KBP–cytoskeleton interactions underlie developmental anomalies in Goldberg–Shprintzen syndrome

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Goldberg–Shprintzen syndrome (GOSHS, MIM #609460) is an autosomal recessive disorder of intellectual disability, specific facial gestalt and Hirschsprung’s disease (HSCR). In 2005, homozygosity mapping in a large consanguineous family identified KIAA1279 as the disease-causing gene. KIAA1279 encodes KIF-binding protein (KBP), whose function is incompletely understood. Studies have identified either the mitochondria or the cytoskeleton as the site of KBP localization and interactions. To better delineate the KIAA1279-related clinical spectrum and the molecular mechanisms involved in GOSHS, we studied five new patients from three different families. The homozygous KIAA1279 mutations in these patients (p.Arg90X, p.Ser200X or p.Arg202IlefsX2) led to nonsense-mediated mRNA decay and loss of KBP function. Despite the absence of functional KBP, respiratory chain complex activity in patient fibroblasts was normal. KBP did not co-localize with mitochondria in control human fibroblasts, but interacted with the actin and tubulin cytoskeleton. KBP expression directly affected neurite growth in a neuron-like cell line (human neuroblastoma SH-SY5Y), in keeping with the central (polymicrogyria) and enteric (HSCR) neuronal developmental defects seen in GOSHS patients. The KBP interactions with actin filaments and microtubules (MTs) demonstrated in our study constitute the first evidence that an actin MT cross-link protein is involved in neuronal development in humans.

INTRODUCTION

Goldberg–Shprintzen syndrome (GOSHS, MIM #609460) is an autosomal recessive disorder first reported in 1981 as a multiple congenital anomaly syndrome associated with Hirschsprung’s disease (HSCR or intestinal aganglionosis) (1,2). To date, 23 patients (7 families and 6 sporadic cases) with clinical manifestations related to GOSHS have been described (1–13). Phenotypic overlap exists between GOSHS and others disorders characterized by microcephaly, intellectual disability and HSCR such as Mowat–Wilson syndrome (MWS, MIM #235730). MWS was clinically delineated in 1998 (14) and molecularly defined in 2001 (15,16).
For some of the early diagnosed GOSH patients, such as case 3 as reported by Hurst et al. (4) and the cases reported by Tanaka et al. (8) and by Ohnuma et al. (9), the clinical features seem to match MWS. For some other patients, the clinical reports are insufficient to conclude to GOSH; therefore, the diagnosis should be considered with caution (5,6). The GOSHS disease-causing gene KIAA1279 was identified by homozygosity mapping, in 2005, in a large Moroccan consanguineous family (17). So far, KIAA1279 mutations have been reported in only three families with GOSHS, two of which were characterized by a homozygous mutation (17) and one by only a heterozygous mutation (13) in affected individuals.

The gene KIAA1279 is located at 10q22.1 and comprises seven exons. The longest transcript of about 2.5 kb is ubiquitously expressed (17). KIAA1279 encodes a 621 amino acid protein with a predicted molecular weight of 71 kDa and two tetratricopeptide repeats (TPRs) that are structural motifs mediating protein–protein interactions (18). This protein was named KIF-binding protein (KBP) due to its interaction with the kinesin-like protein KIF1C used as bait in a yeast two-hybrid assay (19). Immunofluorescence experiments suggested that KBP was located in mitochondria and co-localized with KIF1Balpha, a mitochondrial KIF1B isoform exhibiting marked homology with KIF1C (19). In a recent study, however, KBP was rather shown to interact with the microtubule (MT) destabilizing protein SCG10 and was not found in the mitochondria (20). Finally, studies in zebrafish suggest a role of KBP in axonal structure, outgrowth and maintenance (21).

In the present study, we refined and expanded the clinical spectrum of GOSHS studying five new patients from three different families, each with a different homozygous KIAA1279 mutation, whose functional consequences were evaluated. Because all KIAA1279 mutations reported to date are predicted to create premature termination codons, we evaluated whether the underlying molecular mechanism was mRNA degradation by nonsense-mediated mRNA decay (NMD). To assess potential functional consequences of the mutations on mitochondrial function, we measured respiratory chain complex activity in fibroblasts from the patients. We sought to clarify the pathophysiology of GOSHS by determining whether KBP was involved in the mitochondrial and cytoskeletal network of human fibroblasts and of a neuron-like cell line (human neuroblastoma SH-SY5Y). Thus, we studied KBP involvement in cytoskeletal organization and evaluated KBP interactions with MTs and filamentous actin. We confirmed the role of KBP in neuronal expansion via effects on neurite outgrowth. Overall, our results indicate that KBP is a cytoskeleton-interacting protein with a role in neural development, but do not support a role of KBP in mitochondrial function.

RESULTS
KIAA1279 molecular analysis and patient phenotype
We report five new patients belonging to three different consanguineous families, from Morocco, France and Iraq, respectively. Each family had a homozygous KIAA1279 mutation (Fig. 1A) inherited on an autosomal recessive basis (pedigrees in Fig. 1B). Nonsense mutations were identified in the Moroccan (c.268C>T p.Arg90X) and French (c.599C>A p.Ser200X) families and a frameshift mutation (c.604-605delAG p.Arg202IlefsX2) in the Iraqi family (Fig. 1C). The mutations in the French and Iraqi families have not been reported previously, whereas the mutation in the Moroccan family has been described in another Moroccan family as c.303C>T R90X (17). None of the mutations was identified in 200 control chromosomes or reported as non-pathogenic polymorphisms in SNP databases.

The phenotypic characteristics of the patients are listed in Table 1 and the facial gestalt with its changes over time is illustrated in Figure 1D and in Supplementary Material, Figure S1. Distinctive facial features consisted of severe microcephaly (less than third percentile), sparse hair, arched eyebrows, long eyelashes, ptosis, downslanting palpebral fissures, prominent ears, thick earlobes, prominent nasal bridge, thick philtrum, everted lower lip and pointed chin (Fig. 1D). We also noted maxillary hypoplasia, hypodontia, high-arched palate, short neck, small hands, brachydactyly, clinodactyly of the fifth fingers, fetal finger pads and flatfoot. All patients had hypotonia and major developmental delays. Patients older than 3 years produced two- to five-word sentences. They had learned to walk between 26 and 30 months of age. Joint laxity was a feature in one patient (F3, V-2), exaggerated deep tendon reflexes with peripheral hyperadonia in two patients (F1, IV-2 and F2, IV-1) and anxiety with sleep disturbances in one patient (F2, IV-1). None of the patients had seizures. Cerebral magnetic resonance imaging (MRI) showed bilateral generalized polymicrogyria with malformation of the right Sylvian fissure in one patient (F1, IV-2, Fig. 2). Gyration abnormalities (F2, IV-3), corpus callosal hypoplasia (F2, IV-3 and F3, V-2) and subarachnoid space enlargement (F2, IV-1) were noted in other patients. HSCR was diagnosed in four patients and the remaining patient (F3, IV-1) had unexplored constipation. Ocular features were hyperopia in two patients (F2, IV-1 and IV-3) and bilateral megalocornea in one patient (F1, VI-2). Congenital heart defects were present in two patients (ventricular septal defect in F3, V-2 and aortic valve incompetence requiring valve replacement in F2, IV-1). Urogenital features were unilateral multicystic renal dysplasia with contralateral vesicoureteral reflux (F2, IV-3) and bilateral cryptorchidism (F2, IV-1). Skeletal abnormalities consisted of scoliosis (F2, IV-1) and femoral neck anteverision (F2, IV-3). Two patients had recurrent respiratory tract infections (F2, IV-1 and F3, V-2).

Degradation of mutant alleles related to active NMD
To determine whether NMD targeted KIAA1279 null mutation transcripts, we studied fibroblasts from three homozygous patients (c.268C>T p.Arg90X or c.599C>A p.Ser200X or c.604-605delAG p.Arg202IlefsX2) and from two heterozygous parents (c.599C>A p.Ser200X or c.604-605delAG p.Arg202IlefsX2). We first used quantitative reverse transcriptase PCR (RT-PCR) to measure KIAA1279 expression in mRNA products. In all three homozygous patients, the amount of KIAA1279 mRNA was decreased by about 80% (P < 0.0005) when compared with KIAA1279 wild-type (WT) transcripts (Fig. 3A and B). A 30–50% decrease in
the KIAA1279 transcript ($P < 0.05$ and $P < 0.005$) was observed in the heterozygous individuals when compared with a control (Fig. 3A and B). Experiments were triplicates performed on independent fibroblast cultures, with ABL1 as a reference gene. Furthermore, in the heterozygous individuals, sequencing of the RT-PCR product encompassing the mutation established that only the WT transcript was present.

To assess the extent to which the observed decrease in KIAA1279 mRNA levels affected the amount of protein, we quantified KBP in fibroblasts from the same three homozygous patients and two heterozygous parents. Western blotting of total protein extracts was performed in four independent experiments, and calnexin (CNX) was used as a reference (Fig. 3C). In fibroblasts carrying a heterozygous mutation (p.Ser200X or p.Arg202IlefsX2), the amount of full-length KBP was significantly decreased, by 70% ($P < 0.005$ and $P < 0.0005$, respectively) when compared with the control. No truncated proteins were detected in fibroblasts carrying a heterozygous mutation (p.Ser200X or p.Arg202IlefsX2) or a homozygous mutation (p.Arg90X or p.Ser200X or p.Arg202IlefsX2) (Fig. 3C and D).

Finally, to assess the role of NMD in decreasing the mutant transcripts, we exposed the fibroblasts carrying each of the three mutations to emetine, a potent protein synthesis inhibitor. Emetine treatment inhibits the NMD pathway, resulting in stabilization of transcripts carrying a premature stop codon. We measured KIAA1279 transcripts in emetine-exposed and -unexposed fibroblasts, using semi-quantitative RT-PCR normalized for the ABL1 housekeeping gene, in three independent biologic replicates. Emetine exposure was associated with a significant 4-fold ($P < 0.005$) increase in KIAA1279 mRNA in all tested fibroblasts with a homozygous mutation, when compared with unexposed fibroblasts (Fig. 3E and F). These data indicate that NMD played an active role in degrading KIAA1279 mRNA alleles characterized by a premature stop codon.

KBP is not a mitochondrial protein
In human fibroblasts, KBP had a cytosolic distribution with no mitochondrial localization. To further assess the subcellular localization of KBP, we used the semi-intact cell technique with digitonin permeabilization of control fibroblasts (22,23). No co-localization with mitochondria was observed (Fig. 4A and B).

To investigate the potential impact of KBP deficiency on mitochondrial function, we studied various mitochondrial activities. Using spectrophotometry, we measured the activities of the five oxidative phosphorylation complexes, I through V, in fibroblasts carrying KIAA1279 mutations (24,25).
We found no significant differences across homozygous, heterozygous and control fibroblasts (data not shown). Oxidative stress markers, mitochondrial or cytosolic, were next studied in fibroblasts from four individuals homozygous or heterozygous for p.Ser200X or p.Arg202IlefsX2, as well as in a control. Using automated spectrophotometry, we measured the enzymatic activities of the 14 oxidative stress markers [glutathione peroxidase (GPX), cytosolic superoxide dismutase (Cu/Zn-SOD) and mitochondrial superoxide dismutase (Mn-SOD), glucose-6-phosphate dehydrogenase (G6PD), glutamylcysteine synthetase (GCS) and glutathione synthetase (GS), glutathione reductase (GR) and cellular levels of malondialdehyde (MDA), reduced and oxidized glutathione (GSH and GSSG) and adenosine triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP)](26,27). None of these markers differed significantly across homozygous, heterozygous and control individuals (data not shown).

KBP interacts with MTs

In human fibroblasts, KBP was found to co-localize with α-tubulin in untreated cells (Fig. 5A). Digitonin permeabilization confirmed the co-localization of KBP with α-tubulin (Fig. 5A and B). To determine whether KBP localization was related to interactions between KBP and MTs, we performed in vitro co-sedimentation assays (28). We first performed a MT polymerization reaction in vitro. Using high-speed centrifugation, we separated the supernatant fraction containing the non-polymerized tubulin from the pellet fraction containing the MTs (Fig. 5C supernatant and pellet, lane 1). We then centrifuged total protein extracts of control fibroblasts. The soluble proteins, endogenous tubulin and KBP, were found in the supernatant fraction, whereas the MTs and proteins associated with MTs, such as KBP, were in the pellet (Fig. 5C supernatant and pellet, lane 2). Third, in vitro polymerized MTs were incubated with total protein extracts of control fibroblasts containing endogenous KBP. After high-speed centrifugation, the amount of KBP in the pellet fraction containing the MTs (Fig. 5C pellet, lane 3) increased relative to the amount in the supernatant fraction (Fig. 5C supernatant, lane 3). KBP quantification showed that incubation of in vitro polymerized MTs with fibroblast protein extracts resulted in more than 80% of the KBP being captured by the MTs. The soluble proteins, endogenous tubulin and KBP, were found in the supernatant fraction, whereas the MTs and proteins associated with MTs, such as KBP, were in the pellet (Fig. 5C supernatant and pellet, lane 3). We then centrifuged total protein extracts of control fibroblasts. The soluble proteins, endogenous tubulin and KBP, were found in the supernatant fraction, whereas the MTs and proteins associated with MTs, such as KBP, were in the pellet (Fig. 5C supernatant and pellet, lane 3). Third, in vitro polymerized MTs were incubated with total protein extracts of control fibroblasts containing endogenous KBP. After high-speed centrifugation, the amount of KBP in the pellet fraction containing the MTs (Fig. 5C pellet, lane 3) increased relative to the amount in the supernatant fraction (Fig. 5C supernatant, lane 3). KBP quantification showed that incubation of in vitro polymerized MTs with fibroblast protein extracts resulted in more than 80% of the KBP being captured by the MTs. The soluble proteins, endogenous tubulin and KBP, were found in the supernatant fraction, whereas the MTs and proteins associated with MTs, such as KBP, were in the pellet (Fig. 5C supernatant and pellet, lane 3). Third, in vitro polymerized MTs were incubated with total protein extracts of control fibroblasts containing endogenous KBP. After high-speed centrifugation, the amount of KBP in the pellet fraction containing the MTs (Fig. 5C pellet, lane 3) increased relative to the amount in the supernatant fraction (Fig. 5C supernatant, lane 3). KBP quantification showed that incubation of in vitro polymerized MTs with fibroblast protein extracts resulted in more than 80% of the KBP being captured by the MTs (Fig. 5D). These results indicated interactions between KBP and MTs.

**Table 1.** Phenotypic features in patients with GOSHS

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>DD/ID</td>
<td>21 months</td>
<td>15 years</td>
<td>8 years</td>
<td>32 years</td>
</tr>
<tr>
<td>Microcephaly (less than third percentile)</td>
<td>Yes</td>
<td>Moderate</td>
<td>Severe</td>
<td>Severe</td>
<td>Severe</td>
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<tr>
<td>Language skills</td>
<td>Large number</td>
<td>IR</td>
<td>Large number</td>
<td>IR</td>
<td></td>
</tr>
<tr>
<td>Motor skills</td>
<td>Large number</td>
<td>No</td>
<td>Large number</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Sitting (months)</td>
<td>12</td>
<td>30</td>
<td>12</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Walking (months)</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Brain anomalies</td>
<td>Polymicrogyria</td>
<td>Subarachnoid space enlargement</td>
<td>Gyration anomalies</td>
<td>Corpus callosum hypoplasia</td>
<td></td>
</tr>
<tr>
<td>HSCR</td>
<td>Yes</td>
<td>Megalocornea</td>
<td>Yes</td>
<td>Poliosis and hyperopia</td>
<td></td>
</tr>
<tr>
<td>Ocular features</td>
<td>Megalocornea</td>
<td>Piosis and hyperopia</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Congenital heart defects</td>
<td>No</td>
<td>Aortic valve incompetence</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Urogenital anomalies</td>
<td>None</td>
<td>Cryptorchidism</td>
<td>Vesicoureteral reflux and multicystic renal dysplasia</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Skeletal features</td>
<td>None</td>
<td>Oligodontia</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

DD/ID, developmental delay/intellectual disability; NA, not available; IR, irrelevant; HSCR, Hirschsprung’s disease.

**Figure 2.** Brain MRI of a 1-year-old patient with GOSHS (family 1, IV-2). Axial T2-weighted (A) and coronal T1-weighted (B) slices showing generalized polymicrogyria with malformation of the right Sylvian fissure and asymmetric lateral ventricle dilatation (right > left). The corpus callosum is morphologically normal (B).
Co-sedimentation results were consistent with the immuno-fluorescence data and confirmed that KBP protein bound strongly to the MT cytoskeleton in human fibroblasts. No quantitative or structural changes in the α-tubulin protein were observed in fibroblasts from patients with a homozygous KIAA1279 mutation (p.Arg90X or p.Ser200X or p.Arg202IlefsX2) (data not shown).

KBP interacts with filamentous actin (F-actin)

To determine whether KBP interacted with other cytoskeleton components, we tested interactions between KBP and F-actin in human control fibroblasts. KBP and F-actin partially co-localized in untreated fibroblasts (Fig. 6A). After digitonin permeabilization (Fig. 6A and B), both perfect and slightly

Figure 3. Degradation of KIA1279 mutant alleles is a consequence of active nonsense-mediated mRNA decay. Assays were performed with total pre-mRNA, and protein extracts from fibroblasts obtained from patients, parents and controls. Data are the mean + SD of at least three independent measurements performed in triplicate. *P < 0.05; **P < 0.005; ***P < 0.0005. Hm, homozygous; Hz, heterozygous. (A) Representative image of gel electrophoresis of KIAA1279 RT-PCR. (B) Quantitative RT-PCR analysis of KIAA1279 normalized for ABL1 (housekeeping gene). (C) Representative image of western blot analysis. Only the full-length (WT) KBP form was detected. CNX was used for protein normalization. ϕ indicates non-specific bands. (D) Quantification of KBP normalized for CNX. (E) Semi-quantitative RT-PCR assay performed using cultured fibroblasts from controls, patients and parents, untreated (H2O, in blue) or treated with emetine (in red). KIAA1279 transcript (108 bp) was normalized for the ABL1 housekeeping gene (130 bp). Significant quantitative rescue of NMD-sensitive mutant mRNA alleles with emetine treatment is indicated by asterisks (**P < 0.005). (F) Representation of relative KIAA1279 transcript abundance illustrated in E. Areas under the curve of KIAA1279 normalized for ABL1 transcripts are shown in bar graphs.
Figure 4. Mitochondrial analysis on human fibroblasts. (A) Confocal microscopy images of control fibroblasts, either untreated or digitonin permeabilized, and double-labeled with a mitochondrial marker (Mitotracker, in green) and anti-KBP antibody (in red). (B) Enlarged view of semi-intact fibroblasts (digitonin permeabilized) and dual-channel co-localization analysis performed using ImageJ. Scale bars: 10 μm.

Figure 5. KBP protein co-localizes and interacts with MT cytoskeleton in fibroblasts. (A) Confocal microscopy images of human control fibroblasts, either untreated or digitonin permeabilized, and double-labeled with anti-α-tubulin antibody (red) and anti-KBP antibody (green). (B) Enlarged view of semi-intact fibroblasts (digitonin permeabilized) and dual-channel co-localization analysis performed using ImageJ. Scale bars: 10 μm. (C) KBP interacts with in vitro polymerized MTs. Fibroblast extracts were centrifuged, and only the soluble proteins present in the supernatant were used for the co-sedimentation experiments. Western blotting of in vitro polymerized MTs (lane 1), fibroblast extracts containing endogenous KBP (lane 2) and in vitro polymerized MTs and fibroblast extracts showing KBP co-sedimentation with MTs (lane 3). (D) Representation of the relative quantification of KBP interacting with MTs. The upper bar, denoted by a minus sign (−), represents quantification of KBP from the total protein extracts of control fibroblasts, shown in the western blot in Figure 5C (supernatant and pellet, lane 2, first line). The lower bar, denoted by a plus sign (+), represents quantification of KBP from total protein extracts of control fibroblasts incubated with in vitro polymerized MTs, shown in the western blot in Figure 5C (supernatant and pellet, lane 3, first line).
shifted KBP localization to F-actin were observed (Fig. 6B).

To confirm the interaction between these two proteins, we investigated whether KBP bound to in vitro polymerized F-actin (29). After in vitro polymerization of F-actin, high-speed centrifugation was used to pellet the F-actin (Fig. 6C supernatant and pellet, lane 1). Then, total protein extracts of control human fibroblasts were centrifuged. F-actin was not detected in fibroblast total protein extracts. Endogenous KBP was chiefly found in the supernatant fraction (Fig. 6C supernatant and pellet, lane 2). Next, F-actin was polymerized in vitro then incubated with fibroblast total protein extracts. KBP increased in the pellet fraction (Fig. 6C pellet, lane 3) and decreased in the supernatant fraction (Fig. 6C supernatant, lane 3). KBP quantification after co-sedimentation showed that 60% of KBP was associated with F-actin (Fig. 5D). These results indicated an interaction between KBP and F-actin.

Co-sedimentation results were consistent with the immunofluorescence data and confirmed that the KBP protein bound strongly to the F-actin cytoskeleton (Fig. 6D). No quantitative or structural changes in F-actin protein were observed in fibroblasts from patients with a homozygous KIAA1279 mutation (p.Arg90X or p.Ser200X or p.Arg202IlefsX2) (data not shown).

KBP is involved in neurite outgrowth

To evaluate the consequences of KBP dysfunction on neuron-like cell morphology, we performed KBP extinction and overexpression assays in the SH-SY5Y neuroblastoma cell line. Reduction in endogenous KBP by siRNA induced dendritic spine depletion (Fig. 7A). Conversely, KBP overexpression by transient transfection with a KIAA1279 WT-cDNA construct was followed by an increase in dendritic spine length (Fig. 7A). Neurite length decreased significantly (P = 0.0005) after KBP extinction (26 ± 5 μm) and increased significantly (P < 0.0005) after KBP overexpression (60 ± 18 μm) when compared with neurite length of untreated cells (33 ± 6 μm) (Fig. 7B).

DISCUSSION

GOSHs is a condition first described in patients with syndromic HSCR and ascribed to neural-crest developmental anomalies (1). Reports of bilateral polymicrogyria in patients with KIAA1279 mutations suggested that KBP was involved in cerebral cortex development (17). In vivo studies in zebrafish supported a role of KIAA1279 in both enteric and central nervous system development (21). However, patients with
showed that fibroblasts from patients with KIAA1279 null mutation contained no KBP protein. We demonstrated that KBP protein interacted with both MTs and actin filaments. These data suggest that the multiple developmental anomalies seen in GOSHS might originate from disruption of cytoskeletal homeostasis.

**Clinical and diagnostic relevance**

The diagnosis of GOSHS is based on a distinctive facial gestalt combined with intellectual disability, microcephaly and HSCR. We have further refined the phenotypic description of GOSHS by studying the clinical features in 11 patients with KIAA1279 mutations, 5 in this study and 6 reported previously (4, 11, 17). All patients were born from consanguineous parents.

**Morphological signs**

Facial gestalt is one of the main clues to the diagnosis, and almost all patients present with typical facial features, either from early childhood or later on during growth.

**Growth parameters**

Growth parameters may be normal at birth, although most of the patients have neonatal microcephaly. Severe postnatal microcephaly (lesser than third percentile) is a consistent feature. Short stature was described previously (17). In our patients, height was near the 25th percentile, and overweight (95th percentile) was noted in one patient (F2, VI 3) after 7 years of age.

**Neurological signs**

All children had global developmental delay. In older patients, intellectual disability was moderate to severe. In our patients, motor skills continued to improve over time. Expressive language skills showed the greatest impairments, whereas comprehension skills were partly preserved. Seizures were neither observed in our patients nor reported in previous studies.

**Brain anomalies**

Focal or generalized polymicrogyria was noted in half of the patients investigated by cerebral MRI, two reported here and two studied previously (11). Hypoplasia of the corpus callosum was observed in two of our patients, but was not reported previously.

**Hirschsprung’s disease**

In all families reported so far, at least one affected individual had HSCR. Intrafamilial variability regarding the presence of HSCR was observed in two families, one reported here and one studied previously (17).

**Ocular abnormalities**

In our study, ptosis was progressive in two brothers, and both also had hyperopia. Megalocornea was present in one patient, a feature also reported by Brooks et al. in two cases (17). Iris coloboma was observed in one previously studied patient (4, 11), but was not found in any patients in the present study.
**Congenital heart disease**

Two of our patients had congenital heart disease, a feature not reported previously in patients with KIAA1279 mutations.

**Urogenital anomalies**

One patient had cryptorchidism and another unilateral vesicoureteral reflux and multicystic renal dysplasia. Vesicoureteral reflux was also reported previously in another patient (4,17).

**Skeletal features**

Oligodontia, scoliosis and a high-arched palate were each seen in one of our patients. Cleft lip has not been reported to date in patients with KIAA1279 mutations.

The main differential diagnoses are those in which patients present with a distinctive facial gestalt, microcephalia, intellectual disability and HSCR. MWS patients have HSCR and neurological, ocular, cardiac and urogenital features that overlap with GOSHS. However, patients with MWS have different facial characteristics such as thick and horizontal eyebrows and uplifted earlobes with a central depression. Unlike GOSHS patients, MWS patients do not have polymicrogyria or oligodontia. Ptosis is unusual in MWS patients. Common signs of MWS, such as seizures (80%) and hypospadias (50% of boys), have not yet been observed in patients with proven GOSH.

Another differential diagnosis is Baraitser–Winter syndrome (MIM # 243310). Several key features of Baraitser–Winter syndrome (intellectual disability, microcephaly, congenital ptosis, high-arched eyebrows, ocular coloboma and short stature) (30) overlap with those observed in GOSHS patients. Congenital heart defects, urogenital malformations and HSCR have not been reported so far in patients with Baraitser–Winter syndrome.

**KIAA1279 and NMD**

NMD can prevent the expression of potentially dominant negative proteins, thereby exerting a protective effect on heterozygous carriers of premature stop codons, but can also contribute to a disease phenotype by inhibiting the expression of partially functional proteins (31). NMD occurs when a premature stop codon is located more than 50–54 nucleotides upstream of the last exon–exon junction (32). NMD is a complex mechanism that involves several cellular factors such as the exon junction complex and the cytoplasmic poly(A)-binding protein (33). All KIAA1279 mutations reported to date induce premature stop codons. In fibroblasts from three patients, each carrying a homozygous KIAA1279 null mutation (p.Arg90X in exon 1 and p.Ser200X and p.Arg202IlefsX2 in exon 3 near the junction with intron 3), KIAA1279 mRNA levels were drastically diminished (P < 0.0005) when compared with WT transcripts. No truncated KBP proteins were detected by western blotting. Exposure of the patients’ fibroblasts to the NMD inhibitor emetine significantly increased (P < 0.005) the KIAA1279 transcript when compared with unexposed fibroblasts. Taken together, these results demonstrated that NMD occurred in KIAA1279 truncating mutations, suggesting loss of KBP function as the mechanism underlying GOSHS.

**GOSHS is not a mitochondrial disorder**

In 2005, KBP was identified in a brain cDNA library, using a yeast two-hybrid screen with KIF1C as bait (19). Perfect co-localization between KBP and mitochondria was noted in non-transfected NIH3T3 cells, as well as mitochondrial aggregation when a KBP mutant was overexpressed in the same cell line (19). A study involving a yeast two-hybrid assay in an E11 mouse cDNA library showed that KBP interacted with the MT-binding protein SCG10, but did not localize to mitochondria (20). Here, we investigated whether absence of KBP affected mitochondrial function. Respiratory chain complex activity was normal in fibroblasts in all three tested patients. In control fibroblasts, no co-localization of mitochondria and KBP was observed, confirming the previous results (20). Moreover, patient phenotype was not characteristic of mitochondrial diseases that are usually associated with postnatal neurological deterioration and seizures; whereas GOSHS involves prenatal microcephaly and multiple congenital malformations.

**KBP is associated with both MT and actin cytoskeleton**

The previously reported interaction between KBP and SCG10 suggested a role of KBP in MT organization (20). Here, we showed that KBP co-localized with α-tubulin and F-actin in control human fibroblasts. In vitro co-sedimentation assays confirmed that KBP interacted with MTs and filamentary actin. These results suggested that KBP might contribute to cross-linking of MTs and actin cytoskeletons. Of note, MT actin cross-linking factor (or ACF7), an actin and MT interacting factor, has been shown to bind the TPR of rapsyn, a postsynaptic scaffolding protein (34). Both rapsyn and KBP contain TPRs.

Evidence that cytoskeletal proteins are involved in developmental disorders via effects on neuronal development and migration and on brain architecture has accumulated over the last two decades. The role of MTs in brain development has been established by studies linking brain disorders to several genes that are either involved in MT homeostasis (LIS1 and DCX) or encode tubulin (TUBA1A, TUBA8, TUBB2B and TUBB3) (35–41). Pleiotropic phenotypes with neurological impairment, growth failure and parathyroid gland aplasia have been described in patients carrying mutations of the tubulin-specific chaperone gene TBCE responsible for hypoparathyroidism-retardation-dysmorphism syndrome (MIM # 241410) (42).

Recently, the actin-encoding genes ACTB and ACTG1 have been shown to be involved in brain malformations causing Baraitser–Winter syndrome (MIM # 243310) (30). The brain abnormalities and several facial morphological features of Baraitser–Winter syndrome overlap with GOSHS. Proteins interacting with both actin and MTs have not been associated with neurodevelopmental disorders so far. Our finding that KBP interacts with F-actin and MTs constitutes the first evidence that an actin MT cross-link protein is involved in neuronal development in humans.

**KBP is involved in neurite outgrowth**

The cytoskeleton plays a central role in modulating neuron morphology and growth (43–46). We studied the role of
KBP in the neuroblastoma cell-line SH-SY5Y by silencing and overexpressing KIAA1279. The KBP expression level showed a positive correlation with dendritic spine length. KBP silencing resulted in significant ($P < 0.0005$) dendrite shortening and KBP overexpression in significant ($P < 0.0005$) dendrite lengthening. Dendritic spines are major sites of excitatory synaptic input, and their morphological changes have been linked to learning and memory processes (43). Patients with KIAA1279 null mutation consistently exhibit severe intellectual disability and cerebral developmental anomalies. Furthermore, KBP is involved in axonal structure, outgrowth and maintenance in zebrafish (21).

In conclusion, KBP expression directly affected neurite growth, in keeping with the central (polymicrogyria) and enteric (HSCR) neuronal developmental defects seen in GOSHS. The KBP interaction with actin filaments and MTs demonstrated in our study constitutes the first evidence that an actin MT cross-link protein is involved in neuronal development in humans. We found no evidence of mitochondrial dysfunction. Finally, our finding that NMD mediates the abnormalities seen in GOSHS suggests that treatments targeting premature stop-codon transcripts might deserve evaluation.

**MATERIALS AND METHODS**

**Patients and molecular KIAA1279 analysis**

The patients were recruited at the Reims and Amiens hospitals in France and the Saint-Joseph Hospital in Beirut, Lebanon. Their medical records were reviewed, and their referring physicians were contacted for additional clinical data. DNA studies were performed after informed consent was obtained from the parents of each patient.

Direct sequencing of the KIAA1279 gene was performed using the Sanger method. The coding exons and flanking intronic sequences of the KIAA1279 gene (NM_015634) were amplified by PCR from genomic DNA previously isolated using standard procedures. Primer sequences are available on request. PCR products were sequenced using the Big Dye Terminator reaction kit (Applied Biosystems, Foster City, CA, USA) and a 16-capillary ABI Prism sequencer was then analyzed using Applied Biosystems software. DNA mutation numbering was based on the cDNA sequence (NM_015634.3), where +1 corresponds to the A of the ATG translation initiation codon.

**RNA extraction and RT-PCR assays**

Cell lysates were homogenized using QIAshredder columns (Qiagen, Hilden, Germany). Total RNA was isolated using RNAeasy Kit (Qiagen) according to the manufacturer’s instructions, including on-column DNase treatment. Preparation of mRNA-derived cDNA involved reverse transcription of 1 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers.

RT-PCRs of the 5′ coding region were performed using exon 1 KIAA1279 specific primers (sense primer 5′-GCGAAGCCTTCCGCTTCCG-3′ and reverse primer 5′-CCAGGGCTGGACCTC-3′). The RT-PCR products were analyzed by agarose gel electrophoresis.

Allele-specific RT-PCR was performed using primers encompassing the identified KIAA1279 mutations (exon 1 sense primer 5′-GGCGAAGGTCACCCTCCTCG-3′ and exon 7 reverse primer 5′-GCTCTAGCATGGCTATTCCTG-3′). Sanger sequencing of the corresponding fragments was performed.

Real-time RT-PCRs were performed using the Fast SYBR Green Master Mix (Applied Biosystems) and specific KIAA1279 primers hybridizing to exons 4 and 5 (sense primer 5′-AGCGGACCTTGCACATGC-3′ and reverse primer 5′-CACTGCCGCTGCCTCCATAAAGCA-3′). ABL1 was used as the reference gene with specific primers hybridizing to exons 6–7 (sense primer 5′-AGGAGTGCAACCCGGCAGGA-3′ and reverse primer 5′-CCAGGGAGCTTCGGGCAGCA-3′).

Semi-quantitative fluorescent multiplex RT-PCRs were performed using Qiagen Master Mix and the same primers as those used for the real-time RT-PCR, with fluorescence labeling of the forward primers. To determine the log-linear range of the PCR, the optimal conditions were experimentally established at 25 cycles (ABI Veriti thermocycler, Applied Biosystems). Capillary electrophoresis was performed using an ABI3130XL sequencer and GeneMapper 4.0 software (Applied Biosystems). For each PCR fragment (corresponding to KIAA1279 or ABL1 picks), the area under the curve was calculated. KIAA1279 transcript values were normalized for those of a housekeeping gene (ABL1), and at least three independent measurements were performed in triplicate.

**Plasmid constructs and siRNA**

Human KIAA1279 cDNA was subcloned into the expression plasmid pCMV-Myc (Clontech, Mountain View, CA, USA), and the resulting inserts were fully sequenced. Four siRNAs (ON-TARGETplus SMARTpool) were used to extinguish KIAA1279 expression, and a control siRNA (siCONTROL non-targeting) was used to demonstrate the absence of off-target effects (Dharmacon, Lafayette, CO, USA).

**Cell culture, transfection and drug treatment**

To obtain human fibroblasts, dorsal forearm skin biopsies were performed in three patients, two parents and two healthy donors after obtaining their informed consent according to the Declaration of Helsinki. Primary explant cultures were established in 25 cm² culture flasks in MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ atmosphere. Monolayer cultures isolated independently from the different individuals were maintained at 37°C in 5% CO₂. Primary cultured fibroblasts were used between the third and sixth subpassages.

Medium supplemented with 300 μg/ml of emetine (Sigma-Aldrich, Saint-Louis, MI, USA) was used to incubate 10⁴ cells for 6 h, according to published protocols (47).

Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA, USA) were grown in MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) at 37°C in 5% CO₂. Cells were passaged at 5 × 10⁴ per well into 12-well tissue culture plates 48 h before transfection,
Cells were attached to 0.01% poly-L-lysine-treated coverslips, subtraction. (Syngene, Cambridge, UK) by lowest slope background conducted using the GeneGnome BioImaging System antibody (Abnova). Western blotting and quantification were of KBP was quantified using specific anti-full-length KBP resolution western blotting was performed, and the amount mutants (p.Arg90X, p.Ser200X and p.Arg202IlefsX2), high-resolution western blotting was performed, and the amount of KBP was quantified using specific anti-full-length KBP antibody (Abnova). Western blotting and quantification were conducted using the GeneGnome BioImaging System (Syngene, Cambridge, UK) by lowest slope background subtraction.

Quantitative western blotting by densitometry analysis Cells were washed three times in ice-cold PBS1X, then harvested in ice-cold PBS1X, pelleted at 1000 g at 4 °C and lysed in 300 μl RIPA buffer. Equal amounts of protein were subjected to 15% SDS-PAGE. Quantitative western blotting was performed as previously described (48). The antibodies used were anti-α-tubulin (DSHB, University of Iowa, IA, USA), anti-KBP (Sigma-Aldrich and Abnova, Taipei, Taiwan), anti-CNX (Abcam, Cambridge, UK), anti-F-actin (Abcam), anti-β-actin (Abcam) and horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL, USA). To specifically detect the small KBP mutants (p.Arg90X, p.Ser200X and p.Arg202IlefsX2), high-resolution western blotting was performed, and the amount of KBP was quantified using specific anti-full-length KBP antibody (Abnova). Western blotting and quantification were conducted using the GeneGnome BioImaging System (Syngene, Cambridge, UK) by lowest slope background subtraction.

Digitonin treatment and immunofluorescent staining Cells were attached to 0.01% poly-L-lysine-treated coverslips, pretreated with 0.01% digitonin and fixed with paraformaldehyde for 20 min at room temperature. Immunofluorescent staining was performed as previously described (48). The antibodies used were anti-α-tubulin (DSHB, University of Iowa, IA, USA), anti-KBP (Sigma-Aldrich and Abnova, Taipei, Taiwan), anti-CNX (Abcam, Cambridge, UK), anti-F-actin (Abcam), anti-β-actin (Abcam) and horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL, USA). To specifically detect the small KBP mutants (p.Arg90X, p.Ser200X and p.Arg202IlefsX2), high-resolution western blotting was performed, and the amount of KBP was quantified using specific anti-full-length KBP antibody (Abnova). Western blotting and quantification were conducted using the GeneGnome BioImaging System (Syngene, Cambridge, UK) by lowest slope background subtraction.

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Respiratory chain complex enzyme activities Respiratory chain complex activities were measured on digitonin-treated fibroblasts using spectrophotometric methods, as previously described (24,25). Complex I (NADH quinone reductase) was measured by monitoring rotenone (0.8 μM)-sensitive NADH (0.25 mM) oxidation in the presence of 0.3 mM KCN and 100 μM decylubiquinone. Complex V (ATPase) was subsequently measured using the same spectrophotometric cuvette as oligomycin (10 μM)-sensitive NADH oxidation in the presence of 0.5 mM ATP, 2 mM PEP, 5 mM MgCl₂, 20 IU LDH and 20 IU PK. Complex IV (cytochrome c oxidase) was measured by monitoring the oxidation of 10 μM reduced cytochrome c. After addition of 0.3 mM cyanide, succinate-cytochrome c reductase (Complex II + III) activity was measured in the same cuvette in the presence of additional oxidized cytochrome c (10 μM), 8 μM rotenone and 5 mM succinate. After addition of a Complex II-specific inhibitor (5 mM malonate), Complex III (ubiquinol-cytochrome c reductase) activity was measured in the presence of 2 mM EDTA by monitoring antimycin-sensitive reduction of cytochrome c triggered by 50 μM of reduced decylubiquinone.

In addition, automated spectrophotometric measurements of the enzymatic activities of the 14 oxidative stress markers (GPX, Cu/Zn-SOD, Mn-SOD, G6PD, GCS, GS, GPX, GR, MDA, GSH, GSSG, ATP, ADP and AMP) were performed using a Roche Diagnostics/Hitachi 912 analyzer (Meylan, France), based on protocols described by Kramer et al. (49) and Krahenbuhl et al. (50), with adjustments to the analyzer’s requirements.

MT-binding assay Binding of proteins to MTs was assayed using the Microtubule Binding Protein Spin-down assay Kit (Cytoskeleton, Denver, CO, USA) as previously described (28). Cells were lysed in tubulin buffer containing 0.1% Triton X-100. Lysates were centrifuged at 100 000 g for 1 h in a Beckman centrifuge to remove potential aggregates. The resulting supernatant was incubated for 30 min at room temperature with purified Taxol-stabilized MTs that were generated using the Microtubule/Tubulin Biochem Kit (Cytoskeleton). Subsequently, MTs were pelleted by high-speed centrifugation (100 000 g for 1 h). The resulting pellets and supernatant fractions were analyzed by western blotting.

Actin-binding assay Binding of proteins to F-actin was assayed using the Non-Muscle Actin Binding Protein Biochem Kit (Cytoskeleton) as previously described by Hildebrand and Soriano (29). Purified actin was polymerized as instructed by the manufacturer. M1 cytosol prepared in buffer B was diluted with 2 volumes of buffer E (4.5 mM Tris–HCl, pH 8.0, 50 mM KC1, 1 mM MgCl₂, 1 mM ATP and 0.18 mM CaCl₂) and centrifuged at 4 °C for 90 min at 100 000 g to remove potential aggregates. The resulting supernatant was incubated in buffer E with or without F-actin for 30 min at room temperature. Subsequently, each incubated sample (50 μl) was layered on 0.2 ml of cushion buffer (5 mM Tris–HCl, pH 8.0, 50 mM KC1, 2 mM MgCl₂ and 10% (v/v) glycerol) and centrifuged at room temperature for 90 min at 100 000 g. The resulting pellets and supernatant fractions were analyzed by western blotting.

Statistical analysis Data are described as mean ± SEM. Comparisons of two data-sets were performed using the two-tailed Student’s t-test. P-values less than 0.05 were considered significant.

SUPPLEMENTARY MATERIAL Supplementary Material is available at HMG online.
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Conflict of Interest statement. The authors declare no conflict of interest.

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