Haploinsufficiency of *SF3B4*, a Component of the Pre-mRNA Spliceosomal Complex, Causes Nager Syndrome

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Nager syndrome, first described more than 60 years ago, is the archetype of a class of disorders called the acrofacial dysostoses, which are characterized by craniofacial and limb malformations. Despite intensive efforts, no gene for Nager syndrome has yet been identified. In an international collaboration, FORGE Canada and the National Institutes of Health Centers for Mendelian Genomics used exome sequencing as a discovery tool and found that mutations in *SF3B4*, a component of the U2 pre-mRNA spliceosomal complex, cause Nager syndrome. After Sanger sequencing of *SF3B4* in a validation cohort, 20 of 35 (57%) families affected by Nager syndrome had 1 of 18 different mutations, nearly all of which were frameshifts. These results suggest that most cases of Nager syndrome are caused by haploinsufficiency of *SF3B4*. Our findings add Nager syndrome to a growing list of disorders caused by mutations in genes that encode major components of the spliceosome and also highlight the synergistic potential of international collaboration when exome sequencing is applied in the search for genes responsible for rare Mendelian phenotypes.

Nager syndrome (MIM 154400), first described by Nager and De Reynier in 1948,[1] is the prototype for a group of disorders collectively referred to as the acrofacial dysostoses (AFDs), which are characterized by malformations of the craniofacial skeleton and the limbs (Figure 1).[2,3] The major facial features of Nager syndrome include downslanted palpebral fissures, midface retrusion, and micrognathia, the latter of which often requires the placement of a tracheostomy in early childhood. Limb defects typically involve the anterior (i.e., radial) elements of the upper limbs and manifest as small or absent thumbs, triphalangeal thumbs, radial hypoplasia or aplasia, and radioulnar synostosis. Phocomelia of the upper limbs and, occasionally, lower-limb defects have also been reported.[4] The presence of anterior upper-limb defects as opposed to posterior upper-limb defects and the typical lack of lower-limb involvement distinguishes Nager syndrome from Miller syndrome (MIM 263750), another rare AFD.[5] However, distinguishing Nager syndrome from other AFDs, including Miller syndrome, can be challenging, and there are numerous reports of individuals and families that have been difficult for researchers to categorize.

Nager syndrome is rare, and, to date, fewer than 100 cases have been reported.[6] Most cases are sporadic, but both autosomal-dominant[7–9] and autosomal-recessive[10,11] segregation have been reported. This has led to the widespread speculation that Nager syndrome is genetically heterogeneous. The rarity of this syndrome, which typically occurs as a simplex case, and the different modes of inheritance have made identification of the causal gene(s) intractable to conventional gene-discovery approaches. Given the phenotypic overlap between Nager and Miller syndromes and given our recent finding that Miller syndrome is caused by mutations in *DHODH* (MIM 126064),[12] we and others suspected that the disorders might be allelic or caused by genes in the pyrimidine biosynthetic pathway. However, screening of families affected by Nager syndrome ($n > 12$) did not reveal any mutations in these genes (Bamshad et al., unpublished data).

To identify a gene for Nager syndrome, investigators at the University of Calgary as part of the FORGE (Finding of Rare Disease Genes) Canada Consortium and investigators at the University of Washington (UW) as one of the National Institutes of Health Centers for Mendelian

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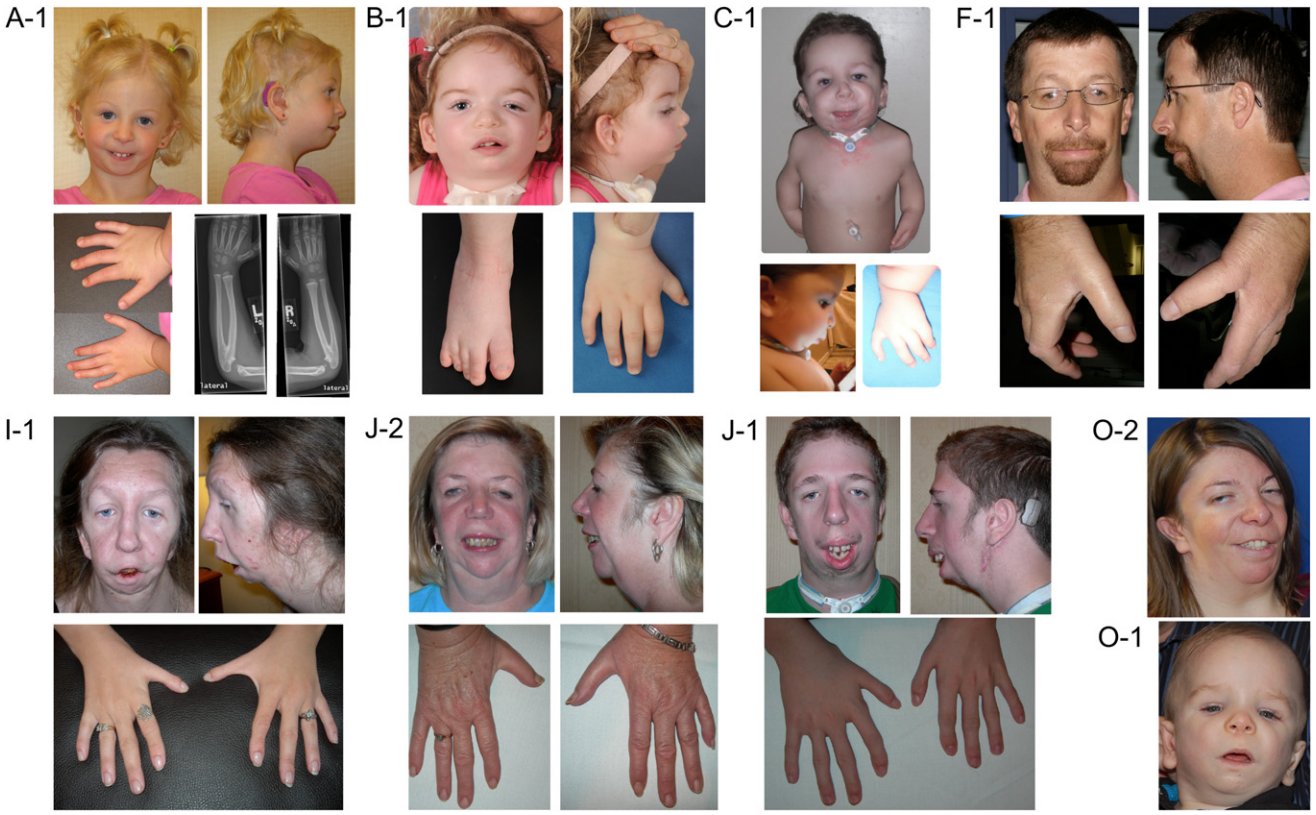

Figure 1. Characteristic Craniofacial and Limb Malformation in Individuals with Nager Syndrome and *SF3B4* Mutations
Typical features of the face include downslanted palpepral fissures, malar flattening, and variable external ear malformations. Hand abnormalities include small thumbs and, in some cases, upper-limb reduction defects (C-1). Labels correspond to those in Tables 1–3, where a detailed description of each individual is provided.

Genomics independently performed exome sequencing.[13] Between both institutions, a total of 35 independent families (a total of 41 affected individuals) afflicted with Nager syndrome were available for study (Figure 1 and Tables 1–3). These families consisted of 28 simplex families, including one in which the parents were consanguineous, and seven multiplex families: two with mother-to-son transmissions, one with a father-to-daughter transmission, two with mother-to-daughter transmissions, one consisting of an affected mother and her two affected sons, and one with a pair of affected monozygotic twins (Table S1, available online). An experienced clinical geneticist was responsible for each diagnosis of Nager syndrome, each participant provided informed consent, and studies were approved by the institutional review boards of the University of Calgary, the University of Washington, and Seattle Children's Hospital.

Exome sequencing of five independent simplex cases was performed by the FORGE Canada team at the McGill University and Genome Québec Innovation Centre according to the manufacturer's (Illumina) standard protocols for the Agilent SureSelect 50 Mb exome enrichment kit, and captured targets were sequenced on a HiSeq2000 sequencer. Reads were aligned to a human reference (hg19) with the Burrows-Wheeler Aligner (BWA),[14] and indel realignment was performed with the GATK.[15] Duplicate reads were then marked with Picard and excluded from downstream analyses. Coverage of consensus coding sequence (CCDS) bases was assessed with GATK, which showed that all samples had >90% coverage of CCDS bases and a depth of at least 10×. For each sample, single-nucleotide variants (SNVs) and short insertions and deletions (indels) were called with the use of SAMtools pileup[16] with the extended base alignment quality (BAQ) adjustment (-E), and they were then quality filtered so that at least 20% of the reads supported the variant call. We annotated variants by using both Annovar[17] and custom scripts to identify whether they affected protein-coding sequence and whether they were previously observed in dbSNP132, 1000 Genomes Project data (Nov. 2011), or ~300 exomes previously obtained at the center.

At the University of Washington, exome sequencing was performed on four simplex cases with Nager syndrome and three unrelated individuals from multiplex families; these three latter individuals included one affected child born to an affected parent and two affected parents, each of whom had at least one affected child. In brief, we performed exome capture on a shotgun library created from 1 μg of genomic DNA by using the ~62 Mb target from Roche Nimblegen SeqCap EZ v2.0 (~300,000 exons and flanking sequence). Exome capture was followed by massively parallel sequencing on a HiSeq 2000 sequencer

Table 1. Clinical Features of Nager-Syndrome Patients A-1 through H-1 with *SF3B4* Mutations

	A-1	B-1	C-1	D-1	E-1	F-1	F-2	G-1	H-1
Inheritance	sporadic	sporadic	sporadic	sporadic	sporadic	sporadic; father of F-2	AD; daughter	sporadic	sporadic
Gender	female	female	male	female	female	male	female	female	female
Age (years)	5	4	2	24	23	38	1	12	5
Downslanted palpebral fissures	+	+	+	+	+	+	ND	–	ND
Absent lower eyelashes	sparse	ND	ND	+	–	ND	ND	–	+
Midface retrusion	+	+	+	+	+	ND	ND	ND	ND
Micrognathia	+	+	+	+	+	+	+	+	+
Ankylosis of TM joint		ND	ND	–	–	ND	ND	+	ND
Abnormal palate	cleft soft palate	–	cleft	–	–	–	cleft	cleft	cleft
Tracheotomy	–	+	+	+	–	–	+	+	ND
Abnormal ears	+	+	+	+	mild	–	ND	ND	ND
Hearing loss	+			+	+	–	+	+	+
Radial ray abnormality	–	+	absent bilateral	–	–	ND	ND	+	+
Abnormal thumbs	stiff bilateral	+	absent bilateral	small right thumb and slender left thumb	hypoplasia	ND	ND	absent bilateral	+
Radioulnar synostosis	bilateral	+	–	unilateral	bilateral	+	+	+	ND
Development	normal	normal	delayed	normal	speech delay only	normal	ND	ND	ND
Other malformations	–	VSD and diaphragmatic hernia	fused 1st and 2nd right metacarpals and bilateral foot deformities	abnormal teeth, partial absence of left fingers 3–5, slender haluces, and hallux valgus	asymmetric face, VPI, and cervical ribs	valgus laxity of knees and ankles	subglottic stenosis	–	–
SF3B4 mutation	c.1A>G[a]	c.864delT[a]	c.1147delC[a]	c.913+1G>A	c.1148dupA	c.1A>G[b]	c.1A>G[b]	c.827dupC	c.1232delC

The following abbreviations are used: TM, temporomandibular; ND, not determined; VPI, velopharyngeal insufficiency; AD, autosomal dominant; and VSD, ventriculoseptal defect.
[a]Confirmed de novo mutation.
[b]Confirmed familial mutation.

(Illumina). BAM files were also aligned to a human reference (hg19) with the BWA,[14] and duplicate reads were removed with Picard. Variant detection and genotyping were performed with the UnifiedGenotyper tool from GATK (refv1.529). Exome completion was defined as having >90% of the exome target at >8× coverage. Typically, this requires that the target have a mean coverage of 60–80×. An automated pipeline, the SeattleSeq Annotation Server, was used for the annotation of variants. Novel variants are defined as those not observed in dbSNP version 134 or in 1,200 exomes drawn from a subset of samples from the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP).

Because the mode of inheritance of Nager syndrome for most cases was unclear, discrete filtering for novel variants in the same gene shared among cases was performed under both autosomal-dominant and autosomal-recessive models. In neither cohort was a gene found to be shared among all the unrelated Nager-syndrome cases under a recessive mode of inheritance, i.e., those who were compound heterozygous for two novel variants or homozygous for a rare novel variant. This suggested that if Nager syndrome were an autosomal-recessive disorder, it would have high locus heterogeneity. Under an autosomal-dominant model, one gene, *TTN* (MIM 188840), in the FORGE cohort and two genes, *MUC12* (MIM 604609) and *MUC16*

Table 2. Clinical Features of Nager-Syndrome Patients I-1 through M-3 with *SF3B4* Mutations

	I-1	J-1	J-2	K-1	L-1	M-1	M-2	M-3	N-1
Inheritance	sporadic	AD; son	sporadic; mother of J-1	sporadic	sporadic	sporadic; father of M-2, M-3	AD; son	AD; son	Sporadic
Gender	female	male	female	female	female	female	male	male	female
Age (years)	28	17	56	15	4	49	20	17	13
Downslanted palpebral fissures	+	+	+	ND	+	+	+	+	+
Absent lower eyelashes	sparse	+	–	ND	+	ND	sparse	+	+
Midface retrusion	+	+	ND	ND	ND	+	+	+	+
Micrognathia	+	+	+	ND	+	–	+	+	+
Ankylosis of TM joint	ND	ND	ND	ND	ND	ND	ND	ND	+
Abnormal palate	–	ND	ND	ND	–	cleft	–	+	high arched; cleft soft palate
Tracheotomy	ND	+	ND	ND	–	–	+	–	+
Abnormal ears	+	+	ND	ND	–	cupped	cupped; narrow canals	cupped; narrow canals	+
Hearing loss	+	+	ND	ND	+	+	+	+	+
Radial ray abnormality	ND	ND	ND	ND	+	–	–	–	–
Abnormal thumbs	small	+	ND	ND	absent left thumb and fused right DIP	small	absent right thumb and small left thumb	small right thumb compared to left	absent right thumb and small and stiff left thumb
Radioulnar synostosis	ND	ND	ND	ND	+	–	right > left	left	+
Development	ND	normal	ND	ND	ND	ND	delayed speech and fine motor	delayed	delayed
Other malformations	–	–	–	–	limited ROM in all extremities at birth, arachnodactly, and small 5th fingers	–	short stature (3–5%), dacryostenosis, and small first toes and first metatarsals	dacryostenosis, short first metatarsals, and sandal gap	limited ROM in elbows and shoulders, camptodactyly, left clubfoot, renal abnormalities, VSD, and dacryostenosis
SF3B4 mutation	c.1060dupC[a]	c.1A>G[b]	c.1A>G[b]	c.452C>A	c.836_837 insGGGTATG[a]	c.1199delC[b]	c.1199delC[b]	c.1199delC[b]	c.88delT

The following abbreviations are used: TM, temporomandibular; ND, not determined; AD, autosomal dominant; ROM, range of motion; VSD, ventriculoseptal defect; and DIP, distal interphalangeal joint.
[a]Confirmed de novo mutation.
[b]Confirmed familial mutation.

(MIM 606154), in the UW cohort had novel variants shared among all unrelated cases. These large genes are frequent sources of false-positive calls and were excluded from consideration.

In both cohorts, several genes had novel or rare variants shared among two, three, or four cases with Nager syndrome, and prioritizing these candidates was not possible. We further filtered the gene list from the FORGE cohort by assigning higher priority to variants predicted to have a greater deleterious impact on protein function (i.e., nonsense, frameshift, initiation-codon, and splice-site variants) and then reanalyzed the list. A single gene, *SF3B4* (MIM 605593), which encodes a component of the U2SNP of the major spliceosome, was found to have different novel variants in two unrelated cases of Nager syndrome (Table S2). In the UW cohort, subsequent discrete filtering limited to only genes with novel variants shared among the three familial-Nager-syndrome cases identified three candidate genes, including *SF3B4*. In two of these cases, the variant identified (c.1A>G [p.M1?]) was identical to one of the *SF3B4* variants found in the FORGE cohort. Sanger sequencing subsequently

Table 3. Clinical Features of Nager-Syndrome Patients O-1 through T-1 with *SF3B4* Mutations

	O-1	O-2	P-1	Q-1	R-1	R-2	S-1	T-1
Inheritance	AD; son	sporadic; mother of O-1	sporadic	sporadic	AD; daughter	AD; mother	sporadic	sporadic
Gender	male	female	male	female	female	female	female	male
Age (years)	1	28	2	1	7	ND	4	2
Downslanted palpebral fissures	+	+	+	ND	+	ND	+	+
Absent lower eyelashes	ND	ND	decreased	minimal	ND	ND	ND	ND
Midface retrusion	ND	ND	+	+	+	+	+	+
Micrognathia	+	+	+	+	+	ND	+	+
Ankylosis of TM joint	ND	ND	ND	ND	ND	ND	ND	ND
Abnormal palate	abnormal soft palate	abnormal soft palate	abnormal soft palate	abnormal soft palate	+	ND	high arched	+
Tracheotomy	ND	ND	ND	ND	ND	ND	+	ND
Abnormal ears	+	+	+	+	+	ND	+	+
Hearing loss	+	+	+	+	+	ND	ND	+
Radial ray abnormality	ND	ND	+	+	short	ND	+	+
Abnormal thumbs	absent right thumb and small left thumb	small	proximally placed and stiff	absent bilateral	stiff bilateral	stiff bilateral	+	+
Radioulnar synostosis	–	bilateral	right	–	ND	ND	ND	+
Development	ND	ND	–	ND	ND	ND	ND	ID
Other malformations	–	–	–	hair extension on cheek, strabismus, mitral valve prolapse, and limited ROM in elbows	–	–	short stature and bilateral syndactyly of the 4th and 5th toes	–
SF3B4 mutation	c.1147dupC[a]	c.1147dupC[a]	c.769delA	c.625C>T	c.1252_1258del CTTCGAG	not tested	c.796dupA	c.661_664dup CCCA

The following abbreviations are used: ID, intellectual disability; TM, temporomandibular; ND, not determined; AD, autosomal dominant; and ROM, range of motion.
[a]Confirmed familial mutation.

confirmed that all four novel variants were present in *SF3B4* and that the variants were de novo in the FORGE simplex cases and inherited from an affected parent (one case) and transmitted to an affected child (one case) in the UW families.

Further evidence supporting the hypothesis that mutations in *SF3B4* cause Nager syndrome was provided by a recent finding that mutations in *EFTUD2* (MIM 603892), which also encodes a component of the spliceosome, cause a mandibulofacial dysostosis with microcephaly (MFDM [MIM 610536]) whose craniofacial findings overlap the symptoms of Nager syndrome.[18] Furthermore, an individual had previously been reported to have an unclassifiable AFD and a 1q21.2 deletion spanning the region containing *SF3B4*.[19] Collectively, these results strongly suggest that mutations in *SF3B4* cause Nager syndrome.

To determine the extent to which *SF3B4* mutations explain cases of Nager syndrome, we used Sanger sequencing to screen *SF3B4* in a validation cohort of 23 additional unrelated families affected by Nager syndrome. We also screened the eight individuals who underwent exome sequencing but in whom no *SF3B4* mutation was found. Collectively, between the initial study cohort and our validation studies, *SF3B4* mutations were found in 20 of the 35 (57%) families, including five of the seven families with more than one individual affected by Nager syndrome. For simplex cases, 15 of 28 (54%) had mutations in *SF3B4*, and in each of the five simplex cases for which DNA was available from both parents, the *SF3B4* mutation was confirmed to have arisen de novo. No *SF3B4* mutation was identified in the single consanguineous family in our cohort. Overall, 25 out of 41 (61%)

Table 4. Summary of *SF3B4* Mutations

Family	Nucleotide	Exon	Peptide	Inheritance	Predicted Effect
A, F, J	c.1A>G	1	p.Met1?	de novo (A) and familial (F and J)	loss of initiator methionine
N	c.88delT	2	p.Trp30Glyfs*10	NA	frameshift
K	c.452C>A	3	p.Ser151*	NA	nonsense
Q	c.625C>T	3	p.Gln209*	NA	nonsense
T	c.661_664dupCCCA	3	p.Asn222Thrfs*265	NA	frameshift
P	c.769delA	4	p.Ile257Tyrfs*63	NA	frameshift
S	c.796dupA	4	p.Met266Asnfs*220	NA	frameshift
G	c.827dupC	4	p.Ser277Ilefs*209	NA	frameshift
L	c.836_837insGGGTATG	4	p.Thr280Glyfs*208	de novo	frameshift
B	c.864delT	4	p.His288Glnfs*32	de novo	frameshift
D	c.913+1G>A	intron 4	NA	NA	splice
I	c.1060dupC	5	p.Arg354Profs*132	de novo	frameshift
C	c.1147delC	6	p.His383Metfs*75	de novo	frameshift
O	c.1147dupC	6	p.His383Profs*103	familial	frameshift
E	c.1148dupA	6	p.His383Glnfs*103	NA	frameshift
M	c.1199delC	6	p.Pro400Leufs*58	familial	frameshift
H	c.1232delC	6	p.Pro411Glnfs*47	NA	frameshift
R	c.1252_1258delCTTCGAG	6	p.Leu418Alafs*38	familial[a]	frameshift

The following abbreviation is used: NA, parental DNAs were unavailable for the confirmation of inheritance.
[a]Mother's sample was not available for testing.

Nager-syndrome-affected individuals in our cohort had mutations in *SF3B4* (Tables 1–3 and Table S1).

A total of 18 unique mutations were identified, and these include 14 frameshift, two nonsense, one splicing, and one missense mutation (c.1A>G [p.Met1?]), the last of which is predicted to abolish the methionine initiation codon and was the only recurrent (i.e., in three unrelated families) mutation found (Table 4 and Figure 2). Four of the Nager-syndrome cases with a *SF3B4* variant that was missed by exome sequencing were found to have indels, and manual inspection of the BAM files revealed that they all occurred in regions with relatively low coverage (i.e., <8×). None of the *SF3B4* missense, nonsense, or splice-site mutations identified in the participants were found in >10,800 chromosomes sequenced as part of the NHLBI-ESP (see Web Resources). Indeed, only 22 rare (minor allele frequency of <0.5%) SNVs, six of which were missense variants, were observed in this ESP dataset. Accordingly, *SF3B4* is highly conserved, and most new mutations are likely to be selected against, consistent with the observation that most of the mutations we identified are de novo events.

All of the *SF3B4* variants identified are predicted to encode a truncated polypeptide chain or an elongated polypeptide with an altered 3′ end in the absence of nonsense-mediated RNA decay (Figure 2C). Together with the observation that a deletion of chromosomal region 1q12–q21.1 or q21.3, which encompasses *SF3B4*, has been reported in a child with characteristics of Nager syndrome,[19] our findings suggest that Nager syndrome probably results from haploinsufficiency of *SF3B4*.

SF3B4 encodes SAP49, a spliceosomal protein that is one of seven core proteins of the mammalian SF3B complex and is highly conserved with two RNA recognition motifs (RRMs) followed by a proline-glycine rich domain (Figure 2B). During assembly of the U2SNP prespliceosomal complex, SAP49 binds to the pre-mRNA just upstream of the branch point sequence but also interacts specifically with other U2 snRNPS, particularly SAP145, suggesting that SAP49 plays a crucial role in tethering the U2 snRNP to the branch site.[20]

Our discovery adds Nager syndrome to an emerging group of disorders caused by mutations in genes that encode subunits of the spliceosome. Mutations in splicing factors were initially reported in patients with autosomal-dominant retinitis pigmentosa.[21–24] Subsequently, biallelic mutations in *RNU4ATAC* (MIM 601428), a component of the minor spliceosome required for excision of the U12 class of introns, were reported to cause lethal microcephalic osteodysplastic primordial dwarfism type I (MIM 210710).[25,26] Most recently, mutations in *EFTUD2* have been reported in individuals with MFDM, a disorder with many features that overlap those of Nager syndrome.[18] Although the limbs are typically normal in MFDM, the

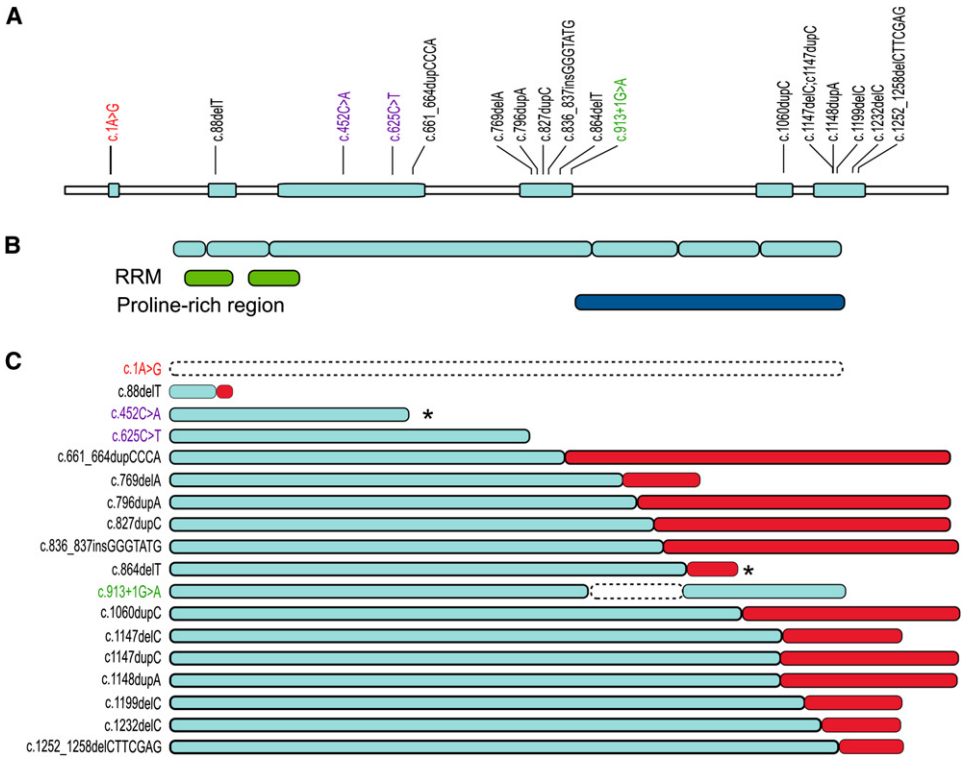

Figure 2. Location of *SF3B4* Mutations that Cause Nager Syndrome
(A) Distribution of mutations. Red text indicates an initiator methionine mutation, purple text indicates nonsense mutations, green text indicates a splice mutation, and black text indicates frameshift mutations.
(B) Domain structure of SAP49. The following abbreviation is used: RRM, RNA recognition motif.
(C) Predicted translational effect of each *SF3B4* mutation. Dotted lines indicate a deletion, the blue box indicates reference amino acid sequences, the red bar indicates altered amino acid sequences, and asterisks indicate potential for nonsense-mediated RNA decay.

fact that some individuals have thumb abnormalities suggests that Nager syndrome and MFDM could be confused with one another.[18] To this end, we screened *EFTUD2* in all of the UW cohort Nager-syndrome individuals who did not have mutations in *SF3B4*, and we identified a single individual with a novel nonsense mutation (c.2495C>G [p.Tyr832*]). This mutation affects the amino acid residue adjacent to an *EFTUD2* mutation (c.2493C>A [p.Tyr831*]) found in one family with MFDM.[18] In retrospect, this individual also had microcephaly, suggesting that MFDM rather than Nager syndrome is the appropriate diagnosis.

It is clear that SAP49 and U5-116KD, encoded by *EFTUD2*, are critical components of the major spliceosome, but their role in the pathogenesis of defects in craniofacial and limb development is unknown. The mouse homolog of *SF3B4*, SAP49 (mSAP49), is highly conserved and shows broad expression in adult tissues.[27,28] Whole-mount in situ hybridization shows high expression at day 11 after conception in limbs and somites, as well as dynamic patterns of expression in the developing heart.[27] These results suggest that mSAP49 expression during development varies in both a time- and tissue-specific manner. Whereas limb malformations are frequently seen in Nager syndrome, the fact that only 2 out of 20 (10%) *SF3B4*-mutation carriers had cardiac defects suggests that *SF3B4* haploinsufficiency has only minor effects on human heart development.

Spliceosomes are not only critical for mediating intron splicing but are also key regulators of alternative splicing[29,30] and, as such, play an important role in the control of gene-expression pathways. Furthermore, alternative splicing of mRNA is a major source of protein diversity, and tissue-specific alternative splicing further increases the diverse cellular functions of proteins. Spliceosomes might directly regulate developmental genes via the control of splicing or tissue specificity. Thus, the defects observed in individuals with Nager syndrome could be due to aberrant splicing of genes involved in craniofacial and limb development. However, this hypothesis is not supported by evidence from *sf3b1*$^{+/-}$-heterozygous-null mice. Isono et al.[31] reported that *Sf3b1*$^{+/-}$ heterozygous mice exhibited skeletal malformations concomitant with ectopic *Hox* expression. Because polycomb group (PcG) proteins are required for the stable repression of *Hox*, Isono et al. measured the transcript levels for five PcG genes and three Hox genes.[31] Despite *Sf3b1* expression levels that were reduced by half in the *sf3b1*$^{+/-}$ mice, transcript levels of the PcG and Hox genes were similar to those of the controls. Thus, the homeotic transformations in *sf3b1*$^{+/-}$ mice appear to be independent of alterations in overall gene transcription.

Further evidence that mutations in *SF3B4* might cause Nager syndrome via a mechanism unrelated to its role in mediating splicing is provided by studies linking SAP49 to bone morphogenetic protein (BMP) signaling. BMP proteins are multifunctional growth factors with roles in early embryogenesis and skeletogenesis. BMP2 and BMP4 are the main sources of BMP signaling in the developing limbs and also play a critical role in chondrogenesis.[32] To find molecules specific to BMP-mediated signal transduction, Watanabe et al.[33] identified SAP49 by using BMPR-IA, a BMP receptor, as a bait protein in a yeast two-hybrid screen. Coimmunoprecipitation and immunoblot analysis confirmed their interaction in mammalian cells.[33] SAP49 is also found in cell-membrane fractions, and overexpression of SAP49 inhibits BMP-2-mediated osteogenic and chondrocytic differentiation. These findings suggest that SAP49, in addition to its role in mRNA splicing, might also specifically inhibit BMP-mediated osteochondral cell differentiation[33]

In summary, we applied exome sequencing across 12 unrelated cases to discover that mutations in *SF3B4* cause Nager syndrome and explain ~60% of the cases in our overall cohort, including five out of seven multiplex families and 54% of simplex cases. The clinical characteristics of individuals with Nager syndrome caused by *SF3B4* mutations were indistinguishable from those without *SF3B4* mutations. Although partial or whole-gene deletions or mutations in noncoding regulatory regions of *SF3B4* might explain some of the *SF3B4* mutation-negative cases in our cohorts, this observation is strong evidence that Nager syndrome is genetically heterogeneous. Our findings also indicate that the phenotypic overlap between AFD Nager syndrome and MFDM is probably due in part to a shared defect in the same biological process and suggests that similar clinical phenotypes for which the cause has yet to be identified might be due to variants in genes that have a similar role.

Supplemental Data

Supplemental Data include two tables and supplemental acknowledgments and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

Exome Variant Server, http://evs.gs.washington.edu/EVS/
FASTX Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/
GATK, http://www.broadinstitute.org/gsa/wiki/
Human Genome Varation, http://www.hgvs.org/mutnomen/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Picard Tools, http://picard.sourceforge.net/
SAMtools, http://samtools.sourceforge.net/
SeattleSeq, http://snp.gs.washington.edu/SeattleSeqAnnotation/

Accession Numbers

The RefSeq accession number for the *SF3B4* sequence reported in this paper is NM_005850.4.

References

1. Nager, F., and Reynier, D. (1948). Das Gehorogan bei den angeborenen Kopfmissnildungen. Pract. Otorhinolaryngol. (Basel) *10*, 1–128.
2. Halal, F., Herrmann, J., Pallister, P.D., Opitz, J.M., Desgranges, M.F., and Grenier, G. (1983). Differential diagnosis of Nager acrofacial dysostosis syndrome: Report of four patients with Nager syndrome and discussion of other related syndromes. Am. J. Med. Genet. *14*, 209–224.
3. Opitz, J.M. (1987). Nager "syndrome" versus "anomaly" and its nosology with the postaxial acrofacial dysostosis syndrome of Genée and Wiedemann. Am. J. Med. Genet. *27*, 959–963.
4. Le Merrer, M., Cikuli, M., Ribier, J., and Briard, M.L. (1989). Acrofacial dysostoses. Am. J. Med. Genet. *33*, 318–322.
5. Miller, M., Fineman, R., and Smith, D.W. (1979). Postaxial acrofacial dysostosis syndrome. J. Pediatr. *95*, 970–975.
6. Schlieve, T., Almusa, M., Miloro, M., and Kolokythas, A. (2012). Temporomandibular joint replacement for ankylosis correction in Nager syndrome: Case report and review of the literature. J. Oral Maxillofac. Surg. *70*, 616–625.

7. Aylsworth, A.S., Lin, A.E., and Friedman, P.A. (1991). Nager acrofacial dysostosis: Male-to-male transmission in 2 families. Am. J. Med. Genet. *41*, 83–88.
8. Hall, B.D. (1989). Nager acrofacial dysostosis: Autosomal dominant inheritance in mild to moderately affected mother and lethally affected phocomelic son. Am. J. Med. Genet. *33*, 394–397.
9. McDonald, M.T., and Gorski, J.L. (1993). Nager acrofacial dysostosis. J. Med. Genet. *30*, 779–782.
10. Chemke, J., Mogilner, B.M., Ben-Itzhak, I., Zurkowski, L., and Ophir, D. (1988). Autosomal recessive inheritance of Nager acrofacial dysostosis. J. Med. Genet. *25*, 230–232.
11. Kennedy, S.J., and Teebi, A.S. (2004). Newly recognized autosomal recessive acrofacial dysostosis syndrome resembling Nager syndrome. Am. J. Med. Genet. A. *129A*, 73–76.
12. Ng, S.B., Buckingham, K.J., Lee, C., Bigham, A.W., Tabor, H.K., Dent, K.M., Huff, C.D., Shannon, P.T., Jabs, E.W., Nickerson, D.A., et al. (2010). Exome sequencing identifies the cause of a mendelian disorder. Nat. Genet. *42*, 30–35.
13. Bamshad, M.J., Ng, S.B., Bigham, A.W., Tabor, H.K., Emond, M.J., Nickerson, D.A., and Shendure, J. (2011). Exome sequencing as a tool for Mendelian disease gene discovery. Nat. Rev. Genet. *12*, 745–755.
14. Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754–1760.
15. McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., and DePristo, M.A. (2010). The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. *20*, 1297–1303.
16. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R.; 1000 Genome Project Data Processing Subgroup. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078–2079.
17. Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. *38*, e164.
18. Lines, M.A., Huang, L., Schwartzentruber, J., Douglas, S.L., Lynch, D.C., Beaulieu, C., Guion-Almeida, M.L., Zechi-Ceide, R.M., Gener, B., Gillessen-Kaesbach, G., et al; FORGE Canada Consortium. (2012). Haploinsufficiency of a spliceosomal GTPase encoded by EFTUD2 causes mandibulofacial dysostosis with microcephaly. Am. J. Hum. Genet. *90*, 369–377.
19. Waggoner, D.J., Ciske, D.J., Dowton, S.B., and Watson, M.S. (1999). Deletion of 1q in a patient with acrofacial dysostosis. Am. J. Med. Genet. *82*, 301–304.
20. Champion-Arnaud, P., and Reed, R. (1994). The prespliceosome components SAP 49 and SAP 145 interact in a complex implicated in tethering U2 snRNP to the branch site. Genes Dev. *8*, 1974–1983.
21. McKie, A.B., McHale, J.C., Keen, T.J., Tarttelin, E.E., Goliath, R., van Lith-Verhoeven, J.J., Greenberg, J., Ramesar, R.S., Hoyng, C.B., Cremers, F.P., et al. (2001). Mutations in the pre-mRNA splicing factor gene PRPC8 in autosomal dominant retinitis pigmentosa (RP13). Hum. Mol. Genet. *10*, 1555–1562.
22. Tanackovic, G., Ransijn, A., Ayuso, C., Harper, S., Berson, E.L., and Rivolta, C. (2011). A missense mutation in PRPF6 causes impairment of pre-mRNA splicing and autosomal-dominant retinitis pigmentosa. Am. J. Hum. Genet. *88*, 643–649.
23. Vithana, E.N., Abu-Safieh, L., Allen, M.J., Carey, A., Papaioannou, M., Chakarova, C., Al-Maghtheh, M., Ebenezer, N.D., Willis, C., Moore, A.T., et al. (2001). A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). Mol. Cell *8*, 375–381.
24. Zhao, C., Bellur, D.L., Lu, S., Zhao, F., Grassi, M.A., Bowne, S.J., Sullivan, L.S., Daiger, S.P., Chen, L.J., Pang, C.P., et al. (2009). Autosomal-dominant retinitis pigmentosa caused by a mutation in SNRNP200, a gene required for unwinding of U4/U6 snRNAs. Am. J. Hum. Genet. *85*, 617–627.
25. He, H., Liyanarachchi, S., Akagi, K., Nagy, R., Li, J., Dietrich, R.C., Li, W., Sebastian, N., Wen, B., Xin, B., et al. (2011). Mutations in U4atac snRNA, a component of the minor spliceosome, in the developmental disorder MOPD I. Science *332*, 238–240.
26. Nagy, R., Wang, H., Albrecht, B., Wieczorek, D., Gillessen-Kaesbach, G., Haan, E., Meinecke, P., de la Chapelle, A., and Westman, J. (2011). Microcephalic osteodysplastic primordial dwarfism type I with biallelic mutations in the RNU4ATAC gene. Clin. Genet. Published online August 4, 2012. 10.1111/j.1399-0004.2011.01756.x.
27. Ruiz-Lozano, P., Doevendans, P., Brown, A., Gruber, P.J., and Chien, K.R. (1997). Developmental expression of the murine spliceosome-associated protein mSAP49. Dev. Dyn. *208*, 482–490.
28. Nishanian, T.G., and Waldman, T. (2004). Interaction of the BMPR-IA tumor suppressor with a developmentally relevant splicing factor. Biochem. Biophys. Res. Commun. *323*, 91–97.
29. Johnson, T.L., and Vilardell, J. (2012). Regulated pre-mRNA splicing: The ghostwriter of the eukaryotic genome. Biochim Biophys Acta. Published online January 9, 2012.
30. Blaustein, M., Pelisch, F., and Srebrow, A. (2007). Signals, pathways and splicing regulation. Int. J. Biochem. Cell Biol. *39*, 2031–2048.
31. Isono, K., Mizutani-Koseki, Y., Komori, T., Schmidt-Zachmann, M.S., and Koseki, H. (2005). Mammalian polycomb-mediated repression of Hox genes requires the essential spliceosomal protein Sf3b1. Genes Dev. *19*, 536–541.
32. Robert, B. (2007). Bone morphogenetic protein signaling in limb outgrowth and patterning. Dev. Growth Differ. *49*, 455–468.
33. Watanabe, H., Shionyu, M., Kimura, T., Kimata, K., and Watanabe, H. (2007). Splicing factor 3b subunit 4 binds BMPR-IA and inhibits osteochondral cell differentiation. J. Biol. Chem. *282*, 20728–20738.