Developmental and Degenerative Features in a Complicated Spastic Paraplegia

M. Chiara Manzini, PhD,1 Anna Rajab, PhD,2 Thomas M. Maynard, PhD,3 Ganeshwaran H. Mochida, MD, MMSc,1,4,5 Wen-Hann Tan, BMBS,5 Ramzi Nasir, MD, MPH,6 R. Sean Hill, PhD,1 Danielle Gleason, BA,5 Muna Al Saffar, MBChB, CCGC,1,5 Jennifer N. Partlow, MS, CGC,1,5 Brenda J. Barry, MS, CGC,1,5 Mike Vernon, MSc,3 Anthony-Samuel LaMantia, PhD,3 and Christopher A. Walsh, MD, PhD1,5

Objective: We sought to explore the genetic and molecular causes of Troyer syndrome, one of several complicated hereditary spastic paraplegias (HSPs). Troyer syndrome had been thought to be restricted to the Amish; however, we identified 2 Omani families with HSP, short stature, dysarthria and developmental delay—core features of Troyer syndrome—and a novel mutation in the \(\text{SPG20}\) gene, which is also mutated in the Amish. In addition, we analyzed \(\text{SPG20}\) expression throughout development to infer how disruption of this gene might generate the constellation of developmental and degenerative Troyer syndrome phenotypes.

Methods: Clinical characterization of 2 non-Amish families with Troyer syndrome was followed by linkage and sequencing analysis. Quantitative polymerase chain reaction and in situ hybridization analysis of \(\text{SPG20}\) expression were carried out in embryonic and adult human and mouse tissue.

Results: Two Omani families carrying a novel \(\text{SPG20}\) mutation displayed clinical features remarkably similar to the Amish patients with Troyer syndrome. \(\text{SPG20}\) mRNA is expressed broadly but at low relative levels in the adult brain; however, it is robustly and specifically expressed in the limbs, face, and brain during early morphogenesis.

Interpretation: Null mutations in \(\text{SPG20}\) cause Troyer syndrome, a specific clinical entity with developmental and degenerative features. Maximal expression of \(\text{SPG20}\) in the limb buds and forebrain during embryogenesis may explain the developmental origin of the skeletal and cognitive defects observed in this disorder.

Hereditary spastic paraplegias (HSPs) comprise several disorders commonly divided into 2 subgroups: “pure” HSPs characterized by progressive spasticity in the lower limbs due to pyramidal tract degeneration and “complicated” HSPs, where lower limb spasticity is associated with a variety of other neurological signs and clinical features. Complicated HSPs are clinically heterogeneous, mainly autosomal recessive syndromes, frequently described and mapped in sporadic families within inbred populations.1–3 Because of this heterogeneity, diagnosis.
Troyer syndrome (Online Mendelian Inheritance in Man #275900) is a complicated HSP associated with short stature, skeletal abnormalities, dysarthria, and developmental delay, first described in the Old Order Amish.\textsuperscript{3,5} Since the original description in 1967, several Troyer-like syndromes have been reported,\textsuperscript{6-9} but they often differed from classical Troyer syndrome in their neurological or skeletal features. The Amish founder mutation is a single nucleotide deletion in the SPG20 gene,\textsuperscript{10} leading to the loss of the spartin protein;\textsuperscript{11} however, no additional SPG20 mutations were subsequently identified,\textsuperscript{5} and it was suspected that Troyer syndrome may be restricted to the Amish.

We identified an Omani kindred presenting with clinical features resembling Troyer syndrome. All affected Omani individuals had a novel homozygous null mutation in SPG20, and their clinical descriptions matched closely to those of Amish Troyer syndrome individuals of comparable ages. Because Troyer syndrome is associated with developmental features, such as short stature, skeletal abnormalities, and global developmental delay, we investigated the sites of action of SPG20 during development in humans and mice. \textit{SPG20}/\textit{Spg20} expression in the adult is relatively modest and widespread in the nervous system; however, maximal and focal expression is observed in the embryonic limb buds, face, and forebrain during early morphogenesis, which might explain the developmental phenotypic changes involving the extremities, face, and brain.

\textbf{Subjects and Methods}

\textbf{Enrollment and Clinical Studies}

The subjects belonged to 2 families residing in a remote region of Oman and were originally ascertained by a local clinical geneticist. Detailed family and medical histories were obtained by a genetic counselor, who is a native Arabic speaker, and a developmental pediatrician, also a native Arabic speaker, conducted a developmental assessment. A pediatric neurologist performed a standard neurological examination, and a second clinical geneticist obtained a detailed family and medical history by a genetic counselor, who is a native Arabic speaker, and a developmental pediatrician, also a native Arabic speaker, conducted a developmental assessment.

A pediatric neurologist performed a standard neurological examination, and a second clinical geneticist obtained anthropometric measurements. The height of all subjects and head circumference of subjects under the age of 36 months were plotted on the Centers for Disease Control and Prevention 2000 growth charts,\textsuperscript{12} and head circumference of subjects above 36 months and all other anthropometric measurements were plotted on standard charts.\textsuperscript{13} Written, informed consent was obtained from the subjects or their legal guardians. The Ministry of Health in Oman and the institutional review board of Children’s Hospital Boston approved this study.

\textbf{Linkage Analysis}

Genomic DNA was purified from lymphocytes separated from peripheral blood using commercial kits (Qiagen, Valencia, CA). Six hundred nanograms of genomic DNA was used to hybridize Affymetrix Human SNP Array 6.0 at the Genomic Analysis Core of the University of North Carolina (UNC) Neuroscience Center of the UNC School of Medicine, Chapel Hill, North Carolina. After removal of low-quality calls and of Mendelian and non-Mendelian errors using Merlin software,\textsuperscript{14} the single nucleotide polymorphism (SNP) data were analyzed using Allele software\textsuperscript{15} under a fully penetrant autosomal recessive model. For microsatellite analysis, highly polymorphic microsatellite markers were chosen from the Marshfield database in the University of California at Santa Cruz Genome Browser. Fluorescently labeled polymerase chain reaction (PCR) primers (Applied Biosystems, Foster City, CA) were used to amplify DNA samples using standard conditions, and PCR products were resolved on an Applied Biosystems 3130xl Genetic analyzer.

\textbf{SPG20 Sequencing Analysis}

Sequencing of \textit{SPG20} (NM_015087; NM_001142294-6) coding region was performed by SeqWright (Houston, TX) on PCR products after amplification of genomic DNA. PCR primers were designed for each exon including at least 50bp of flanking intronic sequences. Primer sequences are available on request. Once a putative change was identified, at least 96 control DNAs (192 alleles) were tested to exclude the possibility of a benign polymorphic change.

\textbf{Patient Cell Lines and Mutated Protein/mRNA Detection}

Transformed lymphoblastoid cell lines were established from peripheral blood of affected and unaffected individuals at the Partners Center for Personalized Genetic Medicine (Cambridge, MA). Cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum, glutamine (2mM) and penicillin/streptomycin (100U/100 \mu g) (all from Gibco, Grand Island, NY). Protein lysates were obtained by boiling a cell pellet in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Western blotting was performed using standard protocols on Bio-Rad equipment. Presence of spartin was assessed using a rabbit anti-SPG20 antibody (Proteintech Group, Chicago, IL) and a goat anti-rabbit IRDye-800CW secondary antibody (LI-COR Biosciences, Lincoln, NE) detected on an Odyssey Imager (LI-COR Biosciences).

Total RNA was purified from patient cell lines using the mirVana RNA Isolation Kit (Ambion Applied Biosystems, Foster City, CA). Random primed cDNA was generated using reverse transcriptase (Promega, Madison, WI) and analyzed by quantitative PCR (qPCR) using SYBR Green reagents (Applied Biosystems) on an ABI 7500 qPCR platform as previously described.\textsuperscript{16} Primer sequences are available in Supplementary Table.

\textbf{SPG20/Spg20 Expression Analysis}

cDNA samples from human fetal and adult postmortem brain tissue were obtained commercially (BioChain Institute, Hayward, CA and Clontech, Mountain View, CA). cDNA from embryonic and postnatal mouse brains was generated as described above. qPCR was performed as described above (see Supplementary Table for primer sequences). DNA templates for in situ hybridization probes were cloned using a 550bp fragment of the \textit{Spg20}
coding sequence using the following primers: Spg20-BamHI 5'-ctacagatctatacgcagagacagcotaacgcgaagcc-3' and Spg20-Sall 5'-gccccgccgcctgtcctgtcacctgtcctggtac-3'. Digoxigenin-labeled riboprobes were synthesized and in situ hybridization was performed as previously described.17 Video images of the hybridized sections were obtained on a Wild Leitz (Heerbrugg, Switzerland) photomicroscope or Leica (Nussloch, Germany) DMR microscope under consistent illumination conditions.

Results
Identification of a Novel SPG20 Mutation in an Omani Kindred
We examined 2 related Omani families presenting with short stature, spasticity, dysarthria, and developmental delay. Affected individuals are in black and unaffected in white. The status of individual 2-8 (in gray) could not be determined, because she is too young to properly assess neurological symptoms. All numbered individuals were examined, but genomic DNA was only collected from individuals for whom microsatellite analysis is shown. Microsatellite analysis revealed common maternal (in yellow) and paternal (in blue) haplotypes in all affected. Microsatellite markers in the linkage region identified by the single nucleotide polymorphism analysis are in bold. One homozygous marker (D13S219) is common to all affected individuals (highlighted in orange). (B) The homozygous region contains 6 genes, including SPG20 (in orange). (C) The affected individuals carried a homozygous 2bp deletion in SPG20, which was present in heterozygosity in the carriers. (D) Western blot analysis of patient cell lines showed that full-length SPG20 protein is missing in the affected individuals (A) compared with a nonaffected noncarrier sibling (NA). (E) Quantitative polymerase chain reaction analysis of cDNA from the patient cell lines indicated that SPG20 mRNA is not present in the affected individual. deltaRN = signal magnitude expressed by the difference in the normalized reporter (RN) values.

FIGURE 1: Affected individuals carry a homozygous null mutation in the SPG20 gene. (A) Pedigree of 2 related Omani families affected with short stature, spasticity, dysarthria, and developmental delay. Affected individuals are in black and unaffected in white. The status of individual 2-8 (in gray) could not be determined, because she is too young to properly assess neurological symptoms. All numbered individuals were examined, but genomic DNA was only collected from individuals for whom microsatellite analysis is shown. Microsatellite analysis revealed common maternal (in yellow) and paternal (in blue) haplotypes in all affected. Microsatellite markers in the linkage region identified by the single nucleotide polymorphism analysis are in bold. One homozygous marker (D13S219) is common to all affected individuals (highlighted in orange). (B) The homozygous region contains 6 genes, including SPG20 (in orange). (C) The affected individuals carried a homozygous 2bp deletion in SPG20, which was present in heterozygosity in the carriers. (D) Western blot analysis of patient cell lines showed that full-length SPG20 protein is missing in the affected individuals (A) compared with a nonaffected noncarrier sibling (NA). (E) Quantitative polymerase chain reaction analysis of cDNA from the patient cell lines indicated that SPG20 mRNA is not present in the affected individual. deltaRN = signal magnitude expressed by the difference in the normalized reporter (RN) values.
Genomic DNA was obtained from most individuals in the pedigree and hybridized to Affymetrix Human SNP Array 6.0 chips for genome-wide genotyping. We suspected the presence of a homozygous ancestral mutation because the families resided in an isolated mountain village and large stretches of homozygous DNA identified in all individuals suggested some degree of consanguinity; however, we could not ascertain any shared ancestry as far as 5 generations removed from the probands by pedigree analysis. Thus, the SNP data was first analyzed following an autosomal recessive model assuming no consanguinity. Linkage analysis identified 1 region of 7.0 Mb flanked by SNPs rs11619306 and rs9576104 on chromosome 13q12.3 (log of odds = 3.6). Microsatellite analysis confirmed a shared maternal and paternal haplotype in all affected individuals (see Fig 1A). Individual 1-4, who was unaffected, was homozygous for the maternal haplotype throughout most of the linkage region, excluding marker D13S219, which was homozygous on all affected individuals. At this locus, the SNP data indicated an 844 kb region of homozygosity between markers rs1418987 and rs9315443, shared only by affected but not unaffected individuals. Among the 6 genes in this region (see Fig 1B), SPG20 was the strongest candidate gene, because individuals carrying an SPG20 mutation were affected with a remarkably similar phenotype, a complicated form of hereditary spastic paraplegia associated with short stature, dysarthria, and developmental delay, called Troyer syndrome. Since the SPG20 mutation in the Amish (c.1110delA) was reported to be a null mutation, we generated lymphoblastoid cell lines from 2 affected (1-3 and 1-5) and 1 unaffected individual (2-5). We found that the full-length spartin protein was absent in the affected individuals (see Fig 1D). We also carried out qPCR for SPG20 on cDNA samples from 2 of the lymphoblastoid lines and could not detect any mRNA in the affected individual (see Fig 1E), indicating that this mutation is a null allele.

Clinical Characterization of Patients Carrying a Novel SPG20 Mutation
To ascertain phenotypic similarities or differences among individuals carrying SPG20 mutations, we compared the clinical presentations of the Omani individuals with the clinical description of 21 Amish patients by Proukakis et al (Table). All affected individuals in the Omani cohort presented with short stature and dysarthria, and were delayed in reaching motor and cognitive developmental milestones. They all had some difficulties walking, with clumsy, mildly spastic gait, which their parents reported to be worsening over time. The most common physical features were relative hypertelorism and overgrowth of the maxilla leading to overbite, as well as hand and feet anomalies such as brachydactyly (5/6 patients), hammer toes, and pes cavus (Fig 2). Additional nonspecific skeletal malformations observed in the hands were clinodactyly, camptodactyly, and hypoplastic 5th middle phalanges (see Fig 2E, F). Most affected individuals had persistent cognitive deficits and poor performance in school, but no emotional lability was reported. Detailed neuropsychological testing was not available.

The neurological examination revealed distal amyotrophy (4/6 patients), dysmetria in the upper extremities (3/6 patients), hyperreflexia, which was more severe in the lower limbs (4/6 patients), and ankle clonus (3/6 patients). Heel cords were tight in the 4 older affected individuals. Brain magnetic resonance imaging (MRI) was performed in 2 individuals (1-7 at 14 years and 2-3 at 19 years). Both individuals had mild atrophy of the cerebellar vermis, mild white matter volume loss, and revealed periventricular white matter hyperintensity in T2-weighted images, consistent with gliosis (see Fig 2I, J).

The mean age of our cohort (16.6 years) was younger than the Amish cohort, which might explain the milder and more variable phenotype observed in the Omani individuals; therefore, we subdivided the Amish cohort into a younger subset (Amish I) comparable in age to our cohort (<27 years; mean age, 15 years) and an older subset (Amish II) (>28 years; mean age, 43.5 years). Our cohort closely matched the description of the Amish I group, whereas the Amish II group was more severely affected, consistent with the progressive, degenerative aspects of Troyer syndrome (see Table).

Expression of Spg20 in the Developing and Adult Nervous System
There appear to be 2 major components in Troyer syndrome: an early developmental aspect and a neurodegenerative process. The combination of phenotypes, including limb, craniofacial, and behavioral anomalies, is common in complex genetic disorders affecting early morphogenetic events, and the relevant genes are often specifically expressed in the embryonic rudiments of the affected adult structures. We first analyzed SPG20 expression in the developing and adult human brain using qPCR. SPG20 was expressed at modest levels in the fetal
and adult human brain compared to a highly expressed neurally specific gene, synaptophysin (SYP; Fig 3A, B). Levels of SPG20 were highest in the amygdala, cortex, and thalamus, and lowest in the hippocampus and cerebellum. Although clearly detectable, SPG20 was expressed at substantially lower levels than SYP. Expression levels and distribution of the mouse orthologue of SPG20, Spg20, in the adult mouse brain parallel those of the human, with some divergence; particularly, relative expression of Spg20 in the hippocampus was slightly elevated compared with humans. In addition, we analyzed Spg20 expression in mouse spinal cord and brainstem and found that it was highest in the spinal cord of all brain regions measured. As in the human, Spg20 was expressed at comparatively lower levels than Syp. Spg20 is not a brain-specific gene, as it is also expressed in several other organs.10,19 It is, however, developmentally regulated, as expression was maximal at midgestation and embryonic day (E)10, and declined precipitously thereafter (see Fig 3C–F).

We then used in situ hybridization to localize Spg20 in adult, fetal, and embryonic mouse brain (Fig 4). Spg20 was expressed at relatively low levels in neurons and glia throughout the adult brain, including glia in fiber tracts. Modestly elevated expression was seen throughout hippocampal stratum pyramidale of the CA fields and dentate gyrus and the transitional parahippocampal/entorhinal cortex. Low expression was seen in the cerebral cortex with no apparent laminar or cell class specificity. The only additional site of elevated expression in the forebrain was the habenular complex, including the habenular recess appended to the corpus callosum. Spg20 is expressed throughout the cerebellum in Purkinje cells, granule cells, and scattered cells in the molecular layer. In the brain-

<table>
<thead>
<tr>
<th>TABLE: Clinical Features of the Omani Troyer Syndrome Individuals and Comparison with the Amish Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Height (cm)</td>
</tr>
<tr>
<td>%</td>
</tr>
<tr>
<td>OFC (cm)</td>
</tr>
<tr>
<td>Developmental milestones and cognition</td>
</tr>
<tr>
<td>Talked at</td>
</tr>
<tr>
<td>Walked at</td>
</tr>
<tr>
<td>Poor school performance</td>
</tr>
<tr>
<td>Emotional lability</td>
</tr>
<tr>
<td>Neurological exam</td>
</tr>
<tr>
<td>Dysarthria</td>
</tr>
<tr>
<td>Tongue dyspraxia</td>
</tr>
<tr>
<td>Distal amyotrophy</td>
</tr>
<tr>
<td>Hyperreflexia</td>
</tr>
<tr>
<td>Upper</td>
</tr>
<tr>
<td>Lower</td>
</tr>
<tr>
<td>Ankle clonus</td>
</tr>
<tr>
<td>Clumsy, spastic gait</td>
</tr>
<tr>
<td>Dysmetria</td>
</tr>
<tr>
<td>Skeletal abnormalities</td>
</tr>
<tr>
<td>Overbite</td>
</tr>
<tr>
<td>Hypertelorism</td>
</tr>
<tr>
<td>Hand/foot abnormalities</td>
</tr>
</tbody>
</table>

OFC = occipitofrontal circumference; NA = not available.
stem, there is robust expression in large neurons, probably
motor neurons, and in the facial nucleus. Spg20 is also
expressed in cells distributed throughout the spinal cord
with no noticeable discontinuities. As predicted from the
qPCR data, expression in the fetal brain is relatively low,
with no apparent regional distinctions. Aside from ele-
vated expression in the lens placode and pigment epithe-
lum, cochlear epithelium, and condensing mesenchyme
at sites of myogenic or cartilage formation, there is little
focal expression at fetal stages (see Fig 4L–N; E12.5 and
E14.5 are shown).

In contrast to the later stages, and, as predicted from
the qPCR analysis, Spg20 expression in the midgestation
embryo is elevated and highly patterned. In the E10.5
embryos, shortly after neural tube closure, Spg20 is spe-
cifically expressed in the initial frontonasal mass/forebrain,
craniofacial structures, aortic arch/heart primordium, and
limb buds during morphogenesis, with lowest expression
in the heart (Fig 4O, P). qPCR analysis confirms this dis-
tribution; expression levels in limb buds, branchial arches,
and frontonasal mass/forebrain are substantially elevated
compared with the heart as well as with whole E10.5 em-
bryo (Fig 4R). Our observations suggest that Spg20 has its
most specific and maximal activity in the limbs, face and
forebrain during early morphogenesis. This is remarkable,
because the sites of phenotypic manifestations in the af-
fected members of the Omani Troyer syndrome kindred
described here include anomalies of the limb extremities,
face, and brain.

Discussion

Our identification of a novel, disease-associated SPG20
mutation in an Omani kindred indicates for the first time
that Troyer syndrome is not restricted to the Amish, as
previously proposed.\textsuperscript{2} Complicated autosomal recessive
HSPs are heterogeneous disorders often lacking clear clinical
and molecular diagnostic guidelines. Of the 17 distin-
tinct loci for complicated HSP identified to date, more
than half have been described in single families or isolated populations.\(^1,2\) In the past 4 decades, several HSP syndromes with Troyer-like features have been reported, but none matched the clinical presentation in the Amish cases.\(^5\) Some of these disorders were later mapped to different loci such as SPG26,\(^6,20\) SPG39,\(^21\) and ARSACS.\(^22,23\) Even within the isolated region where these Omani families reside, we had described a similar extended pedigree with dysarthria, mental retardation, cerebral palsy, and microcephaly.\(^24\) Linkage analysis ruled out the SPG20 locus in the second kindred, confirming that the 2 disorders are separate clinical and genetic entities (A.R., R.S.H., C.A.W., unpublished data). Four individuals in this extended family (N = 45 individuals) also were heterozygous carriers for the SPG20 mutation, highlighting its presence in this isolated population. Thus, this novel mutation in SPG20 is geographically and genetically distinct from that in the Amish population; nevertheless, both mutations result in the phenotypic spectrum associated with Troyer syndrome.

**FIGURE 3:** Quantitative polymerase chain reaction analysis of SPG20/Spg20 expression in human and mouse tissues. (A) There is modest variation in SPG20 local expression in the adult human brain when distinct regions are compared to whole brain samples. (B) Human SPG20 expression is substantially lower than that of the brain-specific gene synaptophysin (SYP) across all brain regions with the exception of the amygdala. (C) A similar profile of regionally variable Spg20 expression, with some modest differences, is seen in the mouse brain. (D) Lower Spg20 expression is observed when compared with Syp. (E) Spg20 is expressed in several murine tissues beside the brain. (F) Spg20 is developmentally regulated in the mouse embryo with highest expression levels in the whole embryo at E10.5. DeltaCT = signal magnitude expressed by the difference in CT values. Hippoc = hippocampus; Mesen = mesenchyme; Cerebel = cerebellum; Sp Cord = spinal cord; Sk Muscle = skeletal muscle; Rel. = relative.
Although a clear Troyer syndrome diagnosis is difficult to reach in young subjects due to the variability and mildness of their symptoms, direct comparison of clinical features showed that the Omani cohort closely resembled the age-matched Amish Troyer syndrome group (Amish I), suggesting that \textit{SPG20} null mutations cause a well-defined phenotype. As in the Amish, we observed short stature, skeletal abnormalities in the extremities, developmental delay, and dysarthria of possible cerebellar origin from an early age. Therefore, a diagnosis of Troyer syndrome must be considered when this constellation of phenotypes is present in young children. Facial dysmorphism

![Image](image_url)
and skeletal features are subtle, and in single cases in non-consanguineous populations this disorder could be initially diagnosed simply as cerebral palsy. Spasticity and distal amyotrophy appeared in the teenage years and worsened slowly over time. In the Amish, brain MRI revealed white matter abnormalities, which were less severe in the youngest patient examined (age 15 years), suggesting a progressive worsening of the condition. MRI analysis indicates that the Omani Troyer syndrome individuals of comparable ages also have mild white matter abnormalities, supporting the hypothesis that white matter degeneration accompanies disease progression, although direct neuronal degeneration cannot be ruled out. In addition, we identified mild cerebellar atrophy, which is consistent with the cerebellar signs. Serial MRI scans of the Omani individuals might resolve whether progressive neurological symptoms are correlated with increased white or gray matter degeneration. Despite many similarities to the Amish cohort, a few specific differences were observed in the affected Omani individuals, including the presence of hypertelorism and a pronounced overbite, and the absence of inappropriate emotional responses. As new cases are identified it will be interesting to assess whether these traits have variable penetrance or are population specific.

Functional studies on spartin, the protein encoded by *SPG20*, have identified multiple roles in protein ubiquitination and lipid droplet formation, and a possible link to endothelial growth factor receptor signaling. Previous expression analyses by Northern blot or qPCR identified widespread *SPG20*/spartin localization in the brain and in other tissues, however, it was unclear whether regional and cellular dynamism in *SPG20* expression could explain the phenotypes observed in the affected individuals. Our study shows that whereas *SPG20*/Spag20 expression is modest and virtually ubiquitous in the adult and developing brain, early stages of embryonic development show maximal levels of *Spag20* in the limbs, face, and forebrain primordia. This localized expression suggests a parallel role for *SPG20*/Spag20 in morphogenesis and differentiation at these phenotypic sites; accordingly, a loss of function mutation may contribute to the phenotypic spectrum of Troyer syndrome.

Acknowledgment
This work was supported by the NINDS (grant RO1 NS 35129, C.A.W.), NICHD (grant RO1 HD029178, A.-S.L.), the Dubai Harvard Foundation for Medical Research (C.A.W.), the Manton Center for Orphan Disease Research (C.A.W.), the Muscular Dystrophy Association (Development Grant, M.C.M.), and the University of North Carolina (Reynolds Faculty Fellowship, A.-S.L.). C.A.W. is an Investigator of the Howard Hughes Medical Institute. We thank the families for their cooperation and their hospitality; the local nurses and doctors for their assistance; Megumi Aita at the University of North Carolina In Situ Core for help with the in situ analysis; Daniel Rakic at Children’s Hospital, Boston for help with the sample preparation and analysis; and the following colleagues at Children’s Hospital Boston for helpful discussion on the clinical features of the patients: Hope E. Dickinson, a speech pathologist from the Department of Otolaryngology and Communication Enhancement, and Janice Ware, a child psychologist and associate director of the Developmental Medicine Center.

Authorship
M. Chiara Manzini and Anna Rajab contributed equally and are co-first authors.

Potential Conflicts of Interest
Nothing to report.

References


