REPORT

Compound Heterozygosity of Low-Frequency Promoter Deletions and Rare Loss-of-Function Mutations in *TXNL4A* Causes Burn-McKeown Syndrome

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Mutations in components of the major spliceosome have been described in disorders with craniofacial anomalies, e.g., Nager syndrome and mandibulofacial dysostosis type Guion-Almeida. The U5 spliceosomal complex of eight highly conserved proteins is critical for pre-mRNA splicing. We identified biallelic mutations in *TXNL4A*, a member of this complex, in individuals with Burn-McKeown syndrome (BMKS). This rare condition is characterized by bilateral choanal atresia, hearing loss, cleft lip and/or palate, and other craniofacial dysmorphisms. Mutations were found in 9 of 11 affected families. In 8 families, affected individuals carried a rare loss-of-function mutation (nonsense, frameshift, or microdeletion) on one allele and a low-frequency 34 bp deletion (allele frequency 0.76%) in the core promoter region on the other allele. In a single highly consanguineous family, formerly diagnosed as oculo-oto-facial dysplasia, the four affected individuals were homozygous for a 34 bp promoter deletion, which differed from the promoter deletion in the other families. Reporter gene and in vivo assays showed that the promoter deletions led to reduced expression of *TXNL4A*. Depletion of *TXNL4A* (*Dib1*) in yeast demonstrated reduced assembly of the tri-snRNP complex. Our results indicate that BMKS is an autosomal-recessive condition, which is frequently caused by compound heterozygosity of low-frequency promoter deletions in combination with very rare loss-of-function mutations.

Burn-McKeown syndrome (BMKS [MIM 608572]) is a rare condition with normal intellectual development and the characteristic combination of choanal atresia, sensorineural deafness, cardiac defects, and typical craniofacial dysmorphisms, consisting of narrow palpebral fissures, coloboma of the lower eyelids, a prominent nose with high nasal bridge, short philtrum, cleft lip and/or palate, and large and protruding ears.[1–3] It has been suggested that oculo-oto-facial dysplasia (OOFD [MIM 610332]), reported in a large consanguineous native Alaskan family, is part of the same phenotypic spectrum.[4–6] BMKS has been proposed to be inherited in an X-linked or autosomal-recessive inheritance mode, with a single affected individual reported with a ring chromosome 18.[1]

To identify the genetic basis of BMKS, we investigated affected individuals from 11 families (BMKS001–BMKS011) (Table 1, Figure 1, and Figure S1 available online) by whole-exome sequencing (WES) and whole-genome sequencing (WGS) and tested for gene dosage alterations by microarray and MLPA analysis. We obtained a written informed consent from the families for participation in this study. The study was performed according to

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Table 1. Clinical Findings in Individuals with BMKS

Family	BMKS001	BMKS001	BMKS002	BMKS004	BMKS005	BMKS006	BMKS007	BMKS007	BMKS008	BMKS009	BMKS010	Total
Individual	III/1	III/2	II/1	II/1	II/4	II/1	II/1	II/2		II/1	II/1	14
Sex	M	M	M	F	F	F	M	M	2 F, 2 M	F	M	6 F, 8 M
Positive family history	+	+	–	–	–	–	+	+	+	–	–	5/14
Hypertelorism	+	+	+	+	+	+	+	+	2/4	+	+	12/14
Short palpebral fissures	+	+	+	+	+	+	+	–	4/4	+	+	14/14
Defect of lower eyelids	+	+	+	–	+	–	+	–	4/4	+	+	12/14
Prominent nasal bridge	+	+	+	+	–	+	+	+	4/4	+	+	13/14
Short philtrum	+	+	+	+	+	+	+	+	2/4	+	+	12/14
Thin lips	+	(+)	–	–	+	–	+	+	1/4	+	+	8/14
Cleft lip or palate	–	–	+	+	+	–	–	–	3/4	+	+	8/14
Bifid uvula	+	–	–	–	–	–	–	–	1/4	–	–	2/14
Bilateral choanal atresia/stenosis	+	+	+	+	+	+	+	+	4/4	+	+	14/14
Prominent ears	+	+	–	+	+	–	+	+	2/4	+	+	10/14
Preauricular tags	–	+	–	+	+	+	–	–	4/4	–	–	8/14
Micrognathia	–	–	–	+	–	–	+	+	4/4	+	+	9/14
Cardiac defect	–	–	–	+	+	–	+	–	1/4	–	–	4/14
Hearing loss	+	–	+	+	–	–	+	+	4/4	+	–	10/14
Normal psychomotor development	+	+	+	(+)	+	+	+	+	4/4	+	+	13/14
Short stature	–	–	–	+	+	–	+	–	0/4	–	–	2/14
Others		unilateral renal agenesis		bilateral aplasia of lacrimal ducts, amblyopia on the left, microstomia	absent l cochlear nerve, reduced right (implant not possible)		inguinal hernia, right eye: herniation into right chamber	inguinal hernia			syndactyly toes II/III	

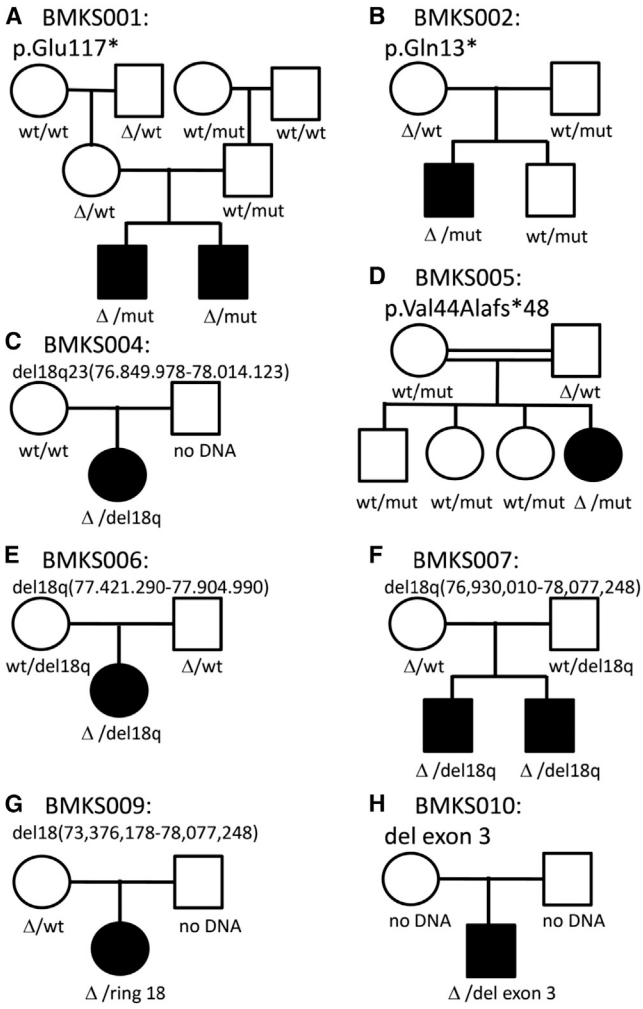

Figure 1. Pedigrees of Individuals with BMKS and Biallelic Mutations in *TXNL4A*

(A) Family BMKS001 with two affected brothers (III/1 and III/2) with a maternally inherited promoter deletion and a paternally inherited frameshift mutation, p.Glu117* (previously published in Wieczorek et al.[2]).

(B) Family BMKS002 with an affected male individual (II/1) carrying a maternal promotor deletion and a paternal frameshift mutation, p.Gln13*.

(C) Family BMKS004 with an affected female individual (II/1) carrying a promoter deletion on one and a 1.2 Mb terminal deletion 18q23 (chr18: 76,854,774_78,077,248del) on the other allele. Mother does not carry both deletions; father's DNA is unavailable.

(D) Family BMKS005 with an affected female individual (II/4) with a paternal promoter deletion and a maternal frameshift mutation, p.Val44Alafs*48.

(E) Family BMKS006 with an affected female individual (II/1) carrying a paternal promoter deletion and a large maternally inherited interstitial 18q deletion (chr18: 77,421,290_77,904,990del).

(F) Family BMKS007 with two affected brothers (II/1 and II/2) carrying the maternal promoter deletion and a large terminal deletion 18q (chr18: 76,841,645_78,077,248del) (previously published in Burn et al.[1]).

(G) Family BMKS009 with an affected female individual with a maternal promoter deletion and ring chromosome 18 resulting in a 18q deletion (chr18: 73,376,178_78,077,248del) (published in Burn et al.[1]). Paternal DNA was not available.

(H) Family BMKS010 with an affected male individual with promoter deletion and single exon deletion 3. DNA was not available from both parents.

the Declaration of Helsinki protocols and was approved by the local institutional review board (ethical votum 12-5089-BO for CRANIRARE).

The clinical data of the two affected brothers (III/1 and III/2) of German family BMKS001 were published in 2003 (Figures 2A and 2B).[2] The affected individual of BMKS002 is a 34-year-old Swiss male who had the initial clinical diagnosis of Treacher Collins syndrome (TCS [MIM 154500]). He had bilateral choanal atresia and bilateral cleft lip and palate. No internal malformations have been reported (Figures 2C and 2D). The girl of family BMKS003 is the first child of Turkish, nonconsanguineous parents. She had facial dysmorphism and cleft palate compatible with BMKS (Figures S2A and S2B, Table S1). In addition, she had imperforate anus, persistent ductus arteriosus, deafness, fifth finger clinodactyly, and hallux valgus. The affected girl of family BMKS004 originates from Vietnam. She had bilateral choanal atresia and a patent foramen ovale, which closed spontaneously (Figures 2E and 2F). The affected girl of family BMKS005 is the fourth child of healthy, consanguineous parents of Pakistani origin. The possibility of a right-sided cleft lip and palate was noted on the 20 weeks antenatal ultrasound scan. This diagnosis was confirmed at birth (Figures 2G and 2H). She was considered to have membranous choanal atresia, but no intervention was required. The neonatal hearing test revealed significant hearing loss. In Dutch family BMKS006, the girl is the only affected in her family. She had breathing problems because of bilateral choanal atresia. She presented with short palpebral fissures, lower eyelid coloboma, slight left ptosis, high vaulted palate, and three preauricular tags at left side (Figures 2I and 2J). Family BMKS007 is one of the original Burn-McKeown syndrome families, case 1 and case 2, previously published by Burn et al.[1] Current photographs are shown in Figures 2K and 2L. Pedigree BMKS008 is the family previously published with oculo-oto-facial dysplasia.[4] The index of family BMKS009 is case 5 of the original Burn-McKeown syndrome paper[1] with ring chromosome 18 (Figures 2M and 2N). BMKS010 is a French boy aged 3 years, 8 months, born to healthy parents. He had bilateral choanal atresia and a unilateral cleft lip. No internal malformations have been reported. The girl of family BMKS011 was born to healthy Brazilian parents. She had bilateral choanal atresia and a patent ductus arteriosus. Prominent ears and hearing loss were also diagnosed (Figures S2C and S2D; Table S1).

Whole-genome microarray or MLPA analysis was performed in 11 families. Array analysis for the individuals of families BMKS001, BMKS002, BMKS003, BMKS004, and BMKS011 was performed with CytoScanHD Array (Affymetrix) and evaluated with Affymetrix Chromosome Analysis Suite v.2.0. In family BMKS006, an Agilent 180 K custom HD-DGH microarray was performed according to manufacturer's instructions. Array results were confirmed by qPCR assays, designed with the Universal ProbeLibrary Assay Design Tools (Roche Applied Science). The qPCR analyses were performed in the parents—if

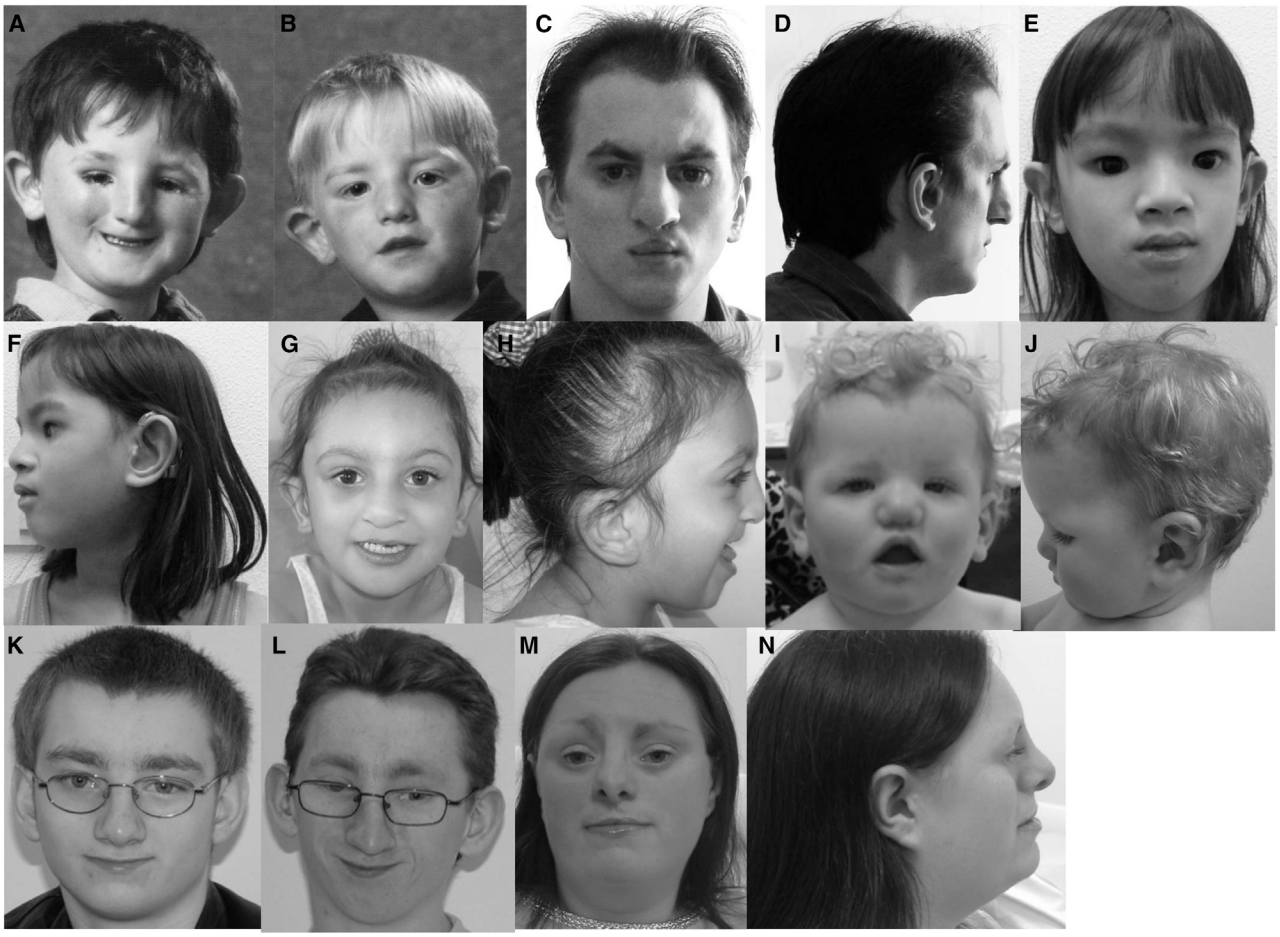

Figure 2. Facial Phenotype of Individuals with BMKS
(A and B) Persons III/1 and III/2 of family BMKS0013.
(C and D) Person II/1 of family BMKS002.
(E and F) Person II/1 of family BMKS004.
(G and H) Person II/4 of family BMKS005.
(I and J) Person II/1 of family BMKS006.
(K and L) Persons II/1 and II/2 of family BMKS007.
Note the craniofacial similarities including narrow palpebral fissures with coloboma of lower eyelids, high nasal bridge, short philtrum, small mouth, small chin, and large and protruding ears.
(M and N) Person BMKS009 in adulthood with narrow and upslanting palpebral fissures, high nasal bridge, and short philtrum.
Consent for the publication of photographs was obtained for these individuals.

DNA was available—as well. Array analysis for the individuals of families BMKS005, BMKS008, and BMKS010 was performed with Genome-Wide Human SNP Array v.6.0 (Affymetrix) according to the manufacturer's protocol. Genotypes and copy number data were generated within the Affymetrix Genotyping Console (v.4.1.3.840) via the Birdseed V2 algorithm and SNP 6.0 CN/LOH algorithm, respectively. For families BMKS007 and BMKS009, array analysis was undertaken on the Agilent 8x60k oligo array (ISCA v.2.0, Agilent Technologies UK) and analyzed with BlueFuse Multi v.3.3. We developed a multiplex ligation-dependent probe amplification (MLPA) assay for *TXNL4A* and its promoter region. It contains one probe for each of the gene's three exons as well as a probe targeting the promoter distal to the 34 bp deletion (Figure S3). Oligonucleotides for MLPA were purchased at MWG Eurofins. MLPA reagents were from MRC-Holland. MLPA reactions were performed according to standard protocols as provided by MRC-Holland, and analysis of data was carried out as described.[7]

Copy-number analyses by microarray analysis revealed five heterozygous microdeletions at 18q23, all including *TXNL4A* (MIM 611595) (Table 2, Figures 3 and S3). Two identical terminal deletions of 1.2 Mb were found in families BMKS004 and BMKS007 and an interstitial deletion of 0.48 Mb in family BMKS006. In a person originally described by Burn and colleagues[1] (BMKS009), the ring chromosome 18 is associated with a terminal deletion of 4.7 Mb. In addition, we hypothesized that smaller deletions might be also present in individuals with BMKS and established an MLPA. This revealed a single heterozygous exon 3 deletion of *TXNL4A* in family BMKS010.

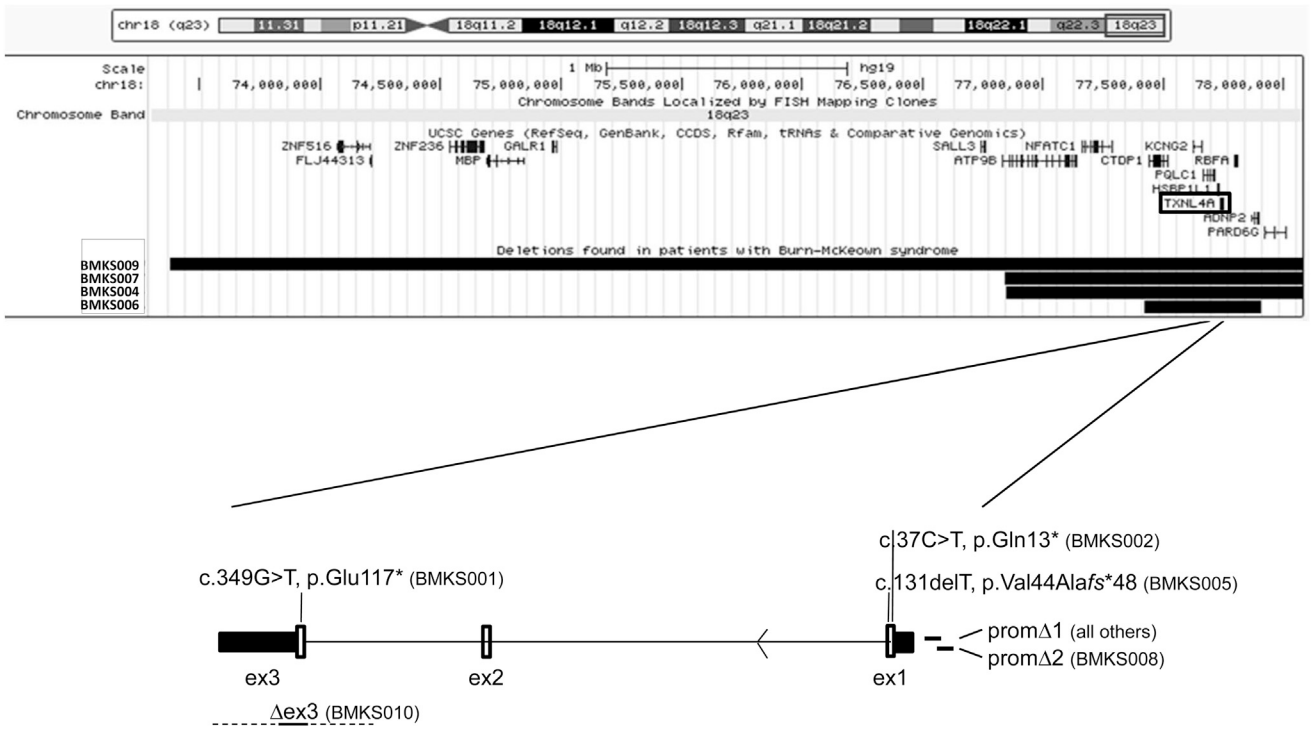

Figure 3. Gene Structure of *TXNL4A* with Identified Mutations and Deletions
The heterozygous deletions comprising *TXNL4A* identified in the index individuals of families BMKS009, BMKS007, BMKS004, and BMKS006 with a size of 4.7 Mb, 1.147 Mb, 1.164 Mb, and 0.484 Mb, respectively, are shown at the top of the figure. The gene structure with the identified mutation in families BMKS001, BMKS002, and BMKS005 are shown at the bottom as well as the exon 3 deletion of family BMKS010.

Parental DNA was available in two families (BMKS007 and BMKS006), and the respective deletions were inherited from a healthy parent.

To identify the causative gene under the assumption of an autosomal-dominant mode of inheritance with reduced penetrance, we then investigated five affected individuals from four families by exome sequencing[8] (BMKS001, BMKS002, BMKS003, and BMKS005) in whom 18q23 microdeletions had been excluded. We identified heterozygous *TXNL4A* loss-of-function mutations in three families: nonsense mutations were identified in families BMKS001 and BMKS002 (RefSeq accession number NM_006701.2; c.349G>T [p.Glu117*] and RefSeq NM_006701.2; c.37C>T [p.Gln13*], respectively). A frameshift mutation was present in family BMKS005 (RefSeq NM_006701.2; c.131delT [p.Val44Alafs*48]) (Table 2, Figure 3). All mutations in the index persons were confirmed and the segregation analyses within the families were performed by Sanger sequencing. None of these mutations were present in the 1000 Genomes Project[9] data, in dbSNP, or in 3,000 in-house control exomes from individuals with unrelated diseases. However, as in the case of the microdeletions, all intragenic *TXNL4A* mutations were inherited and also found in healthy parents, grandparents, and siblings of the affected individuals investigated.

The recurrent observation of heterozygous *TXNL4A* loss-of-function mutations in unaffected family members (Figure 1) made haploinsufficiency unlikely as the disease-causing mechanism. Moreover, the unaffected siblings in the consanguineous family, BMKS005, shared a different haplotype across the *TXNL4A* locus compared to the affected child, suggesting that a second variant at this locus remained undetected.

We then set out to search for genomic noncoding variants contributing to BMKS and performed whole-genome sequencing (WGS) in six affected individuals from five families (BMKS001, BMKS002, BMKS004, BMKS005, and BMKS008).[4] Libraries for genome sequencing were prepared with PCR-free protocols (TruSeq DNA PCR-Free, Illumina). Sequencing was performed as 100 bp paired-end reads on HiSeq2000/2500 systems (Illumina). For the genomes, we generated on average 150 Gb of sequence resulting in an average depth of coverage of 45 with 98% of RefSeq coding region covered at least 20 times. Image analysis and base calling was performed with Illumina Real Time Analysis. Reads were aligned against the human assembly hg19 (GRCh37) via Burrows-Wheeler Aligner (BWA v.0.6.2). We performed variant calling specifically for the regions targeted by the exome enrichment kit, by using SAMtools (v.0.1.18), PINDEL (v.0.2.4t), ExomeDepth (v.0.1), and custom scripts. In case of genome sequences, we used CNVnator (v.0.2.7) and BreakDancer (v.1.1.2) instead of ExomeDepth to detect structural variants. Variant quality was determined with the SAMtools varFilter script. We used default parameters, with the exception of the minimum p value for base quality bias (−2), which

Table 2. Mutations and Deletions Identified in *TXNL4A*

Pedigree and Individual	1st Mutation	Origin	2nd mutation	Origin
BMKS001, III/1 & III/2	chr18: g.77,733,765C>A, c.349G>T (p.Glu117*), exon 3	pat (pat GM)	promoter Δ **1**	mat (mat GF)
BMKS002	chr18: g.77,748,356G>A, c.37C>T (p.Gln13*), exon 1	pat	promoter Δ **1**	mat
BMKS003	Ø		Ø	
BMKS004	WGS[hg19] 18q23(76,854,774–78,077,248)x1 = > Δ 1.222 Mb, del(18)(q23→qter)	? (not mat)	promoter Δ **1**	? (not mat)
BMKS005	chr18: g.77,748,262delA, c.131delT (p.Val44Alafs*48), exon 1	mat	promoter Δ **1**	pat
BMKS006	arr[hg19] 18q23(77,421,290–77,904,990)x1, Δ 0.484 Mb	mat	promoter Δ **1**	pat
BMKS007, II/1 & II/2	WGS[hg19] 18q23(76,841,645–78,077,248)x1 = > Δ 1.235 Mb, del(18)(q23→qter)	pat	promoter Δ **1**	mat
BMKS008	promoter Δ 2	mat, pat	promoter Δ **2**	pat, mat
BMKS009	46,XX,r(18)(p14q23) arr[hg19] 18q23(73,376,178–78,077,248)x1 = > Δ 4.701 Mb, del(18)(q23→qter)	de novo	promoter Δ **1**	mat
BMKS010	Δ of exon 3 of *TXNL4A* (MLPA)	? (par. n. avail.)	promoter Δ **1**	? (par. n. avail.)
BMKS011	Ø		Ø	

The WGS sequences extend up to 78,017,154 where the terminal repeat region starts. The position of the endpoints for the terminal deletions are set to 78,077,248 according to the length of chromosome 18 (CRCh37/hg19). *TXNL4A* reference sequence is RefSeq NM_006701.2.
promoter Δ **1**: WGS[hg19] chr18: g.77,748,581_77,748,614delCGCCGTGCGTGCTGACGGCATGCGCGCGCGCTAG
promoter Δ **2**: WGS[hg19] chr18: g.77,748,604_77,748,637delGCGCGCGCTAGCGCCGTGCGTGCTGACGGCATGT
The overlap of the promoter Δ **1** and promoter Δ **2** deletions is underlined.
Abbreviations are as follows: pat, paternal; mat, maternal; GF, grandfather; GM, grandmother: Ø, no mutation found; WGS, whole-genome sequencing.

we set to 1×10^{-400}. Additionally, we applied a custom script to mark all variants having adjacent bases of low median base quality. All variants were then annotated with custom Perl scripts. Annotation included information about known transcripts (UCSC Known Genes and RefSeq genes), known variants (dbSNP v.135), type of mutation, and—if applicable—amino acid change in the corresponding protein. The annotated variants were inserted into an in-house database. To discover putative disease-causing variants, we queried the database to show only variants with a minor allele frequency less than 1%. To reduce false positives, we filtered out variants that had a variant quality of less than 30 or didn't pass the filter scripts. We then manually checked the raw read data of the remaining variants with the Integrative Genomics Viewer (IGV). In the affected individuals of families BMKS001, BMKS002, BMKS004, and BMKS005, we identified a common heterozygous 34 base pairs (bp) deletion (chr18: 77,748,581–77,748,614, hg19) in the putative promoter region of *TXNL4A* (Figure S4). We denote this deletion as type 1 Δ. The putative promoter is comprised of two identical 22 bp motifs (CATGCCGTCAGCACGCACGGCG, in the direction of transcription), which are separated by a 12 base pair spacer (CTAGCGCGCGCG) (Figure S5). This 56 bp region (chr18: 77,748,581–77,748,636, hg19) maps 49 to 105 bp upstream of the putative transcription start site (RefSeq NM_006701.2) and contains multiple potential transcription factor binding sites. A 34 bp deletion of the same type was also found by amplification of the *TXNL4A* promoter in the affected individuals from the remaining four families with heterozygous microdeletions (primer sequences and PCR conditions are available on request). Parental samples of nine affected individuals were available for segregation analysis. In each family, the common 34 bp deletion was inherited from the unaffected parent who did not carry the null mutation (Table 2 and Figure S6).

Interestingly, the affected individual from family BMKS008[5] was homozygous for a slightly different 34 bp deletion in the putative promoter region. It comprised the proximal 33 bp of the 56 bp region plus the preceding A nucleotide (chr18: 77,748,604–77,748,637, hg19) (Figure S5). We denote this deletion as type 2 Δ. Segregation analysis in this family showed that the homozygous promoter deletion segregated with the phenotype, whereas all heterozygotes appeared healthy (Figure S6). It is worth noting that the two resulting sequences of the deletions differed by only one nucleotide, which is the A or G, respectively, that precedes the 22 bp repeat. Deletions in the same region were not present in the 1000 Genomes Project data. This is most likely due to insufficient representation of this GC-rich region in genomic libraries that were prepared with protocols containing PCR-amplification steps. Thus, for example, this region was not covered in 200 low-coverage genomes (4- to 8-fold) sequenced in-house. We therefore analyzed 3,165 population-based samples of German and 178 of South Asian origin by PCR amplification and agarose gel electrophoresis. We identified 45 heterozygous and a single homozygous type 1 deletions as well as a single heterozygous type 2 deletion pointing to a German allele frequency of approximately 0.76%. Data about the craniofacial phenotype

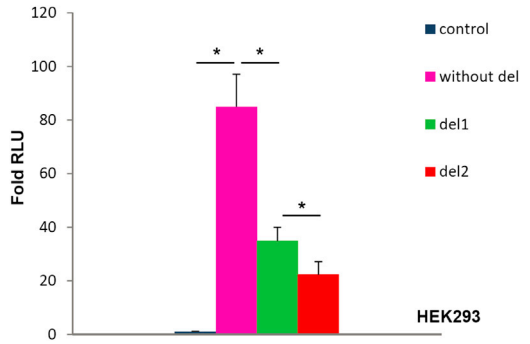

Figure 4. The 34 bp Deletion Reduces the Activity of the Putative *TXNL4A* Promoter in Dual Luciferase Assays
HEK293 cells were transfected with the reporter vector carrying the wild-type promoter without the deletion (without del) of *TXNL4A*, with either type of the 34 bp deletion (type 1 Δ or type 2 Δ), or the reporter vector without insert (control). The vector without the deletion (magenta) functioned as promoter, enhancing the luciferase expression by 85-fold in comparison with the control vector (blue). However, the vector with the type 1 Δ (green) reduced promoter's activity by 59% and with the type 2 Δ (red) by additional 13%, resulting in a total reduction of 72% in comparison with the vector without the deletion. Data represent the means and standard deviations of six technical replicates of three independent experiments and demonstrate the fold change of the relative luciferase units (RLU) compared to the control vector. Asterisks indicate statistically significant differences, with *$p < 0.05$ (Wilcoxon rank sum test).

were not available for the control samples. Haplotype analysis of the genome-sequencing data revealed that the promoter deletions were located on different haplotypes and, thus, that they occurred most likely due to recurrent events rather than a founder effect (Figure S7). Interestingly, 11 control individuals (allele frequency 0.17%) were found to be heterozygous for a 34 bp insertion (chr18: 77,748,581_77,748,614dup) into the promoter region (data not shown). The inserted sequence is identical to the 34 bp cassette that resembles the type 1 Δ (chr18: 77,748,581–77,748,614) and thus must be regarded as a head-to-tail tandem duplication. This results in a 90 bp sequence consisting of three 22 bp motifs separated by two 12 bp spacers. These results support the notion that this region is vulnerable to recurrent mutational events.

To test the hypothesis that the region affected by the upstream deletions exerts promoter function, we performed dual luciferase reporter gene assays in HEK293 cells (Figure 4). A 585 bp construct (chr18: 77,748,330–77,748,914) containing 382 bp of the wild-type promoter region, the 140 bp 5′-UTR, and the first 21 codons (63 bp) of the *TXNL4A* gene enhanced luciferase expression 85-fold compared to the empty control vector. However, promoter activity of constructs containing the type 1 Δ (del1) and type 2 Δ (del2) was reduced by 59% and 72%, respectively, compared to the wild-type construct. These data indicate that the 56 bp region (chr18: 77,748,581–77,748,637) indeed exerts promoter activity and that the entire region is essential for normal activity because the identified deletions result in a considerable reduction of expression. Furthermore, the severe attenuation of *TXNL4A* expression in case of the type 2 Δ might be sufficient to cause the phenotype in homozygous individuals in family BMKS008.

We further investigated the in vivo effect of the promoter deletion on *TXNL4A* allelic expression ratios. By sequence analysis of reverse-transcribed *TXNL4A* from persons and relatives heterozygous for a nonsense mutation, we noted that these mutant transcripts escaped nonsense-mediated mRNA decay (NMD) (not shown). This finding was not surprising. The UAG generated by stop mutation c.37C>T is followed by two *in-frame* AUGs that can function as alternative translation start sites. The c.131delT introduces a frameshift into the mRNA. However, it leads to a premature stop not before the novel codon 92, which is encoded in the last exon of *TXNL4A*. Thus, it probably escapes NMD as does the transcript with the c.349G>T (p.Glu117*) in exon 3. Primer extension analysis revealed that steady-state transcript levels of the allele carrying the wild-type ORF and the type 1 promoter deletion are reduced compared to transcript levels of the allele with the mutant ORF and wild-type promoter (Figure S8). This provides further evidence that the deletion in the promoter region negatively affects transcription.

The U5 spliceosomal complex of eight highly conserved proteins is critical for pre-mRNA splicing.[10] The orthologous yeast gene, which is also known as *DIB1/SNU16*, encodes a highly evolutionarily conserved 138 amino acid protein that is an essential component of the U4/U6•U5 tri-snRNP complex required for pre-mRNA splicing.[11] In yeast, null mutations are lethal. Thus, homozygous null mutations in humans might not be compatible with life. To investigate how reduced expression of the yeast *DIB1* gene influenced the assembly of spliceosome complexes, a haploid yeast strain was constructed with *DIB1* under control of the *GAL1* promoter. Turning off *DIB1* expression resulted in reduced assembly of the U4/U6•U5 tri-snRNP complex (Figure 5). Successful *DIB1* depletion of yeast cells is shown in Figure S9. Because *DIB1* is highly homologous to *TXNL4A*, it is likely that reduced expression of *TXNL4A* in humans would also cause reduced tri-snRNP assembly. We hypothesize that reduced tri-snRNP assembly influences the splicing of a specific subset of pre-mRNAs, resulting in the phenotypic spectrum associated with BMKS.

Here, we show that either compound heterozygosity of a promoter deletion combined with a null mutation or a homozygous promoter deletion at the *TXNL4A* locus cause the majority of cases (9 of 11) of Burn-McKeown syndrome. The index persons of two families (BMKS003 and BMKS011) had the initial diagnosis of BMKS because of overlapping clinical findings. They are clinically not distinguishable from the mutation-positive individuals (Table S1). Consistent with genetic heterogeneity for BMKS, by direct sequencing of the coding region of

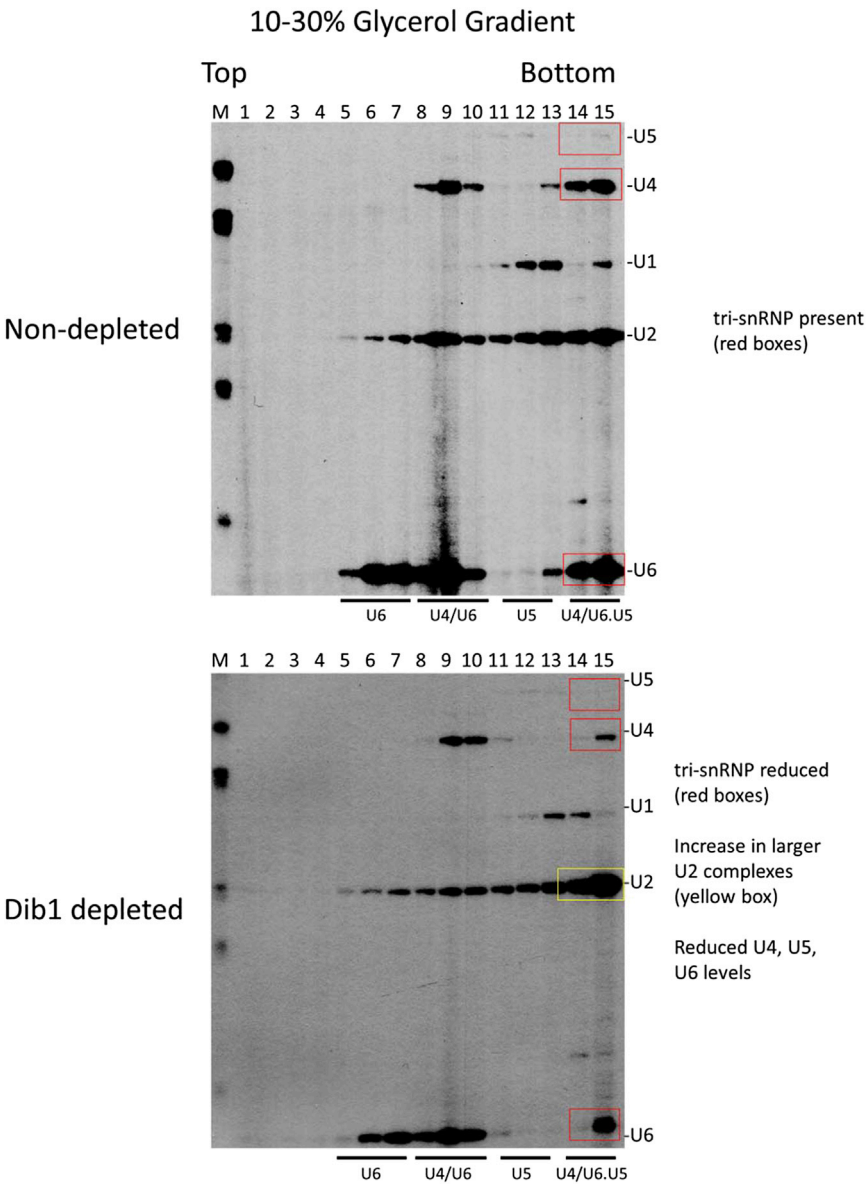

Figure 5. Depletion of *DIB1*, the Yeast Ortholog of *TXNL4A*, Results in Defective Tri-snRNP Assembly

Yeast whole-cell splicing extract from strain DIB1GALHA grown in galactose (nondepleted) or glucose (Dib1 depleted) was fractionated through a 10%–30% glycerol gradient to separate spliceosome complexes. RNA isolated from fractions was subjected to primer extension to detect the levels of the U1, U2, U4, U5, and U6 snRNAs in each fraction. The fraction numbers are indicated at the top of each panel. The locations of the U6 snRNP, U4/U6 di-snRNP, U5 snRNP, and U4/U6.U5 tri-snRNP in the fractions are indicated below each panel.

TXNL4A, amplification of the promoter region, microarray analysis, and MLPA in two families (BMKS003 and BMKS011), we did not identify *TXNL4A* mutations (not shown).

The predicted frequency of homozygous carriers of the type 1 promoter deletion of 1 in 17,300 most likely excludes this genotype as cause of BMKS. Investigations of larger cohorts are required. This genotype might lead to a mild phenotype, which escapes clinical notice. In addition, this observation indicates that the homozygous type 2 deletion in pedigree BMKS008 has a stronger effect than homozygous type 1 deletions, which is consistent with the results in the reporter gene assays.

Among 14 individuals with BMKS, 8 presented with cleft lip and/or cleft palate. To evaluate whether *TXNL4A* mutations also contribute to nonsyndromic forms of cleft lip and/or cleft palate, one of the most frequent congenital human malformations, we examined 1,148 persons with nonsyndromic orofacial clefting (994 with nonsyndromic cleft lip with or without cleft palate, 154 with nonsyndromic cleft palate only) (MIM 119530) for *TXNL4A* mutations. All individuals had been clinically assessed prior to inclusion in the study as previously described.[12–14] Persons with possible underlying syndromes, intellectual disability, or possible prenatal contributory factors such as maternal ingestion of known teratogenic substances were not included in the study. The ethnic background of affected individuals and controls had been assessed on the basis of the grandparents' descent. Controls were ethnically matched. We identified 11 heterozygous type 1 deletions, four 34 bp duplications, and one 68 bp duplication. Thus, there is no significant difference between the frequency of promoter deletions/duplications among individuals with nonsydromic clefting and the control cohort.

Because the phenotype of the Treacher Collins syndrome[15] overlaps with that of BMKS, and indeed some of the BMKS individuals had the tentative diagnosis of TCS, we also screened 50 individuals with TCS who do not have mutations in any known TCS gene for mutations and deletions at the *TXNL4A* locus. We identified one person with a heterozygous type 2 promoter deletion, which is in agreement with the rate of heterozygotes in our control cohort. This individual, however, did not have a second *TXNL4A* mutation as determined by sequence and MLPA analysis.

Our results further expand the number of inherited disorders resulting from mutation of components of the spliceosome, which besides others include Nager syndrome (MIM 154400)[16] and mandibulofacial dysostosis type Guion-Almeida (MIM 610536),[17] both also

characterized by craniofacial malformations. Because all three syndromes caused by mutations in spliceosomal components have facial dysmorphism in common, one can assume that craniofacial development is highly sensitive to dysfunction of the spliceosome.

The unusual combination of a low-frequency regulatory variant and a rare null mutation on the other allele has previously been described, for example for *RBM8A* (MIM 605313), as the cause of thrombocytopenia-absent radius syndrome (MIM 274000).[18,19] It is tempting to speculate that this mechanism of obligate compound heterozygosity might underlie a number of other rare recessive conditions where complete loss of function might be incompatible with life.

Supplemental Data

Supplemental Data include nine figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.10.014.

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Web Resources

The URLs for data provided herein are as follows:

1000 Genomes, http://browser.1000genomes.org
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
HGMD Professional, http://www.biobase-international.com/product/hgmd
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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