNA. Similarly, the 2 bp deletion occurs in a stretch of nucleotides parallel to the P4 domain. This may have an even more severe affect on active site binding and processing of 5.8S rRNA. The reason for a lack of effect of the other point mutations is unclear, though they exist in predicted stem loop structures distal to the P4 domain. One possibility is
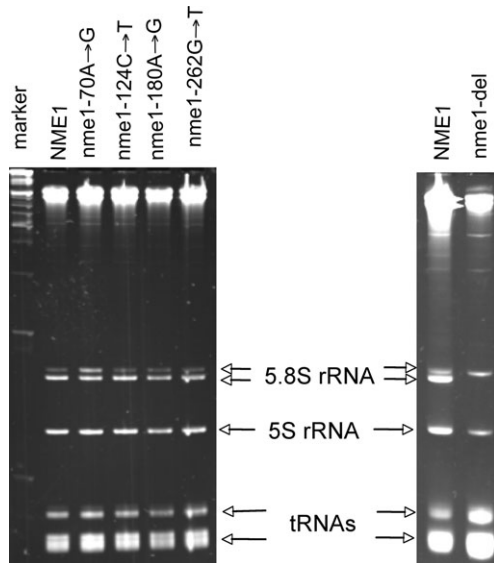
Figure 6. Pre-ribosomal processing of mutant *nme1* strains. Two micrograms of total RNA isolated from diploid yeast strains was separated on a 6% PAA, 7 M urea gel. *nme1*$^{70A>G}$, *nme1*$^{2\ bp-del}$ showed a defect in pre-ribosomal processing resulting in an alteration of the ratio 5.8S$_L$:5.8S$_S$ rRNA, which is usually 1:10. The ratio of the *nme1*$^{70A>G}$ was changed to 2:3, whereas the *nme1*$^{2\ bp-del}$ lacked the short form of the 5.8S rRNA completely.

that divergent functions may have arisen in mammals that require these distal structures that are non-essential in yeast. Alternatively, the yeast RMRP catalytic function may be less sensitive to these substitutions. In fact, while these substitutions affect the local stem loop structure in the yeast RNA, they do not appear to affect the P4 domain structure locally.

To gain insight into how such a defect might be translated in the human situation, we analyzed the transcriptional profile of CHH leukocytes. Interestingly, we saw up-regulation of cytokine family members and cell cycle regulatory genes, which is consistent with the *in vitro* studies performed on T-cells isolated from CHH patients (4). Our longitudinal analysis in one patient suggests that these changes persist over time and reflect the underlying *RMRP* genotype rather than environmental factors at the time of sampling. Some of the up-regulated genes may reflect an underlying common pathogenic mechanism in chondrocytes and lymphocytes. For example, up-regulation of IL8 and GRO-α is observed in osteoarthritic cartilage and is correlated with activation of the p38/MAPK pathway. This activation is also known to promote hypertrophic chondrocyte differentiation and apoptosis, and has been described as one of the cellular alterations in osteoarthritis (52). Altered hypertrophic chondrocyte differentiation would correlate with the primary clinical finding of metaphyseal chondrodysplasia. In addition, transglutaminase 2 is stimulated. TG2 among other transglutaminases modulates differentiation and calcification of chondrocytes and is a mediator of tissue repair (53).

The expression level of *IL8* and *IL6* are even higher in rheumatoid arthritis cartilage (54). In addition, it has been shown that IL8 is increased in several tumors (55,56). Therefore, IL8 up-regulation may be an important biomarker of both skeletal and hematopoietic disease, as CHH patients have

not only metaphyseal changes but also an increased risk for developing lymphomas. Another candidate gene in the pathogenesis of CHH is the *putative lymphocyte G0/G1 switch gene 2* (*G0S2*). *In vitro* studies of T-cells of CHH patients have shown a defect in the cell cycle transition of the G0 to G1 phase (57). Up-regulation of G0S2 leads to an arrest of cells in the G0 phase (58), which is a more permanent phase of end-differentiated cells. Increased STAT1, STAT5 and p21^{Cip1} protein levels are observed in the pre- and hypertrophic chondrocyte zone in achondroplasia and thanaphoric dysplasia. Moreover, expression level was correlated to the severity of the disease (59).

The studies in yeast suggest that there might be yet other functions of RNase MRP that it may have adopted during evolution. Studies in yeast suggest that it may act as a site-specific ribonuclease to cleave *CLB2* mRNA (26). However, we did not observe alterations in *CLB2* expression levels in the microarray studies performed here. The DNA sequence might be conserved among different species but not all functions might be. Not surprisingly, not all human or yeast protein subunits that bind *RMRP* and *NME1*, respectively, have clear orthologs in different species (31,60). These data suggest that while some functions are conserved between yeast and humans, others might have diverged. *RMRP* mutations can affect ribosomal processing in yeast. The human mutations are likely hypomorphic in that they do not abolish essential functions. Moreover, up-regulation of specific genes including *IL8* and *G0S2* may help explain the common effects on T-cell proliferation, malignancy risk and altered chondrocyte hypertrophy.

MATERIALS AND METHODS

RMRP expression pattern

In situ hybridization. WT mouse embryos were collected at specified time points and fixed in 4% paraformaldehyde at 4°C for 12 h. The paraffin-embedding, sectioning and *in situ* hybridization were carried out as described by Albrecht *et al.* (61). The *Rmrp* gene was amplified using total RNA isolated from an E15.5 embryo as a template in the RT–PCR reaction. A *Not*I linker was added to the primers PH-21-Xho and PH-22-Pst, and the PCR product was subcloned into the *Not*I site of pBluescript SK– (Stratagene).

Northern blot. To elucidate the expression pattern of the human *RMRP* gene, a human multiple tissue northern blot (RNWAY Laboratories Inc., Korea) was hybridized with a *RMRP* probe amplified with the primers RMRP5 and RMRP6 at an annealing temperature of 61°C. Primer sequences are available in Supplementary Material. The PCR reaction was supplemented with DMSO to a final concentration of 2%. The PCR product was labeled with random hexamers (Invitrogen). 10^6 CPM probe was added per milliliter of Church-buffer and hybridized over night at 68°C. The blot was then rinsed twice with 5×SSC, 0.1% SDS at 68°C for 20 min followed by two washes with 2×SSC, 0.1% SDS also at 68°C, then twice with 1×SSC, 0.1% SDS and lastly once with 0.5×SSC, 0.1% SDS. A radiographic film was exposed over night at room temperature.
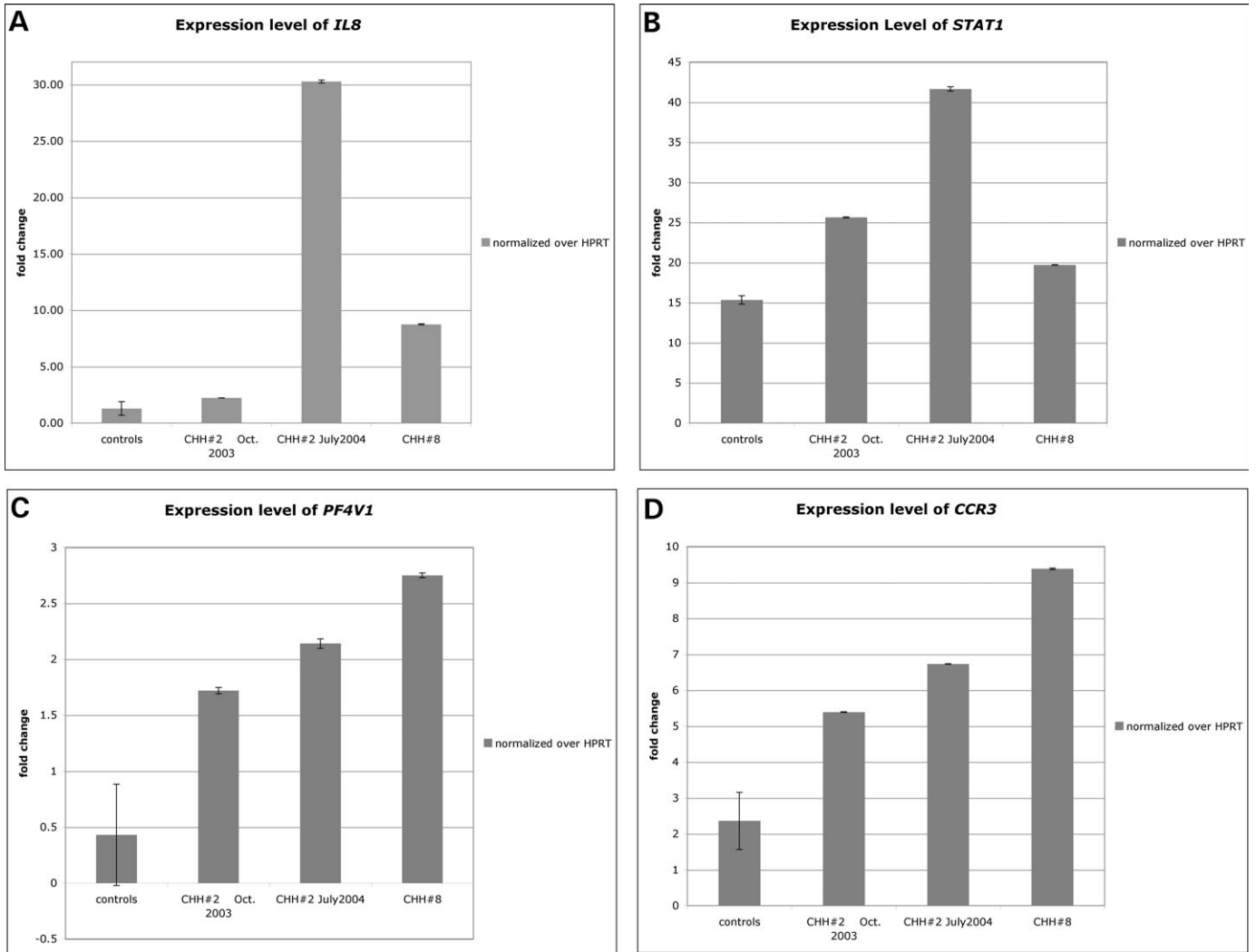
Figure 7. Verification of microarray data via quantitative real-time-RT–PCR. *HPRT* was used as a control housekeeping gene to normalize for expression level. The up-regulation of the genes tested correlates with the microarray analysis.

Transfections

The *RMRP* promoter was amplified from human genomic DNA with the primers RMRP8-*Eco*RI and RMRP14-*Cla*I. The second promoter included an additional 500 bp of upstream sequence, was 841 bp in length, and was amplified with the primers RMRP8-*Eco*RI and RMRP9-*Cla*I. Primer sequences are available in Supplementary Material. The annealing temperature for the PCR reaction was 61°C for both primer pairs. The PCR products were subcloned into the *Eco*RI and *Cla*I sites of the vector pSilencer U6 (Ambion) containing an RNAi oligo targeted against the luciferase reporter gene of the vector pGL3 Control (Promega). The U6 promoter of this vector was removed with a *Kpn*I and *Apa*I digest. The remaining vector was then religated. The sequence for the RNAi oligo was provided by Steve Elledge at Baylor College of Medicine in Houston, TX, USA. The shRNA was cloned into the *Eco*RI and *Hin*dIII sites of the vector. Cos7 cells were plated at 0.6×10^6 cells/well in six-well plates just before transfection. Transfections were done in triplicates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Luciferase and beta-galactosidase activities were assayed 48 h after transfection as described by Zhou *et al*. (62). The transfections were repeated twice in independent assays.

RNA isolation of whole blood and quantitative real-time PCR

Blood from CHH patients was collected in ACD, Solution A yellow top BD Vacutainers™ (BD, Franklin Lakes, NJ, USA) after informed consent was obtained. Total RNA from whole blood was isolated using the Versagene RNA isolation kit from Gentra according to the manufacture's protocol. Four micrograms of the total RNA was incubated for 90 min in an RT-reaction mix that consisted of 5 μM random hexamers (Invitrogen), 1.8 mM dNTPs (Roche), 40 U RNase Inhibitor (Invitrogen), first strand synthesis buffer (Invitrogen), 20 mM DTT (Sigma) and 40 U Reverse Transcriptase (Invitrogen).

The LightCycler® 1.1 Instrument and reagents from Roche Applied Science (Indianapolis, IN, USA) were used for the quantitative PCR and the recommendations of the manufacturer were followed exactly. Twenty microliter reactions are prepared with dNTP concentrations of 1200 mM, MgCl₂ 4 mM buffer and thermostable enzyme and Sybr green as part of the proprietary mastermix. cDNA samples were assayed by fluorometry on a BMG Fluostar plate reader using Ribo-green assay (Molecular Probes). Template concentration was ascertained over multiple dilutions and normalized to 40 ng/µl of which a 1:8 dilution was used for Lightcycler amplification. Two microliters of the 1:8 dilution (12 ng) was added to each 20 µl reaction and fluorescence monitored for 45 cycles. Crossing points were determined by a second derivative algorithm intrinsic to the Lightcycler software. Primer and primer sequence information are available in Supplementary Material.

Yeast strains and studies

The genetic background for the yeast strains used in Figure 5 is YPH275: *MAT**a**/MATα, ura3-52, lys2-80, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1*, CF [TRP1, SUP11, CEN4] (Source: Peter Hieter). YPH1: *MAT**a**/MATα, nme1-Δ::kanR/ NME1ura3-52/ura3-52, lys2-80/lys2-80, ade2-101/ade2/101, trp1-Δ1/trp1-Δ1, his3-Δ200/his3-Δ200, leu2-Δ1/leu2-Δ1*, CF pPH1[*NME1 URA3CEN*] was generated using the PCR-based strategy described by Wach *et al.*(63). YPH1 was transformed with pPH1 (pNME1 URA3 Cen), which contains NME1 and 259 and 205 bp of upstream and downstream sequence subcloned into pYClac33 to generate YPH2. YPH2 was sporulated to generate YPH3: *MAT**a** nme1-Δ::kanR/ NME1ura3-52, lys2-80, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1*, CF [TRP1, SUP11, CEN4] pPH1[*NME1 URA3 CEN*]. Transformation of mutant *nme1* plasmids was done as described by Gietz *et al.* (64).

Viability testing was performed by growing the yeast strains in −leu-ura media to late log phase at 30°C. Ten-fold serial dilution series were plated on −leu-ura and −leu 5-FOA (Fluoroorotic acid) plates and incubated for 3 and 6 days at 30°C, respectively.

For the experiment shown in Figure 7, TLG119: Mat a/ alpha ade2-1/ade2-1 ura3-52/ura3-52 leu2-3,112/leu2-3,112 LYS2/lys2-1 his3/his3 cce1::HIS3/cce1::HIS3 trp1Δ-1/trp1Δ-1 nme1Δ2::TRP1/nme1Δ2::TRP1 pMES127[*URA3 NME1 CEN*] was transformed with mutant *nme1* plasmids and pMES127 evicted by growth on media containing 5-FOA. Strains were grown to saturation and total RNA was isolated as described by Schmitt *et al*. (65).

NME1 PCR mutagenesis

The different *nme1* point mutations were introduced into pPH2, which contains *NME1* and flanking sequences as described earlier subcloned into pRS415, via a two-step PCR described by Higuchi *et al.* (1988). The flanking primers for each mutation were *NME1-3* and *NME1-5*. The mutant primer were 70A → G, 124C → T, 180G → A and the 2 bp deletion. Primer sequences are available in Supplementary Material.

Microarray

The total RNA from leukocytes from two CHH patients and two matched controls was isolated using the RNAeasy Midi Prep kit from QIAGEN. The quality and the concentration of the RNA were determined using an Agilent 2100 Bioanalyzer. The HG-U133A and HG-U133B oligonucleotide arrays from Affymetrix were selected for the microarray analysis. Technical replicates were performed on samples from both patient and control subjects.

Samples were labeled using the standard Affymetrix T7 oligo(dT) primer protocol. Total RNA was reverse transcribed to produce double-stranded cDNA. The cDNA product was used as a template for the *in vitro* transcription reaction, producing biotin-labeled cRNA. The labeled cRNA was quantified using the NanoDrop® ND-1000 instrument. 15.0 µg of the labeled cRNA was fragmented and re-checked for concentration. A hybridization cocktail containing Affymetrix spike-in controls and fragmented labeled cRNA was loaded onto a GeneChip® array. The array was hybridized overnight at 45°C with rotation at 60 r.p.m. then washed and stained with a strepavidin, R-phycoerythrin conjugate stain. Signal amplification was done using biotinylated antistreptavidin. The stained array was scanned on the Affymetrix GeneChip® Scanner 3000. The images were analyzed and quality control metrics recorded using Affymetrix GCOS software version 1.1.2.

The signals of the chips were normalized using GCRMA (Robust Multi-array Analysis) from the statistical analysis software package, Bioconductor (http://128.32.135.2/users/ bolstad/ComputeRMAFAQ/ComputeRMAFAQ.html). We averaged technical replicates and then computed a two sample *T*-test for the patient and control data using the technical averages for each biological replicate as the input. We used the empirical Bayes methods in the R package limma to generate moderated T-statistics and *P*-values for differential expression between control and CHH individuals for each Affymetrix probe set. The empirical Bayes method for computing T-statistics improves power by considering each gene's variance as a sample from a population of gene variances. An annotated gene or gene ontology list was generated using Affymetrix's web site at http://www.affymetrix.com/ index.affx. The list of up- and down-regulated genes was created with Limma at http://bioinf.wehi.edu.au/l.imma/ and filtered in Excel. As cut-off, a 2-fold or higher up-regulation and down-regulation, respectively, was used as the logic to generate the gene lists.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENT

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