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ARTICLE

Further delineation of the *SATB2* phenotype

Dennis Döcker[1], Max Schubach[2], Moritz Menzel[2], Marita Munz[3], Christiane Spaich[1], Saskia Biskup[1,2,3,4] and Deborah Bartholdi*[,1,4]

***SATB2* is an evolutionarily highly conserved chromatin remodeling gene located on chromosome 2q33.1. Vertebrate animal models have shown that *Satb2* has a crucial role in craniofacial patterning and osteoblast differentiation, as well as in determining the fates of neuronal projections in the developing neocortex. In humans, chromosomal translocations and deletions of 2q33.1 leading to *SATB2* haploinsufficiency are associated with cleft palate (CP), facial dysmorphism and intellectual disability (ID). A single patient carrying a nonsense mutation in *SATB2* has been described to date. In this study, we performed trio-exome sequencing in a 3-year-old girl with CP and severely delayed speech development, and her unaffected parents. Previously, the girl had undergone conventional and molecular karyotyping (microarray analysis), as well as targeted analysis for different diseases associated with developmental delay, including Angelman syndrome, Rett syndrome and Fragile X syndrome. No diagnosis could be established. Exome sequencing revealed a *de novo* nonsense mutation in the *SATB2* gene (c.715C>T; p.R239*). The identification of a second patient carrying a *de novo* nonsense mutation in *SATB2* confirms that this gene is essential for normal craniofacial patterning and cognitive development. Based on our data and the literature published so far, we propose a new clinically recognizable syndrome – the *SATB2*-associated syndrome (SAS). SAS is likely to be underdiagnosed and should be considered in children with ID, severe speech delay, cleft or high-arched palate and abnormal dentition with crowded and irregularly shaped teeth.**

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INTRODUCTION

The *special AT-rich sequence-binding protein 2* (*SATB2*) gene (OMIM *608148, first described as *KIAA1034*),[1] is located in a relatively gene-poor region on chromosome 2q33.1. It encodes a protein of 733 amino acids, which shows a high degree of evolutionary conservation.[2] The SATB2 protein is involved in chromatin remodeling and transcriptional regulation through its ability to bind AT-rich DNA sequences known as matrix attachment regions (MAR).[3,4] Depending on the locus, it can either repress or activate transcription of specific genes.[5]

Chromosomal translocations disrupting *SATB2* have been associated with cleft palate (CP) and learning difficulties (OMIM #119540).[6–9] *SATB2* is deleted in the contiguous 2q32q33 deletion syndrome, which is characterized by intellectual disability (ID), feeding difficulties with growth retardation, dysmorphic features, thin and sparse hair, cleft or high-arched palate, tooth abnormalities and behavioral problems.[10–16] Subsequently, smaller deletions of 2q33.1 with similar phenotypes have been described (all of them encompassing *SATB2*), suggesting that haploinsufficiency of this gene might be causative for the core phenotype of the 2q32q33 microdeletion syndrome (see Figure 1).[10,17]

Only one patient carrying a nonsense mutation in *SATB2* has been reported in the literature to date.[18] This individual was identified by screening of 59 patients with craniofacial dysmorphisms with or without ID. Recently, a patient carrying a missense mutation in *SATB2* which was judged as probably damaging was identified in screening a cohort of patients with ID for *de novo* variants.

Here, we report and characterize a second patient carrying the same *de novo* nonsense mutation in *SATB2* as the patient described previously (c.715C>T; p.R239*). The girl displays a specific pattern of craniofacial malformations and severe speech impairment. We propose the delineation of a clinically recognizable syndrome – the *SATB2*-associated syndrome (SAS), which is likely to be underdiagnosed. This report should enable a targeted diagnostic approach in similar patients.

CLINICAL REPORT

The index patient is a 3-year-old girl, the first child of healthy non-consanguineous parents of central European descent. She was born after an uneventful pregnancy by vaginal delivery at 42 weeks gestation. APGAR scores were 9/10/10, and the pH value of the umbilical cord was 7.34. Birth measurements were within normal limits (length 52 cm (50th centile), weight 3320 g (25th–50th centile), head circumference 35 cm (50th centile)). Physical examination after birth showed Pierre Robin sequence with micrognathia and cleft soft and hard palate. Apart from apnea attacks, a weak suck and mild hypotonia, the clinical course was unremarkable during the first months. There were no feeding difficulties, and growth was between the 50th and 25th centile. CP was surgically corrected at the age of 8 months. The girl continued to display muscular hypotonia. At the age of about 12 months, the parents noticed stagnation and then mild regression of her general development. Walking was achieved at 22 months. Her gait was unsteady and atactic and she has not developed precise pincer grip to date. The most noticeable point with regard to

[1]Institute of Clinical Genetics, Klinikum Stuttgart, Stuttgart, Germany; [2]CeGaT GmbH, Tübingen, Germany; [3]Hertie Institute for Clinical Brain Research, Tübingen, Germany
*Correspondence: Dr D Bartholdi, Institute of Clinical Genetics, Klinikum Stuttgart – Olgahospital, Bismarckstrasse 3, Stuttgart D-70176, Germany. Tel: 0049 711 278 74001; Fax: 0049 711 278 74000; E-mail: d.bartholdi@klinikum-stuttgart.de
[4]These authors contributed equally to this work and share senior authorship.
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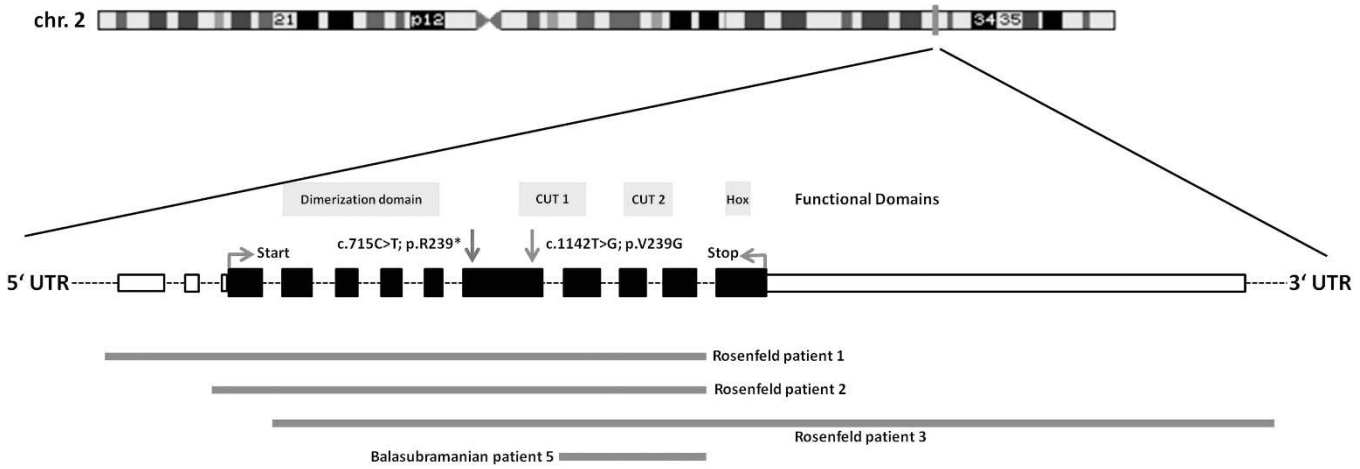

Figure 1 Synopsis of the patients carrying microdeletions restricted to *SATB2* published so far.[10,17] Extent and position of the deletions are derived from the respective original articles. Exons are shown in real relative size (according to NM_001172517.1), whereas intronic areas are standardized and sized down. Larger deletions encompassing neighboring genes and translocations disrupting *SATB2* are not shown. A comprehensive overview of larger deletions encompassing the *SATB2* locus can be found in Balasubramanian *et al.*[10] (cave: new genes have been annotated to the 2q33.1 region, which are not listed in that publication). Only one *de novo* nonsense mutation has been described before,[18] affecting the exact same nucleotide position as the one described in this report (blue arrow). In addition, a probably pathogenic missense mutation has been reported (grey arrow).[28] Indicated in yellow are the functional domains of *SATB2*, according to the Uniprot database. There are a dimerization domain, two CUT domains and one homeodomain (Hox), which constitute crucial DNA-binding motifs. A full color version of this figure is available at the *European Journal of Human Genetics* journal online.

development was the severely delayed speech: at the age of 3 years, she was able to pronounce a few syllables, but no words, and her receptive abilities were very limited. Assessment by the Munich developmental test battery at the age of 34 months showed that her skills for the different subtests were between 13 and 19 months. She had severe sleeping disturbances and showed restlessness/hyperactivity, as well as recurrent episodes of temper tantrums. An MRI scan of the brain at the age of 24 months did not show any abnormalities. Dentition started at the beginning of the second year. The teeth are of abnormal shape and positioning.

On examination at the age of 2 11/12 years (see Figure 2), weight was 12 kg (10th centile), height 94.5 cm (25th centile) and head circumference 49 cm (40th centile). The girl showed facial hypotonia with salivation, a broad forehead and microretrognathia. Palpebral fissures were downslanting and she had an upturned nose with a broad tip and a long and poorly modulated philtrum. She had irregularly shaped and crowded teeth.

Genetic testing including conventional chromosomal analysis, chromosomal microarray analysis and targeted molecular testing (analysis for Angelman syndrome, Rett syndrome and Fragile X syndrome) returned normal. After obtaining written consent of the parents (according to the German law on genetic testing), the patient was included into diagnostic trio-exome sequencing.

MATERIALS AND METHODS

DNA isolation

Genomic DNA was isolated from blood samples using the QIAamp DNA Mini Kit (Qiagen NV, Hilden, The Netherlands; cat. no. 51306) following the manufacturer's instructions.

Exome sequencing

The exomes of the patient and her parents were sequenced using the following protocol: DNA was enriched using the SureSelect 52-Mb exome kit (Agilent, Böblingen, Germany) according to the manufacturer's instructions. Sequencing was performed on a SOLiD 5500xl sequencer (Life Technologies, Darmstadt, Germany). In average, 130 million reads with a length of 75 bp were produced per exome. All raw reads were corrected using the spectral alignment error correction algorithm (SAET) from LifeScope and were mapped with LifeScope 2.5.1 (LifeTechnologies) against the human reference genome (hg19 from UCSC). This yielded an average coverage of 139 for the enriched target regions.

Data analysis

Variant calling – including small insertions and deletions as well as single-nucleotide variants (SNVs) – was performed by LifeScope 2.5.1 (LifeTechnologies), and samtools mpileup 0.1.18 with bcftools and vcfutils.pl.[19] We used high sensitive configurations to reduce false negatives at the cost of an increased false-positive mutation rate. Calls also found in dbSNP (Database of Single-Nucleotide Polymorphisms (Bethesda, MD, USA), National Library of Medicine. Build ID: 137, http://www.ncbi.nlm.nih.gov/SNP/) or the Exome Variant Server database (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, USA; http://evs.gs.washington.edu/EVS/) with an allele frequency $>5\%$ were removed. In addition, frequently observed variants in an in-house database produced from the same sequencing technology and enrichment kit were removed ($>15\%$, minimum number of references: 21).

Transcript and peptide alterations were annotated with NGS-SNP v1.010[20] using the ENSEMBL v69 database.[21] Only variants potentially changing the protein sequence were used for further analysis; intronic, UTR and synonymous mutations were removed.

Trio analysis was conducted to further filter the variant list. The remaining SNVs and insertions or deletions of the index patient were used to check for three cases: index patient (1) is compound heterozygous and each parent carries only one heterozygous mutation; (2) has a homozygous mutation and parents are heterozygous; and (3) has a *de novo* mutation. All three cases were automatically computed by a self-developed tool (unpublished).

Confirmation by Sanger sequencing

The variant in *SATB2* (according to NM_001172517.1, see Results section) was confirmed by Sanger sequencing on an ABI 3730XL sequencer (Life Technologies) following standard procedures. Sequencing was done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Primers used for amplification and sequencing: *SATB2*-E08F: 5′-AGAGGATTTAG AATGGTCAGTGGTTTCTGTC-3′; *SATB2*-E08R: 5′-GGTCCTGAGTTGCATG TTGGGTTC-3′.

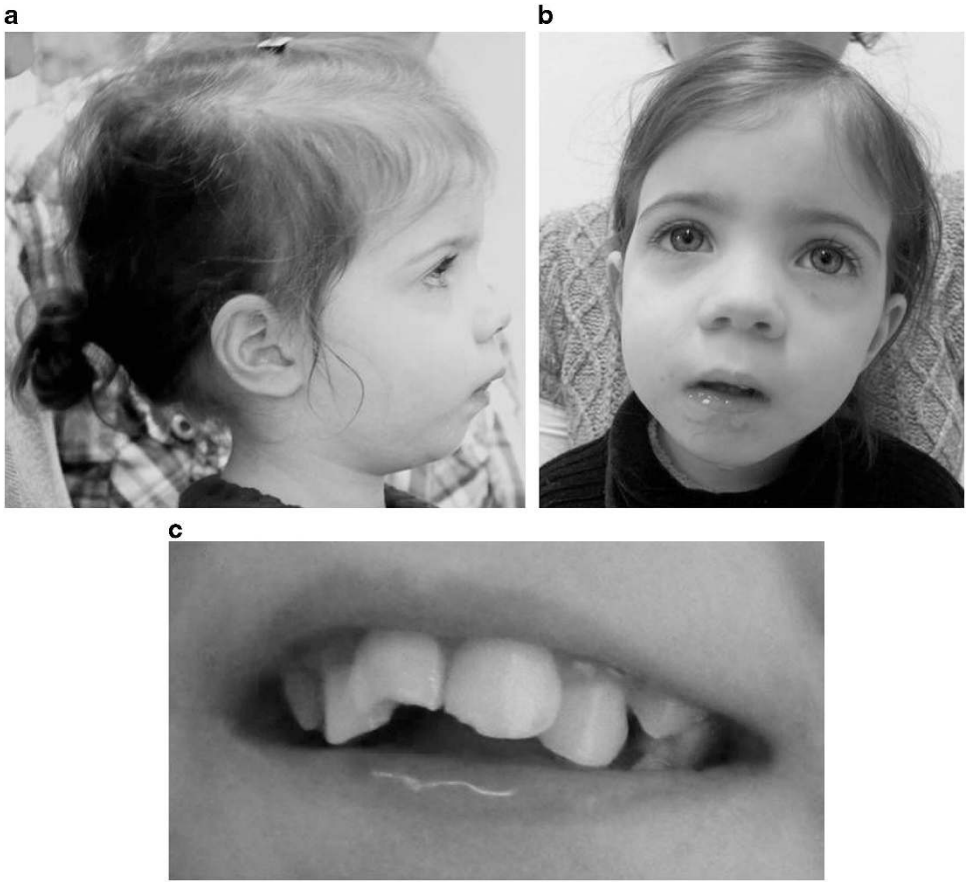

Figure 2 (**a**, **b**) Facial features of the patient at the age of 2 9/12 years. Note the hypotonic face with pronounced salivation. The girl displays hypertelorism, downslanting palpebral fissures, long eyelashes and a slightly upturned nose with a broad tip. She has micrognathia, a long and poorly modulated philtrum, and the ears are low set and dorsally rotated. (**c**) Irregular shape and positioning of the teeth. Consent of the parents for publishing the pictures was obtained.

Confirmation in fibroblasts

In order to test the possibility of mosaicism, we performed skin biopsies in the index patient and her parents and established a fibroblast cell culture according to standard procedures. DNA extracted from these samples was analyzed by Sanger sequencing for the variant in *SATB2* identified in lymphocytes.

RNA sequencing

RNA of fibroblast cultures of the index patient and her parents was isolated using the RNeasy Mini Kit from Qiagen NV (Venlo, The Netherlands; cat. no. 74014) according to the manufacturer's protocol.

cDNA synthesis was done using the Transcriptor High Fidelity cDNA synthesis Kit from Roche Diagnostics (Rotkreuz, Schweiz, cat. no. 05 081 955 001) according to the manufacturer's protocol, using 300 ng of RNA in each case.

RT-PCR was performed with Promega GoTaq Polymerase (cat. no. M3175) using 3 mM $MgCl_2$, Q-solution from Qiagen (betain), and a primer concentration of 0.1 pmol/µl.

Sequencing was done with the BigDye Terminator v3.1 Cycle Sequencing Kit.

The primers used for amplification and sequencing of *SATB2* were as follows: *SATB2*-F: 5′-GGAGCAGTGGAACCATGCCACAGT-3′; *SATB2*-R: 5′-CTGTGGTGAATTTGGCTGTGAGGAG-3′.

RESULTS

Trio-exome analysis in the girl and her parents was performed generating 130 million reads per exome. On average, a coverage of 139 was achieved for the enriched target regions. A total of 3615 potential mutations remained in the index patient after population and protein alteration filtering. The analysis of the trio-exome resulted in 49 mutations (18 genes) in the category 'compound heterozygous mutations', eight homozygous mutations and 536 '*de novo*' mutations (for definitions, see Materials and Methods section). Identified '*de novo*' mutations consisted mainly of sequencing and calling errors because of the sensitive calling approach.

In addition, all variant calls were filtered by a list of 517 genes known to be associated with ID, leaving zero mutations for 'compound heterozygous', one mutation for 'homozygous' and 13 '*de novo*' mutations. These variants were manually inspected and the homozygous call, as well as 12 of the potential *de novo* mutations, turned out to be false positives.

In summary, only one *de novo* mutation in a gene associated with ID/mental retardation – the *SATB2* gene – was detected and confirmed by Sanger sequencing.

The heterozygous point mutation c.715C>T; p.R239* (rs137853127) identified in lymphocytes was also present in the patient's, but absent in her parent's, fibroblast DNA. RNA sequencing of fibroblast mRNA in the affected girl clearly showed the presence of the mutation on RNA level and excluded the mutation in both parents (r.1128c>u; for an overview, see Supplementary Material, Supplementary Figure 3).

DISCUSSION

ID comprises a group of phenotypically and genetically highly heterogeneous entities and represents a major diagnostic challenge in clinical genetics. Trio-exome sequencing has been shown to be a powerful tool in detecting pathogenic mutations in known ID genes and in identifying novel ID genes. Here, we present a patient carrying a *de novo SATB2* point mutation identified by trio-exome sequencing. It is the second patient with a nonsense mutation in this gene reported so far.

SATB2 is located on chromosome 2q33.1 and the NCBI database lists three different transcripts, which all encode the same 733-amino-acid protein.[1] It is a DNA-binding protein that is expressed in vertebrates in the brain, branchial arch derivatives, at sites of bone formation and in various other tissues including kidney and gut.[22–24] *SATB2* was first reported as a CP gene in two individuals carrying chromosomal translocations on 2q32-q33, with the breakpoints mapped to *SATB2*.[6] Interestingly, these two individuals did not display pronounced dysmorphisms or ID, and were therefore classified as 'non-syndromic' CP patients. The role of *SATB2* as a CP gene was further supported by its spatial and temporal expression in the developing vertebrate palate (see below). However, screening of several large cohorts of patients with isolated CP did not reveal pathogenic sequence variants in *SATB2*,[25] questioning its role as an isolated CP gene.

Subsequent to the publication of the translocation patients, several papers reported patients carrying heterozygous deletions of varying extent, encompassing the 2q32q33 or 2q33.1 regions (see Figure 1).[10–17] These deletions, ranging from 20 Mb to 200 kb approximately and all including *SATB2*, were associated with growth retardation, severe ID, dysmorphic features and CP or high-arched palate. As would be predicted from the variation in the extent of deletion and the specific gene content, the patients – although presenting a similar core phenotype – exhibited marked heterogeneity in some of their clinical features.

On the basis of the patient reported in this paper and the previous patient carrying the same nonsense *SATB2* mutation,[18] together with a patient with a small intragenic duplication[26] and four patients carrying deletions restricted to *SATB2*,[10,17] we propose a novel clinical entity termed SAS. SAS is characterized by severe ID with no or only limited speech development, behavioral problems such as restlessness, aggression and sleep disturbances, abnormalities in craniofacial patterning such as micrognathia, cleft or high-arched palate, and problems with teeth/dentition (oligodontia and/or misshaped and crowded teeth). The patients also show subtle dysmorphisms including a hypotonic face and downslanting palpebral fissures (see Table 1 and Figure 2). To our knowledge, this clinical constellation of SAS is distinct from any clinical entity described so far.

Leoyklang *et al*,[18] who published the first patient with a point mutation in *SATB2* back in 2007, proposed that chromosomal deletions associated with haploinsufficiency of *SATB2* cause a less severe phenotype than the nonsense mutation detected in their patient. They suggested that this might be explained by a dominant-negative effect of the truncated or altered SATB2 protein. In our patient, we could detect the mutation at RNA level, indicating that the RNA is stable enough not to undergo immediate nonsense-mediated decay. A very recently published follow-up study of Leoyklang *et al*.[27] including immunoblot analyses further supports

Table 1 Comparison of clinical features of the patients carrying a microdeletion,[10,17] microduplication[26] or *de novo* nonsense mutation[18] only affecting the *SATB2* gene

	Rosenfeld patient 1	*Rosenfeld patient 2*	*Rosenfeld patient 3*	*Balasubramanian patient 5*	*Kaiser patient*	*Leoyklang patient*	*Patient in this report*
Age at time of description	9y 8m	21y	6y	3y	11y	36y	3y
Sex	F	M	F	M	F	M	F
ID	Severe (IQ <50)	Severe	Severe (IQ 32)	Severe	Severe	Severe	Severe
Speech development	20 Words, 5 signs, 1 two-word phrase	Single words	No speech, but some signs	Absent	Nearly absent	1 Word	Absent
Behavior and sleep	Behavior problems	History of aggression	Sleep problems	NR	Challenging behavior	Jovial personality	Behavior and sleep problems
Brain MRI	Normal	NR	NR	NR	NR	No intracranial abnormality	Normal
Dysmorphic features	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Philtrum	Smooth	NR	NR	Short philtrum, high nasal bridge	NR	NR	Smooth
Cleft palate	NR[a]	Yes	No[a]	Yes	Yes	Yes	Yes
Micrognathia	Yes	Yes	NR	Yes	Yes	Yes	Yes
Teeth abnormalities	Delayed dentition	Crowded teeth	Fused central incisors	NR	Oligodontia	Anterior-pointing incisors, oligodontia	Crowded, irregularly shaped
Additional findings	Gestational diabetes; good eye contact; stereotypic hand movements	High pain tolerance; not toilet trained		Gestational diabetes; 1 café-au-lait spot		Seizures, generalized osteoporosis	Astigmatism
Del/dup/mut	Del	Del	Del	Del	Dup	p.R239*	p.R239*

Abbreviations: del, microdeletion; dup, microduplication; ID, intellectual disability; m, months; mut, *de novo* point mutation (position as noted); NR, not reported; y, years.
See Figure 1 for the extents of the deletions.
All of these patients that clearly show the *SATB2*-associated phenotype have severe ID including delayed to non-existent speech development, cleft palate or *assumably high-arched palate (unfortunately, not reported sufficiently), micrognathia and tooth abnormalities.
The *SATB2* missense mutation reported by Rauch *et al*.[28] have not been included into this table because detailed clinical information was not available.

the hypothesis of a possible dominant-negative effect of the truncated SATB2 protein.

Future functional studies of patients with different mutations will hopefully provide deeper insight into the role of normal and altered SATB2 protein during human development. Interestingly, a patient carrying a missense mutation in *SATB2* (c.1142T>G, p.V381G) was recently reported in a large exome sequencing study in individuals with severe sporadic non-syndromic ID. Although the pathogenic role of this specific mutation is not yet clearly established, it seems as if this patient has a mild SAS phenotype.[28]

It also remains to be elucidated, whether the fact that the patient described in this report has the exactly same point mutation as the first patient described in 2007 is due to coincidence or whether there is an underlying pathogenic reason favoring this nucleotide change as a 'hot spot' region.

SATB2 encodes a protein that specifically binds nuclear MARs and thereby has an important role in transcriptional regulation by remodeling chromatin structure. Studies of murine Satb2 show that it is highly expressed in the developing upper and lower jaw including the palatal shelves and teeth buds, as well as in specific areas of the cortex and at sites of bone formation. *Satb2* knock out mice on different backgrounds have been generated to study craniofacial patterning and osteoblast differentiation.[22,24] Heterozygous $Satb2^{+/-}$ mice have a phenotype similar to that of SAS patients, including CP, micrognathia, premaxillary and nasocapsular hypoplasia, and variable incisor hypodontia or adontia. Homozygote $Satb2^{-/-}$ knock out mice are born with exacerbated skeletal malformations and often die perinatally. It has been shown that Satb2 interacts directly with and enhances activity of Runx2 and Atf4, two transcription factors regulating osteoblast formation.[29,30] Furthermore, Satb2 represses the expression of several *Hox* genes including *Hoxa2*, an inhibitor of bone formation and regulator of branchial arch patterning.[22] These studies, together with zebra fish studies,[31,32] have established the crucial role of Satb2 in skeletogenesis, influencing such different processes as patterning, osteoblast differentiation and matrix formation. With respect to humans, it is noteworthy that the first described patient carrying a *de novo SATB2* nonsense mutation, who was 36 years old at the time of reporting, suffered from unexplained very severe osteoporosis.[18]

Interestingly, variants in an adjacent gene, *FONG*, have been linked to increased risk of osteoporosis in a Japanese genome-wide association study.[33] It remains to be elucidated whether there is interaction (or relation, such as co-expression) between *SATB2* and *FONG*.

Apart from its well-established role in craniofacial patterning and skeletogenesis, *Satb2* is expressed in the developing cortex and has an important role in determining the identity of neurons during cortical development.[24,34,35] In the mammalian cerebral cortex, neurons can be divided into two major classes: cortico-cortical projection neurons, which are concentrated in the upper layers of the cortex, and subcortical projection neurons, which are found in the deep layers. Early progenitor cells in the ventricular zone give rise to deep layer neurons while progenitors in the subventricular zone give rise to upper layer neurons. Expression of Satb2 is required in upper layer neurons for the formation of axonal projections that connect the two cerebral hemispheres. In *Satb2* mutants, upper layer neurons fail to migrate to superficial layers and do not contribute to the corpus callosum, but erroneously to the corticospinal tract, which is normally populated by axons of deep layer neurons.[24,32] In addition to *Satb2*, projection identities of cortical neurons are orchestrated by three further genes, *Fezf2*, *Ctip2* and *Tbr1*.[33] These four genes interact with each other, and studies in double mutants led to the hypothesis that cortical neurons undergo a complex genetic switch by mutual repression to produce subcortical or callosal projections. Beyond regulating neuronal projections, Satb2 has also been shown to contribute to a network of genetic repression and derepression in the developing neocortex and thereby to regulate the expression of two disease-related genes, *Auts2* (autism susceptibility gene 2) and *Bhlhb5* (mutated in hereditary spastic paraplegia).[36]

In light of these studies, it is surprising that SAS patients do not exhibit agenesis or hypogenesis of corpus callosum or more subtle malformations of the cortical architecture that would be detectable by MRI (see Table 1). However, the crucial role of *Satb2* in neuronal connectivity very likely explains the severe ID with pronounced impairment of speech development and the seizure disorder in the first patient carrying a nonsense mutation in *SATB2*.

In addition to *SATB2*, numerous other chromatin remodeling genes have been implicated in human neurodevelopmental disorders such as ID, autism spectrum disorder and schizophrenia. Examples include *CBP* and *p300* (Rubinstein–Taybi syndrome), *CHD7* (Charge syndrome), *ATRX* (X-linked alpha-thalassemia mental retardation syndrome) and genes of the *BAF* complex (Coffin–Siris and Kleefstra syndrome).[37] Interestingly, mutations in several of the chromatin remodeling genes implicated in neurodevelopmental disorders are also involved in human cancer, which is also true for *SATB2*. In colorectal cancer, downregulation of *SATB2* is associated with metastasis and poor prognosis.[38,39] Also in laryngeal carcinoma, decreased *SATB2* expression is associated with a poorer outcome.[40] In bone and soft tissue tumors, SATB2 has been proposed to be a novel marker for bone osteoblastic differentiation in benign and malignant mesenchymal tumours.[41] A single study showed an association between *elevated SATB2* mRNA levels and worse outcome in breast cancer patients.[42]

In conclusion, this report confirms *SATB2* as an essential gene for craniofacial patterning and cognitive development in humans and leads to delineation of the SAS. Children with ID and no or severely impaired speech, cleft or high-arched palate, micrognathia and teeth abnormalities should be analyzed for deletions or point mutations in *SATB2*. The identification of further patients with SAS will help to establish the specific role(s) of this gene in human development, determine the long-term prognosis and possibly to identify strategies for therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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