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SOS1 MUTATIONS IN NOONAN SYNDROME: AN UPDATE ON MOLECULAR SPECTRUM, STRUCTURAL INSIGHTS ON PATHOGENIC EFFECTS, AND GENOTYPE-PHENOTYPE CORRELATIONS

Francesca Lepri, Alessandro de Luca, Lorenzo Stella, Cesare Rossi, Giuseppina Baldassarre, Francesca Pantaleoni, Viviana Cordeddu, Bradley J Williams, Maria L Dentici, Viviana Caputo, et al.

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***SOS1* mutations in Noonan syndrome: molecular spectrum, structural insights on pathogenic effects, and genotype-phenotype correlations**

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Abstract

Noonan syndrome (NS) is among the most common non-chromosomal disorders affecting development and growth. NS is caused by aberrant RAS-MAPK signaling and is genetically heterogeneous, which explains, in part, the marked clinical variability documented for this Mendelian trait. Recently, we and others identified *SOS1* as a major gene underlying NS. Here, we explored further the spectrum of *SOS1* mutations and their associated phenotypic features. Mutation scanning of the entire *SOS1* coding sequence allowed the identification of 30 different variants deemed to be of pathological significance, including 13 novel missense changes and in-frame indels. Various mutation clusters destabilizing or altering orientation of regions of the protein predicted to contribute structurally to the maintenance of autoinhibition were identified. Two previously unappreciated clusters predicted to enhance *SOS1*'s recruitment to the plasma membrane, thus promoting a spatial reorientation of domains contributing to inhibition, were also recognized. Genotype-phenotype analysis confirmed our previous observations, establishing a high frequency of ectodermal anomalies and a low prevalence of cognitive impairment and reduced growth. Finally, mutation analysis performed on cohorts of individuals with non-syndromic pulmonic stenosis, atrial septal defects and ventricular septal defects excluded a major contribution of germline *SOS1* lesions to the isolated occurrence of these cardiac anomalies.

Key Words: Noonan syndrome; NS; *SOS1*; mutation analysis; structural analysis; genotype-phenotype correlations.

INTRODUCTION

Noonan syndrome (NS; MIM# 163950) is a relatively common and clinically variable disorder characterized by postnatal reduced growth, facial dysmorphism, and congenital heart defects (CHDs) [Allanson, 1987; Noonan, 1994; van der Burgt, 2007; Tartaglia et al., 2010]. The distinctive and most recurrent facial features consist of a broad forehead, hypertelorism, down-slanting palpebral fissures, ptosis, high arched palate, and low-set, posteriorly rotated ears. Cardiac involvement is present in up to 80-90% of affected individuals, with pulmonic stenosis (PS), septal defects and hypertrophic cardiomyopathy (HCM) occurring most commonly [Burch et al., 1993; Marino et al., 1999]. Other associated features include multiple skeletal defects (chest and spine deformities), webbed/short neck, variable cognitive deficits, cryptorchidism, lymphatic dysplasia, bleeding diathesis, and, rarely, predisposition to certain hematologic malignancies during childhood.

NS is genetically heterogeneous, and, based upon the recent discoveries of the underlying disease genes, is now regarded as a disorder caused by enhanced signal flow through the RAS-MAPK pathway [Tartaglia et al., 2010]. This signaling cascade is known to mediate diverse biological functions, including cell proliferation, survival, fate determination and differentiation. It is activated in response to cytokine, hormone and growth factor stimulation, and is a major mediator of early and late developmental processes including morphology determination, organogenesis, synaptic plasticity processes and growth. In approximately 50% of affected individuals, NS is caused by heterozygous missense mutations in the *PTPN11* gene [Tartaglia et al. 2001, 2002], which encodes a cytoplasmic protein tyrosine phosphatase positively modulating RAS function. Activating mutations in five additional genes coding for transducers or modulatory proteins participating in this signaling pathway (*i.e.*, *KRAS*, *NRAS*, *SOS1*, *RAF1*,

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2
3 and *BRAF*) account for an additional one-fourth of NS cases [Tartaglia et al., 2010]. Mutations in
4 these and other functionally related genes (*i.e.*, *CBL*, *NF1*, *KRAS*, *HRAS*, *BRAF*, *SPRED1*,
5
6 *SHOC2*, *MAP2K1*, and *MAP2K2*) have been reported to underlie clinically related disorders
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8 [Aoki et al., 2008; Tidyman and Rauen, 2009; Tartaglia and Gelb, 2010]. Genotype-phenotype
9
10 correlation surveys have documented that the substantial phenotypic variation characterizing NS
11
12 can be ascribed, in part, to the gene mutated and even to the specific molecular lesion involved.
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17 We and others recently reported that missense mutations in *SOS1* (MIM# 182530) account
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19 for a significant proportion of NS [Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al.,
20
21 2007a]. *SOS1* encodes a guanine nucleotide exchange factor (GEF) responsible for stimulating
22
23 the conversion of RAS from the inactive, GDP-bound to the active, GTP-bound form [Nimnual
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25 and Bar-Sagi, 2002]. *SOS1* is a large multidomain protein characterized by an *N*-terminal
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27 regulatory portion including tandem histone-like folds (HF), which are followed by a Dbl-
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29 homology (DH) domain and a pleckstrin-homology (PH) domain, and a *C*-terminal catalytic
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31 region including the RAS exchanger motif (REM) and CDC25 domains, followed by a tail
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33 providing docking sites for adaptor proteins required for receptor anchoring (Figure 1). The
34
35 majority of NS-causing *SOS1* mutations were observed to affect residues predicted to be
36
37 implicated in the maintenance of *SOS1* in its autoinhibited conformation, and the first
38
39 biochemical characterizations of mutants consistently documented enhanced protein function and
40
41 increased signal flow through RAS [Roberts et al., 2007; Tartaglia et al., 2007]. These surveys
42
43 also indicated that subjects heterozygous for a mutated *SOS1* allele tend to exhibit a distinctive
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45 phenotype that is characterized by ectodermal abnormalities generally associated with an
46
47 absence of cognitive deficits [Tartaglia et al., 2007; Zenker et al., 2007a]. We also observed that
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49 height was less frequently below the 3rd centile compared with the overall NS population
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3 [Tartaglia et al., 2007]. While available information supports the view that *SOS1* is not mutated
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5 in cardiofaciocutaneous syndrome (CFCS; MIM# 115150) [Zenker et al., 2007a], a condition
6
7 clinically related to NS, a few individuals with ectodermal manifestations and distinctive facial
8
9 dysmorphism that might be suggestive of CFCS have recently been reported as having *SOS1*
10
11 mutations [Narumi et al., 2008; Nyström et al., 2008]. In these subjects, cognitive deficits were
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13 generally absent or minor, but at least one case with mental retardation was reported [Narumi et
14
15 al., 2008].
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20 Here, we characterized further the molecular spectrum and distribution of *SOS1* mutations,
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22 as well as the phenotypic features associated with those molecular defects. We also explored
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24 possible involvement of germline *SOS1* mutations in an opportunely selected group of isolated
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26 CHDs that occur as an associated feature in subjects with NS and heterozygous for a *SOS1*
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28 mutation. Our results provide a more accurate description of the spectrum of *SOS1* gene defects,
29
30 their consequence on *SOS1* structure and function, and their associated clinical features.
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36 MATERIAL AND METHODS

37 Patients

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40 Four cohorts were included in the study. The first cohort (group 1) included 143 clinically
41
42 well-characterized patients with NS enrolled in research protocols. Nearly all subjects of this
43
44 cohort were of European ancestry, with the majority being Italian. Within this group, subjects
45
46 were assessed by clinical geneticists experienced with NS and clinically related disorders (G.Z.,
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48 M.C.D., L.M., B.D., G.B.F., M.C.S., A.S., I.K., G.N., M.F.F., A.P., F.S. and O.G.). Clinical
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50 assessment included physical, anthropometric, neurologic and cardiac evaluations, as well as
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52 accurate examination for craniofacial features, ophthalmologic and otorhinolaryngologic
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3 defects, and ectodermal and musculoskeletal anomalies. Clinical features for the majority of
4 these individuals satisfied diagnostic criteria reported for NS [van der Burgt et al. 1994], but a
5 few individuals with a highly suggestive phenotype who lacked sufficient features to receive a
6 definitive diagnosis were also included. Based on scanning of the coding exons by denaturing
7 high-performance liquid chromatography (DHPLC) analysis and/or direct sequencing, no subject
8 within this cohort harbored a mutation in *PTPN11*, *KRAS* or *RAF1*. For approximately half of the
9 cases, mutations in *BRAF*, *MAP2K1*, *SHOC2*, *CBL* and *NRAS* had also been excluded. Besides
10 this large cohort, nine subjects with features fitting CFCS and no mutation in *KRAS*, *BRAF*,
11 *MAP2K1* or *MAP2K2* (group 2) [Sarkozy et al., 2009a] were also included in the study. The third
12 cohort (group 3) (N= 358) comprised anonymous samples from individuals with phenotypes
13 suggestive of NS for whom commercial DNA diagnostic testing was performed. Clinical data
14 were not available for these patients, and *PTPN11* mutations had not systematically been
15 excluded in all the subjects included in this group. While the output obtained from genotyping
16 this cohort of subjects could not be used in genotype-phenotype correlation studies or to estimate
17 *SOS1* mutation prevalence in the NS population, the mutation data were utilized to provide a
18 more detailed picture about the molecular spectrum of disease-causing mutations affecting the
19 *SOS1* gene. Finally, a cohort of 59 subjects with non-syndromic CHDs (PS, N= 21; atrial septal
20 defects (ASDs), N= 23; ventricular septal defects (VSDs), N= 15) was included in the study
21 (group 4). Clinical assessment of patients with isolated CHDs included complete physical
22 evaluation of dysmorphism and malformations, anthropometric measurements, renal
23 ultrasonography, and radiological studies. Cardiac evaluation included preoperative physical
24 evaluation, chest X-ray film, 12-lead electrocardiogram, and two-dimensional transthoracic
25 echocardiography with color flow Doppler. Karyotype analysis was performed in all patients of
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3 this cohort. Inclusion criteria were based on the absence of any association with other clinical
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5 features, and chromosomal anomalies, including the 22q11 deletion.
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8 Informed consent for the genetic analyses was obtained from all patients or their legal
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10 guardians.
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13 14 15 **Mutation analysis**

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17 Genomic DNA was isolated from peripheral blood leukocytes according to standard
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19 procedures. The entire *SOS1* coding sequence, as well as the exon/intron boundaries and flanking
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21 intronic portions were scanned for mutations by direct sequencing (group 3) or DHPLC analysis
22
23 (groups 1, 2, and 4) with the use of the 3100 and/or 3500HT Wave DNA Fragment Analysis
24
25 System (Transgenomic, Omaha, NE), at column temperatures recommended by the Navigator
26
27 version 1.5.4.23 software (Transgenomics), as previously described [Tartaglia et al., 2007].
28
29 Amplimers having abnormal elution profiles were re-amplified, purified (Qiagen, Hilden,
30
31 Germany) and sequenced using the ABI BigDye Terminator Sequencing Kit v.1.1 (Applied
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33 Biosystems, Foster City, CA) and an ABI 3700 Capillary Array Sequencer or ABI 3100 Genetic
34
35 Analyzer (Applied Biosystems). Primer pair sequences as well as PCR and DHPLC analysis
36
37 settings are available upon request. Length of deletions and dinucleotide mutations were
38
39 determined by cloning purified PCR products in a pCR 2.1 TOPO vector (Invitrogen, Carlsbad,
40
41 CA) and sequencing purified clones (Plasmid Mini Kit, Qiagen). Nucleotide numbering of the
42
43 mutations and exonic disease-unrelated variants reflects cDNA numbering with 1 corresponding
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45 to the A of the ATG translation initiation codon in the reference sequence (NM_005633.3), while
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47 position of the intronic variants were numbered according to the reference genomic sequence
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49 (NG_007530.1).
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3 The level of conservation of affected residues among orthologous *SOS1* genes was
4 evaluated by using the NCBI HomoloGene tool (<http://www.ncbi.nlm.nih.gov/homologene>),
5 while the SIFT (Sorting Intolerant From Tolerant, <http://blocks.fhrc.org/sift/SIFT.html>) [Ng and
6 Henikoff, 2001] and PolyPhen (Polymorphism Phenotyping,
7 <http://genetics.bwh.harvard.edu/pph/>) [Sunyaev et al., 2000] software programs were used to
8 predict the biological relevance of the identified missense variants on protein function.
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17 When available, parental DNAs were sequenced to establish whether the identified changes
18 in sporadic cases were *de novo* or to confirm co-segregation between the variant and phenotype
19 in families transmitting the trait. Paternity was confirmed using the AmpDESTER ProfilerPlus
20 kit (Applied Biosystems). All missense changes proven to be *de novo* by parental DNA
21 genotyping were deemed mutations causally linked to the disorder. To exclude the possibility
22 that variants co-segregating with NS were neutral polymorphic changes occurring in the
23 population, at least 300 population-matched DNAs obtained from unaffected subjects were
24 screened (DHPLC analysis and sequencing of variant elution profiles). When parental DNAs
25 were not available, we considered a novel non-synonymous variant as a causative mutation when
26 it affected a residue already reported to be mutated. Novel variants involving residues localized
27 in close proximity to residues mutated in NS, predicted to affect protein structure/function by
28 Polyphen, SIFT and structural evidence, but without sufficient genetic evidence (*i.e.*, cases with
29 unreported ethnicity or missing clinical evaluation and/or genetic testing of other members of
30 their families) were considered as probably pathogenic. These missense changes were found not
31 to occur in controls, and resulted in a non-conservative substitution at an invariant or highly
32 conserved residue (*Homo sapiens* [NP_005624.2], *Pan troglodytes* [XP_515425.2], *Canis*
33 *familiaris* [XP_540157.2], *Bos taurus* [XP_617859.4], *Mus musculus* [NP_033257.2], *Rattus*
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3 *norvegicus* [XP_233820.4], and *Drosophila melanogaster* [NP_476597.2]). Finally, a third
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5 category grouping functionally unclassified variants was considered for those novel changes for
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7 which structural analysis did not allow to infer any functional effect.
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10 The position of mutated amino acid residues was modeled by using the recently generated
11
12 crystal structure of the SOS1 protein truncated at the C-terminus (residues 1-1049) deposited in
13
14 the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>) (PDB ID: 3KSY)
15
16 [Guerasko et al., 2010]. Figures were prepared using the UCSF Chimera 1.5 software package
17
18 (<http://www.cgl.ucsf.edu/chimera/>) [Pettersen et al., 2004]. Electrostatic potential calculations
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20 were performed with the APBS software [Baker et al., 2001].
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27 **Statistical analyses**

28
29 Confidence intervals for proportions were calculated by means of VassarStats software
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31 (<http://faculty.vassar.edu/lowry/VassarStats.html>) using the Newcombe-Wilson method
32
33 including continuity correction [Wilson, 1927; Newcombe, 1998]. Genotype-phenotype
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35 correlations were performed using 2x2 contingency-table analysis. The significance threshold
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37 was set at $P = 0.05$.
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43 **RESULTS**

44 ***SOS1* mutation scanning in NS, CFCS and isolated CHD**

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46 DHPLC analysis and bidirectional direct sequencing of the entire *SOS1* coding sequence
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48 on peripheral blood leukocyte genomic DNA specimens of groups 1 and 3 identified 41 different
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50 heterozygous missense nucleotide substitutions and 3 small in-frame indels in 108 unrelated
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52 subjects. Among them, five were private or common variants unrelated with the trait. Three of
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3 them, c.1964C>T (p.Pro655Leu), c.3032A>G (p.Asn1011Ser) and c.3959A>G (p.His1320Arg,
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5 had previously been reported in unaffected individuals [Roberts et al., 2007; Tartaglia et al.,
6
7 2007]. Similarly, the c.1705C>G (p.Leu569Val) was documented to occur as a homozygous
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9 change in an unaffected parent, and the c.2122G>A (p.Ala708Thr) was found to occur as a
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11 polymorphic change in ethnic-matched unaffected individuals of Hispanic ancestry, and were
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13 therefore regarded as benign polymorphisms. A full list of the disease-unrelated changes,
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15 including silent nucleotide substitutions and intronic variants, is reported in Supp. Table S1.
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20 In these two cohorts, twenty-five *SOS1* sequence variants were deemed to be of
21
22 pathological significance (Table 1). Among these, 17 had previously been established as
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24 causative mutations (Supp. Table S2), whereas 8 were novel. Among the latter, 5 mutations were
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26 single nucleotide substitutions, but three small in-frame indels affecting two mutational hot spots
27
28 within the PH domain (p.Lys427_Asp430delinsAsn and p.Trp432_Glu433del) and the helical
29
30 linker connecting the PH and REM domains (PH-REM linker) (p.Leu554_Met558delinsLys)
31
32 were also identified. The c.1300_1301GG>AA (p.Gly434Lys) and c.1310T>C (p.Ile437Thr)
33
34 changes were demonstrated to occur as *de novo* events by genotyping of parental DNAs. The
35
36 c.2681C>G transition (p.Pro894Arg) was found to co-segregate with the disease, while the
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38 remaining two variants, c.1430A>G (p.Gln477Arg) and c.1655G>T (p.Arg552Met), affected
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40 amino acid residues that had previously been reported to be mutated in NS (Supp. Table S2).
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42 Eight novel missense changes (p.Pro112Arg, p.Ile252Thr, p.Met422Val, p.Glu424Lys,
43
44 p.Gly482Arg, p.Leu490Arg, p.Arg497Gln and p.Thr549Lys) were considered as probably
45
46 pathogenic mutations. The majority of these variants were not observed in more than 300
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48 population-matched unaffected individuals, and were non-conservative, the majority affecting
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50 invariant residues among *SOS1* orthologs and predicted to be “damaging” or have functional
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3 relevance according to the PolyPhen or/and SIFT programs. Moreover, affected residues were
4 located in mutational hot spots or in close spatial proximity to residues mutated in NS and were
5 predicted to have structural/functional consequences on protein structure (see below). For these
6 variants, however, the evidence provided by the theoretical models and structural analysis was
7 not unambiguously supported by genetic evidence. Finally, six missense nucleotide substitutions
8 (p.Thr37Ala, p.Pro478Leu, p.Ile784Thr, p.Arg1131Lys, p.Leu1140Ile and p.Thr1257Ala) were
9 classified as variants of unknown significance. For those, parental DNAs were not submitted for
10 testing or no clinical information was available for the carrier parent, and the predicted amino
11 acid change was generally not considered as having functional relevance according to the
12 PolyPhen or/and SIFT programs. Most of these changes affected the C-terminal portion of the
13 protein for which no structural information is currently available, precluding any consideration
14 regarding possible consequences on protein function.
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32 *SOS1* mutations were identified in 26 of the 143 subjects (18.2%) of group 1, which
33 included patients with clinical features satisfying the diagnostic criteria of NS or highly
34 suggestive for the disorder, and negative for mutations in *PTPN11*, *RAF1*, and *KRAS*. Based on
35 the accurate clinical assessment of this cohort and the relative prevalence of mutations affecting
36 those disease genes in NS, this finding supports the view that mutations in the *SOS1* gene
37 account for approximately 10% of NS cases, which is in line with our previous estimate
38 [Tartaglia et al., 2007], but slightly below those obtained from two other clinically well
39 characterized NS cohorts [Roberts et al., 2007; Zenker et al., 2007a].
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51 DHPLC mutation scanning of coding exons, splice junctions, and flanking intronic portions
52 of *SOS1* did not reveal any putative pathogenic mutation in the nine subjects with a diagnosis of
53 CFCS and negative for mutations in *BRAF*, *KRAS*, *MAP2K1*, and *MAP2K2*. Similarly, no
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3 mutation was identified among the 59 patients with non-syndromic CHDs included in the study,
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5 excluding a major involvement of germline *SOS1* mutations in PS (N= 21; 95% CI= 0.000-
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7 0.192), ASD (N= 23, 95% CI= 0.000-0.178), and VSD (N= 15, 95% CI= 0.000-0.253).
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10 11 12 ***SOS1* mutation diversity, cluster distribution, and molecular modeling analysis**

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15 The present data and available published records (updated to July 2010) (Supp. Table S2)
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17 were utilized to analyze the diversity of NS-causing mutations, their cluster distribution, and to
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19 explore their effects on *SOS1* structure and function. The 183 reported missense mutations
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21 (including those with probably damaging effects) were observed to affect 32 residues, which
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23 were not randomly scattered along the protein but tended to cluster in specific regions (Figure 1).
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26 Approximately forty percent of *SOS1* defects affected four residues located in the PH-REM
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28 linker (Ser548, Thr549, Leu550 and Arg552), with substitutions of residue Arg552 accounting
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30 for one-third of all mutations. Another mutation cluster, involving short stretches within the PH
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32 domain (*i.e.*, Glu424, Trp432, Glu433 and Gly434, and Gln477, Pro478 and Gly482) explained
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34 roughly 20% of mutations. A third functional cluster (see below) resided at the interacting
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36 regions of the DH (Thr266 and Met269) and REM (Lys728, Trp729 and Ile733) domains (16%
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38 of total mutations). Among the most recurrent mutations, the c.2536G>A transition, predicting
39
40 the substitution of Glu846 by lysine within the CDC25 domain, accounted for 10% of defects.
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43 Such a skewed distribution of affected residues implies specific perturbing consequences on
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45 protein function.
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51 *SOS1*'s GEF activity is controlled principally by two binding sites for RAS: the catalytic
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53 site, which is located entirely within the CDC25 domain and promotes GDP release from RAS,
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55 and a distal site, which is bracketed by the CDC25 domain and REM domains and positively
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3 modulates GEF activity through promotion of a conformational change at the active site that
4 allows substrate GDP-RAS to bind [Margarit et al., 2003]. Basally, the catalytic output of SOS1
5 is constrained by the *N*-terminal regulatory HF domain and DH-PH unit [Sondermann et al.,
6 2004; Gureasko et al., 2010], and structural data indicate that this autoinhibitory effect is exerted
7 through direct HF and DH domain-mediated blockade of the allosteric site. An extensive
8 interdomain binding network also involving the PH-REM helical linker and the PH domain has
9 been recognized to stabilize this inhibitory conformation. Following the translocation of SOS1 to
10 the membrane, the inhibitory effect of the HF and DH domains is relieved allowing RAS binding
11 to the allosteric site, which in turn promotes a conformational rearrangement of the CDC25
12 domain allowing RAS binding to the catalytic site. Based on this allosteric mechanism of
13 activation and recent evidence indicating that the HF domain and the DH-PH unit are
14 conformationally coupled to control SOS1's recruitment to the plasma membrane and release of
15 autoinhibition [Gureasko et al., 2010], the distribution of identified mutations in NS supports a
16 picture in which the vast majority of disease-causing lesions affect the stability of the
17 intramolecular interactions that maintain SOS1 in an autoinhibited state by at least two major
18 distinct mechanisms.

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41 A first class of mutations includes lesions predicted to promote conformational
42 rearrangements of domains that reduce the enzyme self-inhibition by impairing proper masking
43 of the distal RAS binding site or by acting on the allosteric control of catalytic activity. Within
44 this class, a first group of mutations (Class 1A: p.Thr266Lys, p.Met269Arg/Thr, p.Lys728Ile,
45 p.Trp729Leu and p.Ile733Phe) involve residues that participate in the autoinhibitory interaction
46 of the DH and REM domains blocking RAS access at the allosteric site, or are closely located to
47 them (Figure 2A). These mutations are predicted to affect the stability of the inactive
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3 conformation of the protein directly by disrupting the inhibitory interdomain bonding network at
4 the distal site. Among these, the most recurrent mutations affect Met269 (10% of total cases),
5 which interacts directly with residues of the REM domain implicated in RAS binding
6 [Sondermann et al., 2004]. Of note, Lys728 and Trp729 are among these, and Ile733 is very
7 close to these residues; therefore, their substitution could also affect the REM's affinity for GTP-
8 bound RAS in addition to the stability of the DH-REM interface. Indeed, the drastic
9 p.Trp729Glu substitution has been shown to inhibit RAS binding to the REM domain
10 [Sondermann et al., 2004]. This might be related to the fact that within this group, mutations
11 affecting residues located at the DH surface appear to be more common (15% of total changes)
12 compared to those affecting the REM surface (2%). Among the probably pathogenetic variants,
13 the p.Ile252Thr substitution could be included in this group. Ile252 is an invariant residue placed
14 within a hydrophobic core of the DH domain, which also involves Thr215, Leu219, Ile249,
15 Tyr295 and Tyr298. Structural perturbation of the DH fold is expected to destabilize the masking
16 of the distal RAS binding site.

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18 A second mutation group includes lesions affecting the interaction between the HF, DH
19 and PH domains, thus perturbing the overall autoinhibited conformation in which the HF and DH
20 domains block the distal RAS binding site [Guerasko et al., 2010] (Class 1B: p.Lys170Glu,
21 p.Tyr337Cys, p.Ile437Thr, p.Cys441Tyr, p.Ser548Arg, p.Leu550Pro, p.Arg
22 552Gly/Thr/Met/Lys/Ser, p.Leu554_Met558delinsLys, and probably pathogenetic variants
23 p.Met422Val, p.Arg497Gln and p.Thr549Lys,) (Figure 2B). Within this group, mutations of
24 residues 548-558 are the most recurrent. This stretch corresponds to the helical linker connecting
25 the PH and REM domains, which contributes structurally to the maintenance of the autoinhibited
26 conformation by interacting with the HF and DH domains [Guerasko et al., 2010]. For

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3 instance, Arg552 interacts directly with the side chains of Asp140 and Asp169 of the HF
4 domain, and mutation of either Arg552 or Asp140 was demonstrated to completely abolish the
5 interaction between these domains [Sondermann et al., 2005]. A similar effect might be predicted
6 for the recurrent Lys-to-Glu change affecting codon 170 within the HF domain, and possibly for
7 the p.Arg497Gln substitution in the PH domain, since the electrostatic interactions between these
8 two residues located at the HF-PH domain interface (but also close to the helical linker)
9 contribute to stabilizing the HF domain's orientation. Similarly, substitution of residues Ile437
10 and Cys441 might affect the PH domain's structure in the region of the domain facing towards
11 the HF domain and possibly cause a reorientation of the latter. Finally, residue Tyr337 is located
12 at the PH-DH interface, and thus probably contributes to stabilizing the DH domain orientation.
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14 The p.Phe78Cys change (and possibly p.Met422Val) can also be included in this group.
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16 Substitution of the highly conserved Phe78, which is an unexposed HF residue contributing to a
17 hydrophobic core (involving Leu55, Leu59, Val74, Val133 and Ile137), might structurally
18 perturb the region of the HF domain at its interface with the helical linker. The p.Met422Val
19 change, on the other hand, might perturb the structure of the PH domain, thus perturbing the PH-
20 HF interaction.
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41 The third group of class 1 mutations is formed by residues of the REM domain, interacting
42 with the helical hairpin of the CDC25 domain, which contributes to the allosteric structural
43 switch [Freedman et al., 2006], and whose conformation is essential for both RAS binding to the
44 active site and nucleotide exchange [Hall et al., 2001; Margarit et al., 2003] (Class 1C:
45 p.Phe623Ile and p.Tyr702His) (Figure 2C). A single mutation has been identified to affect
46 Phe623, a highly conserved residue of the REM domain located at the interface with the CDC25
47 domain. This residue is part of an extended hydrophobic groove of the REM domain that
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3 accommodates the side chains of two hydrophobic residues (Ile956 and Phe958) of the helical
4 hairpin of the CDC25 domain. Such a hydrophobic interaction has been demonstrated to be
5 important for the correct orientation of the helical hairpin, and for SOS1's catalytic activity [Hall
6 et al., 2001]. Based on these observations, it can be hypothesized that the p.Phe623Ile
7 substitution might perturb the catalytic activity of the CDC25 domain by acting either on RAS
8 binding at the catalytic site, on the domain's catalytic efficiency or on allosteric control. A
9 similar effect might be associated with substitution of the invariant Tyr702, which is located
10 within the REM domain at the interface with the CDC25 domain, in close proximity to the
11 region of the latter that undergoes the conformational switch promoted by RAS binding at the
12 distal site.
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27 While the primary anchorage of SOS1 to the plasma membrane is mediated by docking of
28 its C-terminal region to SH3 domain-containing adaptor proteins (*e.g.*, GRB2) that bind to the
29 activated receptors, additional anchorage sites at the membrane are provided by the
30 phosphatidylinositol-4,5-phosphate (PIP₂)- and phosphatidic acid (PA)-binding sites within the
31 PH domain [Chen et al. 1997; Zheng et al., 1997; Zhao et al., 2007], and by an extended
32 positively charged surface of the HF domain that interacts with anionic membranes, and possibly
33 also binds PA and PIP₂ [Guerasko et al., 2010; Yadav and Bar-Sagi, 2010]. These sites appear to
34 have complementary roles: while those within the PH domain mediate SOS1's targeting to the
35 membrane, the latter are thought to activate the GEF at the membrane. In the autoinhibited
36 structure of SOS1, the positively charged site of the HF domain is not oriented in a way that
37 would allow membrane binding [Guerasko et al., 2010]. This finding suggests the possibility that
38 the reorientation of the PA-binding site of the HF domain might be coupled to the destabilization
39 of the auto-inhibited conformation of the protein, permitting SOS1's activation through RAS
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3 binding at the allosteric site [Guerasko et al., 2010; Yadav and Bar-Sagi, 2010]. Class 2
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5 mutations include changes that are predicted to enhance SOS1's catalytic function by membrane-
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7 dependent mechanisms. Within this class, a first group is predicted to perturb the self-inhibiting
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9 orientation of the HF domain by affecting solvent exposed residues located within the positively
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11 charged surface of the HF domain, thus favoring its membrane binding (Class 2A: p.Pro102Arg
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13 and p.Glu108Lys, and probably pathogenetic variant p.Pro112Arg) (Figure 2D). Significantly,
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15 these mutations introduce a positively charged amino acid (arginine or lysine) enhancing the
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17 positive electrostatic potential of the HF surface. In particular, Glu108 is adjacent to the PA-
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19 binding motif and its substitution by lysine generates a contiguous patch of positively charged
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21 residues that was recently demonstrated to potentiate membrane binding [Yadav and Bar-Sagi,
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23 2010]. A similar perturbing effect would be predicted for the c.305C>G and c.335C>G missense
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25 changes affecting the closely located Pro102 and Pro112 residues.
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32 A second group includes mutations affecting the membrane-binding surface of the PH
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34 domain (Class 2B: p.Lys427_Asp430delinsAsn, p.Trp432Arg, p.Trp432_Glu433del,
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36 p.Glu433Lys, p.Gly434Arg/Lys, p.Gln477Arg/His, p.Pro478Arg,
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38 p.Pro481_Gly482insArgLeuPro, and probably pathogenetic variants p.Glu424Lys, p.Gly482Arg
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40 and p.Leu490Arg) (Figure 2E). As anticipated, two sites within the PH domain have been
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42 identified [Chen et al. 1997; Zheng et al., 1997; Zhao et al., 2007]. Both sites contribute to
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44 membrane recruitment of SOS1 and are required for efficient GEF activity. We noticed that the
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46 vast majority of mutations affecting the PH domain involved solvent-exposed residues within
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48 two adjacent regions (*i.e.*, residues 424-436 and 473-493) that do not overlap spatially with the
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50 PIP₂-binding site, which is constituted by residues Lys456, Arg459, Lys472 and Arg489 [Zheng
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52 et al., 1997]. A first cluster of mutations affected residues Gln477, Pro478, Pro481 and
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3 Gly482, which are placed within the PA-binding site encompassing residues 472-483 [Zhao et
4 al., 2007], while the second cluster affect residues Glu424, Lys427-Asp430, Trp432-Gly434 and
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6 Leu490. Of note, the nature of the substitution seemed to be critical since all missense mutations
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8 affecting these regions resulted in the introduction of a positively charged residue. In two cases
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10 (Gly434, Gln477), multiple lesions were predicted to introduce either an arginine or a lysine,
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12 further indicating a specific role for the introduced charged residue. In additional two cases,
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14 including the recurrent c.1297G>A (p.Glu433Lys), the positively charged residue replaced a
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16 negatively charged amino acid. Similarly, the three identified in-frame indels were predicted to
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18 increase the electrostatic potential of the postulated membrane interaction surface. Based on
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20 established evidence supporting an absolute requirement of SOS1's recruitment to the plasma
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22 membrane for RAS activation in response to an extracellular stimulus [Zhao et al., 2007], this
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24 non-random distribution and type of lesions strongly suggest that their pathogenetic effect is
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26 related to a strengthened binding of SOS1 to PA, PIP₂ and/or other membrane phospholipids, and
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28 consequently to a more stable recruitment of the protein at the membrane, which in turn would
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30 enhance the duration and/or amplitude of GEF function.
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39 Only two disease-causing mutations have been found to affect the CDC25 domain so far.
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41 The affected residues (*i.e.*, Glu846 and Pro894) are placed in close proximity to each other, and
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43 distally from the two RAS binding sites and regions implicated in the conformational
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45 rearrangement of the domain promoting RAS's binding to the active site (Figure 2F). The high
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47 recurrence of the p.Glu846Lys change indirectly documents a relevant role of this region in
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49 SOS1's function. This amino acid substitution has recently been documented to profoundly
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51 perturb intracellular signaling [Chen et al., 2010]. Available crystal data, however, do not allow
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53 us to infer any functional effect for these mutations, as well as for unclassified variants
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3 affecting residues in this domain (Ile784, Gln977 and Ser1000) or at the C-terminus (Arg1131,
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5 Leu1140 and Thr1257). We noted that Arg1131 is adjacent to Ser1132, which is one of the
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7 identified growth factor-induced, MAPK-mediated phosphorylation sites of SOS1 [Corbalan-
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9 Garcia et al., 1996]. These sites cluster within a small region that contains the proline-rich SH3-
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11 binding sites implicated in GRB2 binding. Of note, phosphorylation of these sites has been
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13 demonstrated to reduce SOS1's binding affinity for GRB2, and it could be speculated that
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15 mutations altering the recognition motif at these sites might affect phosphorylation and promote
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17 enhanced GRB2 binding. Similarly, Leu1140 and Thr1257 cluster within this region. Based on
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19 evidence suggesting that this region possibly exerts an autoinhibitory effect on SOS1's activity
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21 [Aronheim et al., 1994; Wang et al., 1995], it can be speculated that lesions affecting this region
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23 might perturb such an inhibitory mechanism.
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29 Finally, available structural data did not allow us to identify any functional clue for the
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31 p.Asp309Tyr NS-causing substitution as well as for the unclassified sequence variants affecting
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33 Thr37 and Thr378 residues.
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39 **Phenotypic spectrum and genotype-phenotype correlations of *SOS1* mutations**

40 Extensive clinical information was obtained for 39 subjects with NS and *bona fide SOS1*
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42 mutations, including individuals recruited in this study (group 1, cases NS01 to NS25) and re-
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44 examined subjects of our original study (NS26 to NS39) [Tartaglia et al., 2007] (Supp. Table
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46 S3). Overall, analysis of the clinical data confirmed previous observations from our group and
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48 others indicating that mutations in *SOS1* are associated with a distinctive phenotype that
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50 unambiguously falls within the NS clinical spectrum, but is characterized by a high prevalence of
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52 ectodermal features (keratosis pilaris/hyperkeratotic skin, sparse eyebrows, and sparse,
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3 generally thin and curly scalp hair) (84% of cases), and a relatively low occurrence of cognitive
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5 deficits (11% of cases) compared to what is observed in the NS general population. Of note, in
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7 two cases mental retardation was potentially attributable to critical illness during infancy. In two
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9 additional subjects, the intelligence quotient was border-line to the lower limit of the normal
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11 range with impairment of linguistic skills and oculo-manual coordination in one subject, and
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13 delay of speech in the other (Supp. Table S3). We also confirmed our previous observation
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15 indicating a relatively low prevalence of subjects exhibiting reduced growth (length/stature
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17 below the 3rd centile) (29% of cases). In these subjects, reduced growth was invariably associated
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19 with delayed bone age. Remarkably, more than one-third of subjects with mutated *SOS1* allele
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21 exhibited fetal macrosomia, which, however, did not appear to correlate with the extent of their
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23 postnatal growth, being length/stature in these subjects below the 3rd centile in a comparable
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25 proportion of cases, compared with subjects without this feature (3/14 vs. 7/24, Fisher's exact
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27 probability = 0.45). Of note, *SOS1* mutation-positive subjects displayed a more pronounced
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29 growth failure (45% of cases) as newborns, which was not generally secondary to poor sucking
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31 and/or swallowing.
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39 *SOS1* mutation-positive subjects displayed typical facial features (Figure 3). Macrocephaly
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41 was, however, overrepresented compared to the general NS population (61% vs. 12%) [Sharland
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43 et al., 1992]. Among these subjects, cardiac defects were frequently observed (89% of cases),
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45 with PS, ASD and VSD being the most recurrent anomalies, while prevalence of HCM was
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47 comparable to that observed in *PTPN11* mutation-associated NS, occurring in less than 10% of
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49 cases [Sarkozy et al., 2009b]. Of note, PS was found to be frequently associated with ASD (35%
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51 of cases). Finally, two subjects were documented to present with mandibular multiple giant cell
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3 lesions (MGCLs), which were associated with multiple tumors, including abdominal
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5 rhabdomyosarcoma, cerebral glioma, and skin granular cell tumors in one subject.
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8 The analysis of distribution of the major clinical features among *SOS1* mutation-positive
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10 subjects documented a significantly higher prevalence of fetal macrosomia in subjects with class
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12 1B mutations compared to individuals with class 1A mutations (Fisher's exact probability =
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14 0.024). We failed in identifying any other significant genotype-phenotype correlation. Based on
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16 the relatively small size of the cohort analyzed, however, the occurrence of other associations
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18 between specific features and individual *SOS1* lesions or mutation clusters cannot be ruled out.
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24 DISCUSSION

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27 In this report, we have expanded the available information about the molecular diversity of
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29 *SOS1* mutations underlying NS, and have provided a more comprehensive assessment of the
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31 clinical features associated with those molecular lesions. We also explored systematically the
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33 predicted structural consequences of NS-causing *SOS1* defects, developing a classification of
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35 these gene lesions based on the predicted role of affected residues and functional consequences
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37 derived from the nature of the amino acid change.
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41 Combined with data from previous surveys, our findings indicate that *SOS1* mutations are
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43 almost always missense changes, although we documented that small in-frame indels occur in a
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45 small proportion of cases. Available mutation records (data updated to July 2010) revealed a
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47 complete absence of nonsense, frameshift, and splicing defects, which is consistent with
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49 functional characterization of a panel of NS-causing *SOS1* mutations [Roberts et al., 2007;
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51 Tartaglia et al., 2007; Chen et al., 2010; Guerasko et al., 2010], and further support their
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53 activating role on *SOS1* functional dysregulation in disease pathogenesis. Moreover, the
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3 accumulated data strongly indicated that specificity in the amino acid substitution is relevant to
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5 the functional dysregulation of the protein. Specifically, occurrence of invariant amino acid
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7 changes was observed for several residues and accounted for approximately 50% of total events,
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9 strongly suggesting a specific role for the introduced residue. Exemplifying this is the
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11 observation of positively charged amino acids that were observed invariantly to replace solvent
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13 exposed residues located within the HF and PH domains (*i.e.*, Glu108, Trp432, Glu433 and
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15 Gly434), which provides strong evidence for the electrostatic nature of the perturbing effect of
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17 those mutations on SOS1 functional dysregulation. On the other hand, the identity of substitution
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19 did not seem to be critical in other cases (*e.g.*, most mutations affecting the helical linker
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21 connecting the DH and PH domain), indicating a crucial role for the amino acid residue being
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23 replaced. This is the case of Arg552, for which all but one amino acid substitutions resulting
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25 from a single base change affecting this codon have been documented to cause NS.
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32 Based on SOS1's crystal structure and mechanism of activation [Guerasko et al., 2008,
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34 2010], the non-random distribution pattern of altered residues and nature of substitutions indicate
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36 that NS-causing mutations dysregulate SOS1's GEF function by at least two major mechanisms.
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38 As originally reported by Roberts *et al.* [2007] and Tartaglia *et al.* [2007], a large fraction of
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40 mutations was found to cluster in regions of the protein that participate in the interdomain
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42 binding network that maintain SOS1 in its catalytically inactive conformation (class 1
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44 mutations). Among these, mutations can affect either residues directly implicated in the
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46 autoinhibitory interaction between the DH and REM domains that impair GTP-RAS binding at
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48 the distal site (or residues closely located to them), or residues located within the DH-PH helical
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50 linker or regions of the HF, DH and PH domains that contribute to stabilizing the HF, DH and
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52 REM interdomain binding network that maintains SOS1 in its catalytically inactive
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3 conformation. Of note, this study recognized a third group of class 1 mutations that were found
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5 to specifically affect residues of the REM domain located at the interface with regions of the
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7 CDC25 domain identified to undergo the GTP-RAS binding-induced conformational
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9 rearrangement required for both RAS binding to the active site and nucleotide exchange reaction.
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11 While the effect of these amino acid substitutions on RAS binding at the catalytic site or on the
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13 domain's catalytic efficiency cannot be ruled out, we hypothesize that these mutations might
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15 upregulate SOS1's catalytic activity by weakening the inhibitory allosteric control on the active
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17 site.
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22 Strikingly, our analysis also allowed us to discern a previously unrecognized class of
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24 mutations affecting solvent exposed residues located within the membrane oriented surface of
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26 the PH domain and a positively charged surface of the HF domain, and predicted to enhance
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28 SOS1's catalytic function by distinct membrane-dependent mechanisms. While the complex
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30 process of SOS1's catalytic activation has not been characterized in detail, available structural
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32 and functional data indicate that the two major events controlling SOS1's function, *i.e.*, protein
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34 recruitment to the plasma membrane and release of autoinhibition, are linked. Membrane
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36 translocation of SOS1 is promoted by binding of the protein to activated cell surface receptors
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38 *via* SH3 domain recognition sites at the C-terminus of SOS1 that mediate its interaction with
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40 adaptor proteins. Additional anchorage sites at the membrane, however, are also provided by the
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42 PH and HF domains. Experimental and structural data indicate that two distinct sites within the
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44 PH domains bind to PIP₂ and PA, and contribute significantly to SOS1's targeting to the
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46 membrane [Chen et al., 1997; Zhao et al., 2007]. On the other hand, in the SOS1's autoinhibited
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48 structure, the positively charged surface of the HF domain is not oriented in a way that would
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50 allow membrane binding, suggesting the possibility that reorientation of the HF domain in the
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3 membrane-bound conformation might destabilize HF's interaction with the REM domain,
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5 unmasking the distal RAS binding site [Guerasko et al., 2010; Yadav and Bar-Sagi, 2010]. Based
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7 on this model, the molecular spectrum of disease-causing mutations affecting the PH and HF
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9 domains are predicted to favor the membrane-dependent electrostatic switch leading to a
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11 productive reorientation of the protein in the plane of the membrane and consequent release of
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13 auto-inhibition at the regulatory RAS binding site. In this scenario, class 2 mutations would
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15 enhance the simultaneous engagement of the membrane by the PIP₂- and PA-binding pockets of
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17 the PH domain and the positively charged surface of the HF domain, synergizing to increase the
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19 stability of SOS1 translocation at the membrane and catalytic activation. Specifically, the
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21 pathogenetic effect of this class of mutations affecting the PH domain would be related to a more
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23 stable recruitment of the protein at the membrane, while the introduction of an arginine or lysine
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25 residue within the positively charged surface of the HF domain would favor a spatial
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27 reorientation of the domain weakening its inhibitory function. Consistent with this model,
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29 Guerasko and co-workers [2010] demonstrated that the Glu108Lys substitution promotes
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31 enhanced SOS1's GEF activity in a PIP₂-dependent manner, and elegantly provided evidence for
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33 a role of non-specific electrostatic interactions of the HF domain with the membrane in release of
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35 autoinhibition and activation of SOS1.
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43 The present mutation scanning of a clinically well-characterized cohort of subjects with
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45 features fitting or highly suggestive of NS, and negative for mutations in *PTPN11*, *RAF1* and
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47 *KRAS*, confirmed our previous estimate of *SOS1* mutation prevalence, indicating that defects in
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49 this gene account for approximately 10% of NS. Moreover, detailed clinical examination of 39
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51 mutation-positive individuals, including data from clinical re-examination of 14 subjects
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53 constituting our original cohort [Tartaglia et al., 2007], provided a more complete assessment
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3 of the phenotypic variation associated with these molecular lesions, and strengthened the specific
4 association with ectodermal involvement (84%), and decreased prevalence of cognitive deficits
5 (11%) documented in previous reports [Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al.,
6 2007a; Denayer et al., 2010]. A major finding also regarded the relatively lower prevalence of
7 short stature/length below the 3rd centile in these subjects (29%) compared to the overall NS
8 population, for which an estimate of 70% is generally reported. This percentage is lower than in
9 other studies, in which the prevalence ranged from 31% to 64% [Roberts et al., 2007; Zenker, et
10 al., 2007a; Denayer, et al., 2010]. Based on the different recruitment strategies utilized, this
11 discrepancy might be due, in part, to differences in patient selection (*e.g.*, recruitment performed
12 by pediatric endocrinology units vs. cardiology units). It should be noted, however, that this
13 disagreement might be also related to a sampling bias depending on the age distribution among
14 cohorts. In NS, while birth length is typically normal, growth parameters usually drop below the
15 3rd centile during the first years of life. Since there is often some attenuation and/or delay of the
16 pubertal growth spurt, the prevalence of short stature in NS is expected to be highest during the
17 age of normal puberty. Because of the delay in bone age, however, many patients have some
18 catch-up growth in their late teens. As we documented a delay in bone age in all subjects with
19 length/stature below the 3rd centile, a significant variation in the estimate of the growth status
20 between cohorts differing considerably in age distribution would be expected, since the estimate
21 of reduced growth prevalence in any NS cohort would be sensitive to the composition of the
22 cohort in terms of proportion of pediatric vs adult subjects.

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50 We observed the occurrence of multiple tumors (*i.e.*, MGCLs, abdominal
51 rhabdomyosarcoma, cerebral glioma and granular cell tumors of the skin) in one of the *SOS1*
52 mutation-positive subjects included in the study. It has been established that NS patients are at

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3 increased risk of developing childhood myeloproliferative disease (*i.e.*, juvenile myelomonocytic
4 leukemia) and acute leukemia [Tartaglia and Gelb, 2005], and that this specific association is
5 strictly linked to a specific class of activating germline mutations in the *PTPN11* gene [Tartaglia
6 et al., 2006]. Occurrence of neuroblastoma, glial tumors and rhabdomyosarcoma in subjects with
7 NS, however, has also been reported [Kratz, 2009]. Remarkably, while mutations in *SOS1* have
8 previously been considered to be more benign in terms of risk of malignancy than those in other
9 genes in the RAS/MAPK pathway (*e.g.*, *PTPN11*, *HRAS*, *KRAS* and *NF1*) [Swanson et al.,
10 2008], a significant high occurrence of solid tumors, particularly embryonal rhabdomyosarcoma,
11 has recently been reported in subjects with NS due to a mutated *SOS1* allele [Denayer et al.,
12 2010; Jongmans et al., 2010; Hastings et al., 2010]. The present finding is in line with these
13 recent reports, and provides further evidence that subjects heterozygous for *SOS1* mutations may
14 have a significant risk for certain solid tumors. Of note, MGCLs were observed in one additional
15 *SOS1* mutation-positive subject of the present cohort. MGCLs are benign tumor-like lesions
16 most frequently affecting the jaws but also occurring in other bones or soft tissues. They consist
17 of an osteoblast-like cell population representing the proliferating tumor cells producing
18 cytokines inducing the maturation of a subset of phagocytes into osteoclast-like giant cells [de
19 Lange et al., 2007]. While the incidence of MGCLs in patients with NS is not known, they were
20 originally linked to a narrow spectrum of germline *PTPN11* mutations [Tartaglia et al., 2002;
21 Jafarov et al., 2005; Lee et al., 2005]. In recent years, however, their co-occurrence in NS (and
22 other RAS-opathies) has been extended to other genes encoding transducers with roles in the
23 RAS-MAPK pathway, with *SOS1* being the most frequently mutated gene [Beneteau et al., 2009;
24 Hanna et al., 2009; Neumann et al., 2009], indicating that *SOS1* is a major predisposing gene for
25 MGCLs in NS.
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3 One patient in the present cohort, who was heterozygous for the *de novo* c.1297G>A
4 missense change (p.Gly434Lys), presented with gingival hypertrophy. Interestingly, a *SOS1*
5 frameshift mutation (c.3248-3249insC) predicting a truncated protein lacking the C-terminal
6 region encompassing the SH3 domain-binding and MAPK phosphorylation sites, was previously
7 documented to be responsible for a form of hereditary gingival fibromatosis (HGF1; MIM#
8 135300), a genetically heterogeneous benign gingival overgrowth condition, in a large family
9 [Hart et al., 2002]. Consistent with the negative modulatory role of the C-terminal region on
10 SOS1 function [Wang et al., 1995; Corbalan-Garcia et al., 1998], the SOS1 mutant was
11 demonstrated to localize at the plasma membrane constitutively and sustain the activation of
12 RAS-MAPK signaling, linking the gingival overgrowth to enhanced SOS1-mediated signal flow
13 through RAS [Jang et al., 2007]. While further studies are required to appreciate more precisely
14 the peculiar effect of the HGF1-associated mutation on SOS1 functional dysregulation and its
15 specific effect on gingival growth, the present finding provides further evidence in support of a
16 relation between RAS/MAPK signaling dysregulation and gingival hypertrophy.
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36 Excluding Down syndrome, NS is the most frequent developmental disorder associated
37 with congenital cardiac disease. Among *SOS1* mutation-positive individuals, cardiac defects
38 occurred in the majority of cases (> 85%), which is consistent with previous studies [Roberts, et
39 al., 2007; Tartaglia, et al., 2007; Zenker, et al., 2007a; Denayer, et al., 2010]. Similarly to what
40 observed in NS subjects with *PTPN11* mutations [Tartaglia et al., 2002; Zenker et al., 2004],
41 *SOS1* mutation-positive patients exhibit a high prevalence of PS (68% of cases), which was
42 typically associated with ASD (35% of cases), a relatively high occurrence of atrial and
43 ventricular septal defects (39% of cases), and a low prevalence of HCM (< 10% of cases). Based
44 on these findings and preliminary observations suggesting that a distinct class of mutations in
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3 NS disease might be implicated in isolated CHDs [Pandit et al., 2007; Greenway, et al., 2009],
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5 and given the relatively “mild” developmental and growth related phenotype documented in a
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7 significant proportion of *SOS1* mutation-positive subjects, we evaluated the possible contribution
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9 of germline *SOS1* gene lesions in apparently non-syndromic PS, ASD and VSD. Mutation
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11 analysis revealed no *bona fide* mutation within these cohorts, which does not support the idea of
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13 a major contribution of *SOS1* gene defects in the pathogenesis of these CHDs, in line with other
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15 studies documenting only a minor contribution of germline *PTPN11* mutations in isolated CHDs
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17 and HCM [Sarkozy, et al., 2003, 2005; Roberts, et al., 2005; Weismann, et al., 2005; Limongelli,
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19 et al., 2006].
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25 Subjects heterozygous for a mutated *SOS1* allele present with ectodermal manifestations
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27 and distinctive facial dysmorphism that might be suggestive of CFCS in some individuals
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29 (Figure 3) [Narumi et al. 2008; Nystrom et al. 2008]. In these subjects, however, cognitive
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31 deficits are generally absent or minor [Tartaglia et al., 2007; Zenker et al., 2007a; Narumi et al.
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33 2008; Denayer et al., 2010; present study]. Consistent with these observations, our mutational
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35 screening on a clinically well-characterized CFCS cohort failed in identifying any *SOS1*
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37 mutation, confirming a previous survey indicating that *SOS1* does not represent a major gene for
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39 this disorder [Zenker et al. 2007a]. While the clinical features of *SOS1* mutation-positive subjects
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41 appear to be less severe compared to what is generally observed in CFCS, the identification of
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43 subjects with an “overlapping” phenotype suggests that a clinical continuum might be associated
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45 with defects in *SOS1*, as previously documented for other disease genes implicated in RAS-
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47 opathies [Zenker et al., 2007b; Sarkozy et al., 2009a]. On the other hand, based on the increasing
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49 evidence documenting co-occurrence of mutations in functionally related genes that contribute to
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51 the severity of the phenotype [Nystrom et al., 2009; Tang et al., 2009; Thiel et al., 2009;
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3 Longoni et al., 2010], the possibility that mutations concomitant to those affecting *SOS1* might
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5 modulate the phenotype in these subjects cannot be ruled out. Overall, these findings further
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7 emphasize the difficulty in identifying efficient clinical criteria to define these disorders
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9 nosologically, and make evident the clinical relevance of a molecular-based definition of these
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11 clinically overlapping disorders.
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FIGURE LEGENDS

FIGURE 1. SOS1 domain structure and location of residues altered in Noonan syndrome. (A) Schematic structure of SOS1 and variants identified in the present study. SOS1 protein domains are indicated (DH, DBL homology domain; PH, pleckstrin homology domain; REM, RAS exchanger motif; CDC25, CDC25 domain). Disease-causing mutations and probably pathogenetic/unclassified variants are shown above and below the cartoon, respectively. Residues affected by class 1 mutations/variants are shown in red, while those affected by class 2 and class 3 changes are shown in yellow and green, respectively. Residues affected by substitutions with unpredictable effect on SOS1 function are shown in black. Novel amino acid substitutions are underlined. (B) Location of affected residues in SOS1 represented in its inactive conformation, according to the crystal structure of the protein truncated at the C-terminus (residues 1-1049) (PDB ID: 3KSY) [Guerasko et al., 2010]. C α ribbon trace of the HF (sky blue), DH (sandy brown), PH (plum), REM (dark green), and CDC25 (blue) domains, and the helical linker connecting the PH and REM domains (gray). Mutated residues are indicated with their side chains as thick lines and colored as reported above. Residue Asp309 (uncharacterized mutation p.Asp309Tyr) is shown in purple. Affected residues are listed in Supp. Table S2.

FIGURE 2. Detailed analysis of structural perturbations resulting from Noonan syndrome-causing amino acid substitutions. (A) Class 1 mutations affecting residues at the distal RAS binding site. The cartoon includes the DH (sandy brown) and REM (dark green) domains only.

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3 Affected residues are shown in red. The autoinhibitory binding network includes the
4 hydrophobic interaction between residues Met269 (DH) and Trp729 (REM), both mutated in NS,
5 and between the former and Leu687 (green). Leu690, Val697, Ile718 and Ile736 are hydrophobic
6 residues (cyan) that interact with Ile733 (REM). Mutated residues Thr266 (DH) and Lys728
7 (REM) face each other. Substitution of Thr266 by lysine would create an electrostatic repulsion
8 with Lys728. The unclassified variant Leu252 contributes to a hydrophobic core with residues
9 Tyr215, Leu219, Ile249, Tyr295 and Tyr298 (purple), whose disruption is expected to perturb
10 considerably the DH domain surface interacting with the REM domain. (B) Class 1B mutations.
11 The cartoon includes the HF (sky blue), DH (sandy brown) and PH (magenta) domains and the
12 PH-REM helical linker (grey). Relevant affected residues are shown in red. Met422 and Ile437
13 (PH) participate in a hydrophobic bonding network with residues Ile425, Phe464 and Leu467
14 (cyan, see also the inset). Hydrophobic interaction between Tyr337 and Met538 (green)
15 contributes to the binding network stabilizing the interaction between the PH and DH domains.
16 Other interdomain interactions involve Leu550 and residues of the DH and PH domains, Val225,
17 Leu221, Phe226 and Tyr546 (grey), Arg552 and residues of the HF domain, Asp140 and Asp169
18 (orange), Ser548 and Asp169, and Lys170 and residues of the PH domain, Arg497 and Lys498
19 (blue). Phe78 participates to the hydrophobic interaction involving residues of the HF domain
20 core located close to the PH domain and PH-REM linker (Leu55, Leu59, Val74, Val133 and
21 Ile137; purple). (C) Class 1 mutations affecting the REM domain region (dark green) interacting
22 with the CDC25 domain (blue). The helical hairpin (residues 929-978) implicated in the
23 conformational switch (green) and residues interacting with RAS at the active site (light blue) are
24 shown. Phe623 hydrophobically interact with Ile 601, Leu613, Phe627, Ile956 and Phe958
25 (cyan). The hydrogen bond between Tyr702 and Ser802 (orange) is also shown. (D) Class 2
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3 mutations affecting the HF domain. The left panel shows the HF (light blue), DH (sandy brown)
4 and PH (plum) domains. The HF surface colored according to the electrostatic potential (from
5 red at $-3kT/e$ to blue at $+3kT/e$) is also shown (right panel). Mutations affect solvent exposed
6 residues (yellow side chains, left panel; yellow circles, right panel) located in a region that has a
7 positive electrostatic potential (right panel), and has been implicated in membrane binding. (E)
8 Class 2 mutations affecting the PH domain. The left panel includes the PH domain (plum) only.
9 The PH surface, colored according to the electrostatic potential (from red at $-5kT/e$ to blue at
10 $+5kT/e$) is also shown (left panel). Affected residues are shown in yellow (side chains, left panel;
11 circles, right panel). Residues that are predicted to bind to PIP_2 [Zheng et al., 1997], Lys456,
12 Arg459, Lys472 and Arg489, are shown in green (circled in the right panel), while the PA-
13 interacting region (residues 472-483) [Zhao et al., 2007] is shown in orange (circled in the right
14 panel). (F) Class 3 mutations. The cartoon includes the REM (dark green) and CDC25 (blue)
15 domains only (residues 567-1049). Affected residues are shown in green. Residues implicated in
16 RAS binding at the catalytic site are shown (light blue). Glu846 and Pro894 are placed distally
17 from the active site and regions implicated in the conformational rearrangement of the CDC25
18 domain. Glu846 electrostatically interacts with Arg1026 and Lys 1029 (orange).

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46 **FIGURE 3.** Facial dysmorphism and other features of subjects with Noonan syndrome
47 heterozygous for mutations in the *SOS1* gene. *SOS1* mutation-positive subjects generally exhibit
48 typical facial features, including macrocephaly, hypertelorism, ptosis, downslanting palpebral
49 fissures, sparse eyebrows with keratosis pilaris, a short and broad nose with upturned tip, low-
50 set and posteriorly angulated ears, and high forehead commonly associated with bitemporal
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3 narrowing and prominent supraorbital ridges. Curly hair is present in most of the patients. Other
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5 common features include pectus anomalies (NS10, NS19, NS37), short and/or webbed neck
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7 (NS6, NS10, NS19, NS22, NS38), and cubitus valgus (NS37). Keloid scars (NS16), recurrent
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9 hemorrhages (NS18) and deep plantar creases (NS38) also occur in these subjects. In some
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11 infants, the face is suggestive of cardiofaciocutaneous syndrome due to the coarseness of features
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13 (NS39).
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Table 1. *SOS1* exonic indels and missense changes identified in the study

| Exon | Nucleotide change | Amino acid change | Domain | Notes | Number of cases |
|-------------------------------------|--|---------------------------------|---------------|--------------------------------------|-----------------------------|
| <i>Mutations</i> | | | | | |
| 4 | c.322G>A | p.Glu108Lys | HF | | 2, fam.unknown |
| 5 | c.508A>G | p.Lys170Glu | HF | | 1, sporadic; 2, fam.unknown |
| 7 | c.797C>A | p.Thr266Lys | DH | | 1, sporadic; 3, fam.unknown |
| 7 | c.806T>C | p.Met269Thr | DH | | 3, sporadic; 2, fam.unknown |
| 7 | c.806T>G | p.Met269Arg | DH | | 1, fam.unknown |
| 11 | c.1281_1289delGAATATTGA | p.Lys427_Asp430delinsAsn | PH | | 1, fam.unknown |
| 11 | c.1294T>C | p.Trp432Arg | PH | | 1, sporadic; 1, fam.unknown |
| 11 | c.1294_1299delTGGGAG | p.Trp432_Glu433del | PH | CTRL, NPS | 1, sporadic |
| 11 | c.1297G>A | p.Glu433Lys | PH | | 3, sporadic; 3, fam.unknown |
| 11 | c.1300G>A | p.Gly434Arg | PH | | 2, fam.unknown |
| 11 | c.1300_1301delGGinsAA | p.Gly434Lys | PH | <i>de novo</i> , PPhen+, SIFT+, CON | 1, sporadic |
| 11 | c.1310T>C | p.Ile437Thr | PH | <i>de novo</i> , PPhen++, SIFT+, CON | 1, sporadic; 1, familial |
| 11 | c.1322G>A | p.Cys441Tyr | PH | | 1, fam.unknown |
| 11 | c.1430A>G | p.Gln477Arg | PH | PPhen-, SIFT-, CON | 2, fam.unknown |
| 11 | c.1433C>G | p.Pro478Arg | PH | | 1, sporadic |
| 11 | c.1642A>C | p.Ser548Arg | PH-REM linker | | 4, fam.unknown |
| 11 | c.1654A>G | p.Arg552Gly | PH-REM linker | | 3, sporadic; 8, fam.unknown |
| 11 | c.1655G>A | p.Arg552Lys | PH-REM linker | | 2, sporadic; 2, fam.unknown |
| 11 | c.1655G>T | p.Arg552Met | PH-REM linker | NPS, PPhen++, SIFT+, CON | 2, sporadic |
| 11 | c.1655G>C | p.Arg552Thr | PH-REM linker | | 1, fam.unknown |
| 11 | c.1656G>C | p.Arg552Ser | PH-REM linker | | 2, sporadic; 7, fam.unknown |
| 11 | c.1660_1673delCTTGATGTAACAATinsAA | p.Leu554_Met558delinsLys | PH-REM linker | | 1, fam.unknown |
| 15 | c.2197A>T | p.Ile733Phe | REM | | 1, fam.unknown |
| 17 | c.2536G>A | p.Glu846Lys | CDC25 | | 1, sporadic, 6 fam.unknown |
| 18 | c.2681C>G | p.Pro894Arg | CDC25 | CTRL, PPhen+, SIFT-, CON | 1, familial [§] |
| <i>Possibly pathogenic variants</i> | | | | | |
| 4 | c.335C>G | p.Pro112Arg | HF | NPS, PPhen+, SIFT- | 1, sporadic |
| 7 | c.755T>C | p.Ile252Thr | DH | CTRL, PPhen++, SIFT+, CON | 1, familial |
| 11 | c.1264A>G | p.Met422Val | PH | PPhen++, SIFT-, CON | 1, fam.unknown |

Table 1. *SOS1* exonic indels and missense changes identified in the study (continued)

| Exon | Nucleotide change | Amino acid change | Domain | Notes | Number of cases |
|------------------------|---------------------|---------------------|---------------|--------------------------------|---------------------------------|
| 11 | c.1270G>A | p.Glu424Lys | PH | PPhen+, SIFT+, CON | 1, fam.unknown |
| 11 | c.1444G>C | p.Gly482Arg | PH | PPhen+, SIFT+, CON | 1, fam.unknown |
| 11 | c.1469T>G | p.Leu490Arg | PH | CTRL, NPS, PPhen++, SIFT+, CON | 1, sporadic |
| 11 | c.1490G>A | p.Arg497Gln | PH | CTRL, PPhen+, SIFT+, CON | 1, sporadic* |
| 11 | c.1646C>A | p.Thr549Lys | PH-REM linker | PPhen+, SIFT-, CON | 1, fam.unknown |
| 17 <i>Unclassified</i> | | | | | |
| 18 <i>variants</i> | | | | | |
| 3 | c.109A>G | p.Thr37Ala | HF | PPhen-, SIFT-, CON | 1, fam.unknown |
| 11 | c.1433C>T | p.Pro478Leu | PH | PPhen+, SIFT-, CON | 1, fam.unknown |
| 15 | c.2351T>C | p.Ile784Thr | REM | PPhen++, SIFT+, CON | 1, fam.unknown ^{&} |
| 23 | c.3392G>A | p.Arg1131Lys | C-terminus | PPhen-, SIFT-, CON | 1, fam.unknown |
| 23 | c.3418T>A | p.Leu1140Ile | C-terminus | PPhen-, SIFT-, CON | 1, fam.unknown |
| 24 | c.3769A>G | p.Thr1257Ala | C-terminus | PPhen-, SIFT-, CON | 1, fam.unknown |

Nucleotide numbering reflects cDNA numbering with 1 corresponding to the A of the ATG translation initiation codon in the reference sequence (NM_005633.3). Exon 2 corresponds to the first protein coding exon. Novel mutations are in bold. HF, histone folds; DH, DBL homology domain; PH, plekstrin homology domain; REM, RAS exchanger motif, CDC25, CDC25 domain. Fam.unknown, familial status unknown; NPS, unavailable parental DNA samples. CTRL, variant not occurring in ≥ 300 population-matched unaffected subjects; *de novo*, variant demonstrated to occur *de novo* by DNA genotyping of unaffected parents; PPhen++, amino acid change predicted to be “probably damaging” by PolyPhen; PPhen+, amino acid change predicted to be “possibly damaging” by PolyPhen; PPhen-, amino acid change predicted to be “benign” by Polyphen; SIFT+, amino acid change predicted to “affect protein function” by SIFT; SIFT-, amino acid change predicted to be “tolerated” by SIFT; CON, variant at a conserved residue. §, variant inherited from an affected parent; *, variant inherited from an apparently unaffected parent; &, variant concomitant with the disease-causing p.Met269Arg change.

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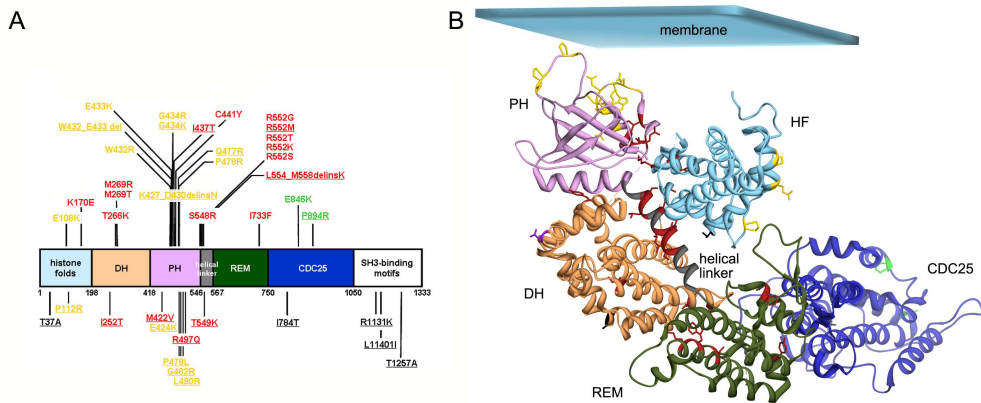


FIGURE 1. *SOS1* domain structure and location of residues altered in Noonan syndrome.

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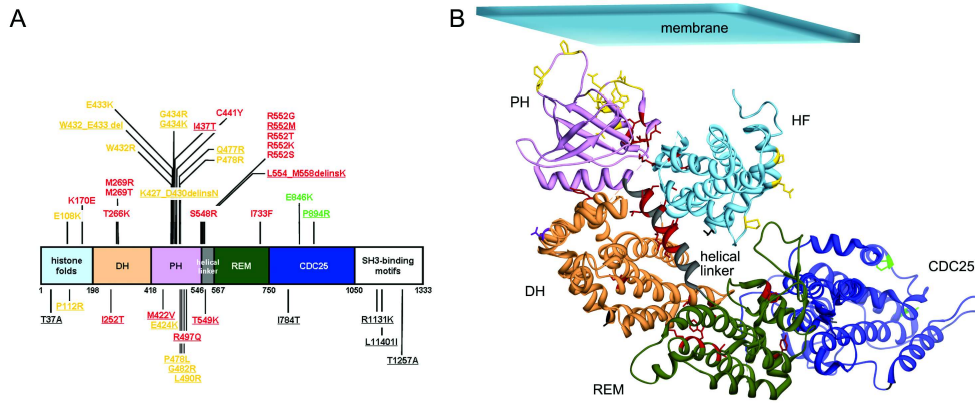


FIGURE 1. *SOS1* domain structure and location of residues altered in Noonan syndrome.

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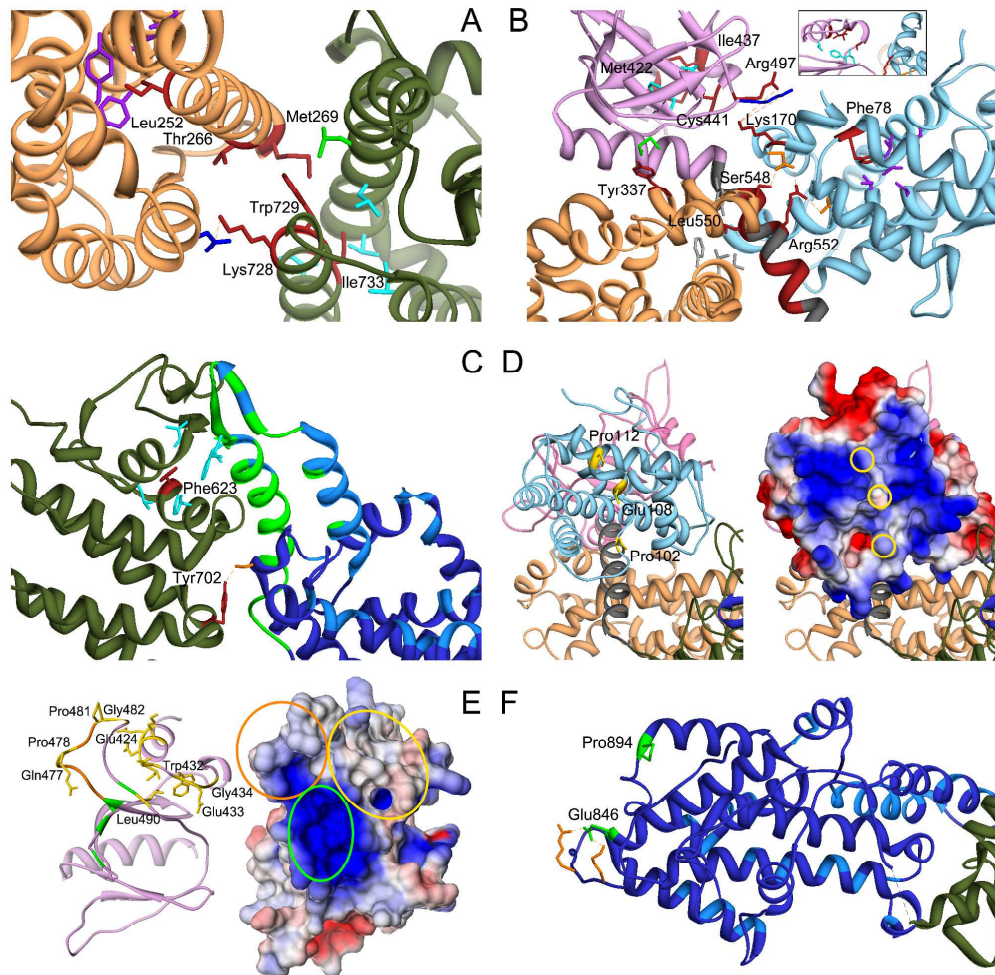


FIGURE 2. Detailed analysis of structural perturbations resulting from Noonan syndrome-causing amino acid substitutions.

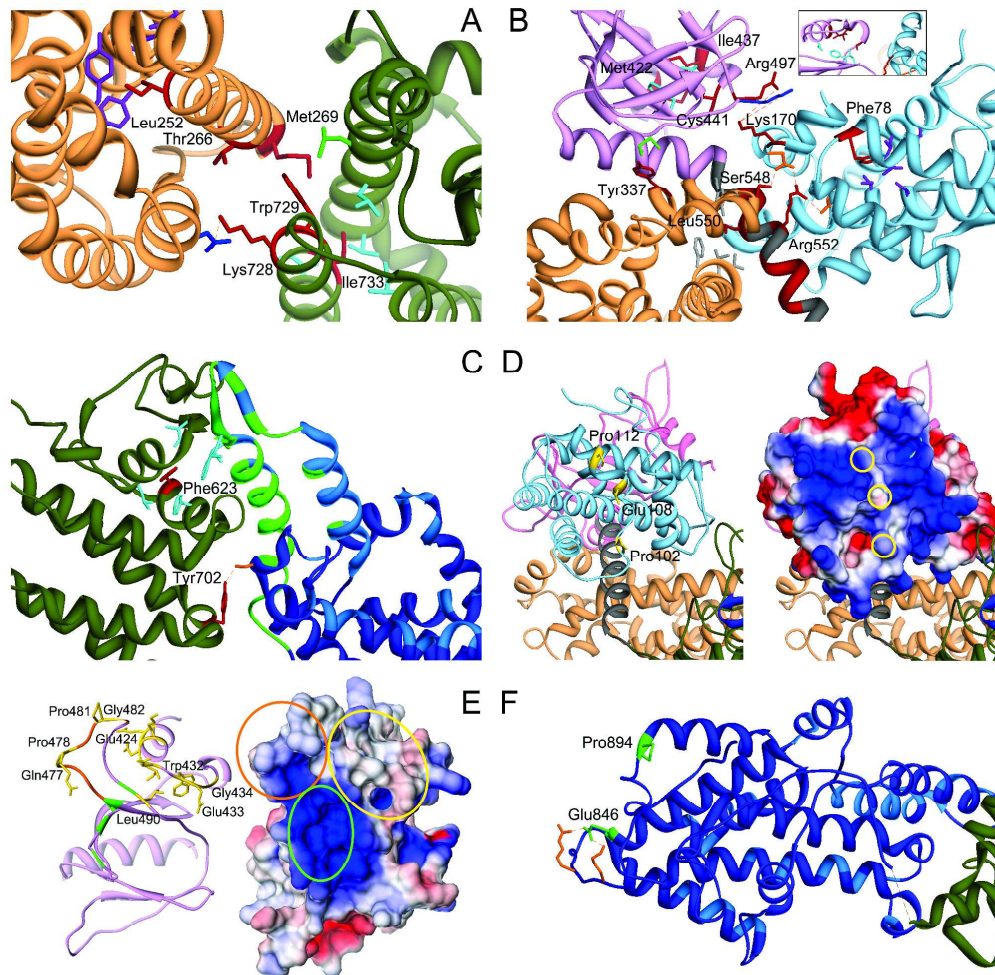


FIGURE 2. Detailed analysis of structural perturbations resulting from Noonan syndrome-causing amino acid substitutions.

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FIGURE 3. Facial dysmorphism and other features of subjects with Noonan syndrome heterozygous for mutations in the SOS1 gene.

Supp. Table S1. List of disease-unrelated *SOS1* sequence variants identified in the study

| Location | Nucleotide change | Amino acid change |
|-----------|-------------------|-------------------------|
| Intron 2 | c.87+86delG | - |
| Intron 4 | c.345+52C>G | - |
| Intron 5 | c.510+24T>C | - |
| | c.510+59C>G | - |
| Intron 6 | c.720+25C>G | - |
| | c.721-69T>C | - |
| | c.721-18G>C | - |
| Intron 7 | c.864+23C>T | - |
| | c.864+24G>A | - |
| Intron 9 | c.1074+5G>C | - |
| | c.1075-25C>G | - |
| Intron 10 | c.1202+25A>T | - |
| | c.1203-20T>C | - |
| Exon 11 | c.1230G>A | Silent change (Gln410) |
| | c.1705C>G | p.Leu569Val |
| | c.1800T>A | silent change (Ile600) |
| | c.1854C>G | silent change (Tyr618) |
| Intron 11 | c.1859-40T>C | - |
| | c.1859-37T>C | - |
| Exon 13 | c.1964C>T | p.Pro655Leu |
| Exon 14 | c.2122G>A | p.Ala708Thr |
| Intron 16 | c.2511-98C>G | - |
| Intron 17 | c.2673+14T>C | - |
| | c.2674-22C>G | - |
| Exon 18 | c.2760G>A | silent change (Arg920) |
| Intron 18 | c.2791+53C>T | - |
| Intron 19 | c.2964+43C>A | - |
| Exon 20 | c.3032A>G | p.Asn1011Ser |
| Exon 21 | c.3093T>C | silent change (Tyr1031) |
| Intron 21 | c.3346+72+73delCT | - |
| | c.3347-143T>C | - |
| | c.3347-127T>C | - |
| Exon 22 | c.3357C>T | silent change (Thr1119) |
| Intron 22 | c.3391+7A>G | - |
| | c.3392-89T>G | - |
| Exon 24 | c.3959A>G | p.His1320Arg |

Nucleotide numbering of the exonic variants reflects cDNA numbering with 1 corresponding to the A of the ATG translation initiation codon in the reference sequence (NM_005633.3). Exon 2 corresponds to the first protein coding exon. Position of intronic variants is numbered according to the reference genomic sequence (NG_007530.1).

Supp. Table S2. List of *SOS1* exonic indels and missense changes (updated to July 2010)

| Nucleotide change | Amino acid change | Status and functional class | N | References |
|-----------------------------|---------------------------------|-----------------------------|----|--------------------------------|
| c.109A>G | p.Thr37Ala | Unclassified variant | 1 | <i>p.s.</i> |
| c.233T>G | p.Phe78Cys | Probably pathogenic, 1B | 1 | 3 |
| c.305C>G | p.Pro102Arg | Mutation, 2A | 1 | 11 |
| c.322G>A | p.Glu108Lys | Mutation, 2A | 4 | 2, <i>p.s.</i> |
| c.335C>G | p.Pro112Arg | Probably pathogenic, 2A | 1 | <i>p.s.</i> |
| c.508A>G | p.Lys170Glu | Mutation, 1B | 5 | 6, 11, <i>p.s.</i> |
| c.755T>C | p.Ile252Thr | Probably pathogenic, 1A | 1 | <i>p.s.</i> |
| c.797C>A | p.Thr266Lys | Mutation, 1A | 8 | 1, 6, 7, 11, <i>p.s.</i> |
| c.806T>C | p.Met269Thr | Mutation, 1A | 9 | 3, 6, 11, <i>p.s.</i> |
| c.806T>G | p.Met269Arg | Mutation, 1A | 8 | 1, 2, 3, 6, 8, <i>p.s.</i> |
| c.925G>T | p.Asp309Tyr | Mutation, uncharacterized | 3 | 1, 3, 5 |
| c.1010A>G | p.Tyr337Cys | Mutation, 1B | 1 | 1 |
| c.1132A>G | p.Thr378Ala | Unclassified variant | 1 | 11 |
| c.1264A>G | p.Met422Val | Probably pathogenic, 1B | 1 | <i>p.s.</i> |
| c.1270G>A | p.Glu424Lys | Probably pathogenic, 2B | 1 | <i>p.s.</i> |
| c.1281_1289delGAATAT TGA | p.Lys427_Asp430de linsAsn | Mutation, 2B | 1 | <i>p.s.</i> |
| c.1294T>C | p.Trp432Arg | Mutation, 2B | 5 | 2, 3, 10, <i>p.s.</i> |
| c.1294_1299delTGGGAG | p.Trp432_Glu433del | Mutation, 2B | 1 | <i>p.s.</i> |
| c.1297G>A | p.Glu433Lys | Mutation, 2B | 14 | 2, 3, 4, 6, 8, 11, <i>p.s.</i> |
| c.1300G>C | p.Gly434Arg | Mutation, 2B | 1 | 1 |
| c.1300G>A | p.Gly434Arg | Mutation, 2B | 3 | 3, <i>p.s.</i> |
| c.1300_1301delGGinsAA | p.Gly434Lys | Mutation, 2B | 1 | <i>p.s.</i> |
| c.1310T>C | p.Ile437Thr | Mutation, 1B | 2 | <i>p.s.</i> |
| c.1322G>A | p.Cys441Tyr | Mutation, 1B | 4 | 6, <i>p.s.</i> |
| c.1430A>G | p.Gln477Arg | Mutation, 2B | 2 | <i>p.s.</i> |
| c.1431G>T; 1433C>T | p.Gln477His; Pro478Leu | Mutation, 2B | 1 | 3 |
| c.1433C>G | p.Pro478Arg | Mutation, 2B | 2 | 3, <i>p.s.</i> |
| c.1433C>T | p.Pro478Leu | Unclassified variant (2B) | 1 | <i>p.s.</i> |
| c.1442_1443insAAGACT TCC | p.Pro481_Gly482 insArgLeuPro | Mutation, 2B | 1 | 5 |

| Nucleotide change | Amino acid change | Status and functional class | N | References |
|---------------------------------------|------------------------------|-----------------------------|----|-----------------------------|
| c.1444G>C | p.Gly482Arg | Probably pathogenic, 2B | 1 | <i>p.s.</i> |
| c.1469T>G | p.Leu490Arg | Probably pathogenic, 2B | 1 | <i>p.s.</i> |
| c.1490G>A | p.Arg497Gln | Probably pathogenic, 1B | 1 | <i>p.s.</i> |
| c.1642A>C | p.Ser548Arg | Mutation, 1B | 8 | 1, 2, 6, <i>p.s.</i> |
| c.1646C>A | p.Thr549Lys | Probably pathogenic, 1B | 1 | <i>p.s.</i> |
| c.1649T>C | p.Leu550Pro | Mutation, 1B | 2 | 2, 8 |
| c.1654A>G | p.Arg552Gly | Mutation, 1B | 29 | 1, 2, 3, 8, 11, <i>p.s.</i> |
| c.1655G>C | p.Arg 552Thr | Mutation, 1B | 2 | 9, <i>p.s.</i> |
| c.1655G>T | p.Arg 552Met | Mutation, 1B | 2 | <i>p.s.</i> |
| c.1655G>A | p.Arg 552Lys | Mutation, 1B | 7 | 2, 3, <i>p.s.</i> |
| c.1656G>C | p.Arg 552Ser | Mutation, 1B | 15 | 2, 6, 9, <i>p.s.</i> |
| c.1656G>T | p.Arg 552Ser | Mutation, 1B | 4 | 3, 5, 8 |
| c.1660_1673delCTTGAT GTAACAATinsAA | p.Leu554_Met558 delinsLys | Mutation, 1B | 1 | <i>p.s.</i> |
| c.1705C>G | p.Leu569Val | Polymorphism | | 11, <i>p.s.</i> |
| c.1867 T>A | p.Phe623Ile | Mutation, 1C | 1 | 3 |
| c.1964C>T | p.Pro655Leu | Polymorphism | | 1, 2, 3, 11, <i>p.s.</i> |
| c.2104T>C | p.Tyr702His | Mutation, 1C | 2 | 2, 3 |
| c.2122G>A | p.Ala708Thr | Polymorphism | | <i>p.s.</i> |
| c.2183A>T | p.Lys728Ile | Mutation, 1A | 1 | 12 |
| c.2186G>T | p.Trp729Leu | Mutation, 1A | 1 | 2 |
| c.2197A>T | p.Ile733Phe | Mutation, 1A | 2 | 2, <i>p.s.</i> |
| c.2351T>C | p.Ile784Thr | Unclassified variant | 1 | <i>p.s.</i> |
| c.2536G>A | p.Glu846Lys | Mutation, 3 | 19 | 1, 2, 3, 5, 8, <i>p.s.</i> |
| c.2681C>G | p.Pro894Arg | Mutation, 3 | 1 | <i>p.s.</i> |
| c.2930A>G | p.Gln977Arg | Unclassified variant | 1 | 2 |
| c.2999G>A | p.Ser1000Asn | Unclassified variant | 1 | 3 |
| c.3032A>G | p.Asn1011Ser | Polymorphism | | <i>p.s.</i> |
| c.3392G>A | p.Arg1131Lys | Unclassified variant | 1 | <i>p.s.</i> |
| c.3418T>A | p.Leu1140Ile | Unclassified variant | 1 | <i>p.s.</i> |
| c.3769A>G | p.Thr1257Ala | Unclassified variant | 1 | <i>p.s.</i> |
| c.3959A>G | p.His1320Arg | Polymorphism | 1 | 2, <i>p.s.</i> |

Nucleotide numbering of the exonic variants reflects cDNA numbering with 1 corresponding to the A of the ATG translation initiation codon in the reference sequence (NM_005633.3). *p.s.*, present study.

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Supp.Table S3. Clinical features of *SOS1* mutation-positive subjects

| Case | NS1 | NS2 | NS3 | NS4 | NS5 | NS6 | NS7 | NS8 | NS9 | NS10 |
|-----------------------------------|-----------------|------------------|------------------|------------------------------------|-------------------------------------|-------------------------------------|-------------------|------------------|------------------|------------------|
| Code | S1021 | S1036 | S1052 | BO-2564 | BO-0138 | BO-4043 | BO-1767 | BO-2905 | BO-0235 | BO-1834 |
| Sex | M | M | F | M | M | M | M | M | M | M |
| Age at evaluation | 11 y | 12 m | 1 y | 12 m | 8 y, 10 m | 5 m, 13 d | 2 y, 1 m | 14 y, 5 m | 4 y, 2 m | 8 y, 2 m |
| Amino acid change | R552K | W432_E433del | R552S | E433K | R552G | E433K | T266K | P478R | M269T | M269T |
| Polyhydramnios | + | - | + | + | - | - | + | - | - | + |
| Fetal macrosomia | - | - | + | + | - | + | - | - | - | - |
| Neonatal/infantile growth failure | - | - | - | - | + | - | - | - | + | + |
| Poor sucking | - | - | + | - | - | - | - | - | + | - |
| Poor swallowing | - | - | + | - | - | - | - | - | - | - |
| Apneas | - | - | - | + | - | - | - | - | - | - |
| Short stature (<3rd centile) | - | - | - | - | - | - | - | + | - | + |
| Height/length (cm) | | | | 71 | 127 | 65 | 98 | 147 | 105 | 113 |
| Age | | | | 12 m | 8 y, 10 m | 5 m, 13 d | 2 y, 1 m | 14 y, 5 m | 4 y, 2 m | 8 y, 2 m |
| Centile | 5 th | 50 th | 75 th | 5 th – 10 th | 25 th – 50 th | 25 th – 50 th | >98 th | <2 nd | 50 th | <2 nd |
| Delayed bone age | | | | | | | | + | | + |
| GH deficiency | | | | | + | | | + | | |
| Craniofacial anomalies | + | + | + | + | + | + | + | + | + | + |
| - Macrocephaly | - | - | - | + | + | - | + | - | - | - |
| - Scaphocephaly | - | - | - | - | - | - | - | - | - | - |
| - High forehead | + | + | - | - | + | + | + | + | + | + |
| - Bitemporal narrowing | + | + | - | - | + | + | + | - | + | + |
| - Prominent supraorbital ridge | - | - | - | - | + | + | + | + | - | + |
| - Downslanting palpebral fissures | + | - | + | + | + | + | + | + | + | + |
| - Hypertelorism | - | - | + | + | + | + | + | - | + | + |
| - Epicanthal folds | + | - | + | + | + | + | + | + | + | + |
| - Palpebral ptosis | + | + | + | + | + | - | + | - | - | + |
| - Flat nasal bridge | + | - | + | + | + | + | + | - | + | + |
| - Broad nasal root | - | - | + | - | + | + | + | - | + | + |
| - Prominent philtrum | + | - | + | - | + | + | + | + | - | + |
| - Thick lips/macrostomia | - | - | - | - | + | - | + | - | - | + |
| - Low-set ears | + | + | + | + | + | + | + | + | + | + |
| - Thickened helix | - | - | + | + | + | + | + | - | + | + |
| - Large, thick ear lobe | - | - | + | + | + | + | + | - | + | + |

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| Case | NS1 | NS2 | NS3 | NS4 | NS5 | NS6 | NS7 | NS8 | NS9 | NS10 |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| - Micrognathia | - | - | + | | - | + | - | + | - | + |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS11 | NS12 | NS13 | NS14 | NS15 | NS16 | NS17 | NS18 | NS19 | NS20 |
|-----------------------------------|-----------|-----------|---------|----------|----------|-------|---------|----------|----------|-------------|
| Code | BO-1651 | BO-0596 | BO-2635 | BO-4338 | HD1256 | HD290 | 05-0175 | 07-0934 | HD1284 | HD696 |
| Sex | M | M | F | M | F | M | M | F | F | F |
| Age at evaluation | 4 y, 11 m | 16 y, 7 m | 26 y | 1 y, 3 m | 9 y, 7 m | 22 y | 13 y | 10 y | 6 y, 6 m | 9 y |
| Amino acid change | W432R | E433K | R552G | E846K | R552S | K170E | L490R | R552G | I437T | R552G |
| Polyhydramnios | - | + | - | + | + | + | - | - | - | - |
| Fetal macrosomia | - | - | - | - | + | - | - | - | - | - |
| Neonatal/infantile growth failure | - | + | + | + | + | - | + | + | + | - |
| Poor sucking | - | - | + | + | - | - | - | - | N.R. | - |
| Poor swallowing | - | - | - | + | - | - | + | - | N.R. | - |
| Apneas | - | - | - | - | - | - | - | - | - | - |
| Short stature (<3rd centile) | - | + | - | + | + | - | - | - | + | - |
| Height/length (cm) | 114 | 161 | 154 | 72 | 123 | 172 | 138.5 | 107 | 93 | 132.5 |
| Age | 4 y, 11 m | 16 y, 7 m | 26 y | 1 y, 3 m | 9 y, 7 m | 22 y | 11 y | 6 y, 8 m | 4 y, 6 m | 8 y, 6 m |
| Centile | 90th-97th | 2nd | 10th | 2nd | < 3rd | 25th | 25th | 3rd | <2nd | 50th – 75th |
| Delayed bone age | - | + | + | | + | | - | | + | - |
| GH deficiency | - | + | - | | - | - | - | | | - |
| Craniofacial anomalies | + | + | + | + | + | + | + | + | + | + |
| - Macrocephaly | + | + | + | + | - | + | + | + | + | + |
| - Scaphocephaly | - | - | - | - | - | - | - | - | - | - |
| - High forehead | + | + | + | + | + | + | + | + | + | + |
| - Bitemporal narrowing | - | - | - | - | + | - | + | + | + | - |
| - Prominent supraorbital ridge | - | + | + | + | + | + | - | - | + | - |
| - Downslanting palpebral fissures | + | + | + | + | - | + | + | + | + | + |
| - Hypertelorism | + | + | + | + | - | + | + | + | + | + |
| - Epicanthal folds | - | + | + | - | + | - | + | - | + | + |
| - Palpebral ptosis | + | + | + | + | + | + | + | + | - | - |
| - Flat nasal bridge | - | - | - | - | + | - | + | + | + | - |
| - Broad nasal root | - | + | - | - | + | - | - | + | + | + |
| - Prominent philtrum | - | + | - | - | + | - | + | + | - | + |

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| Case | NS11 | NS12 | NS13 | NS14 | NS15 | NS16 | NS17 | NS18 | NS19 | NS20 |
|--------------------------|------|------|------|------|------|------|------|------|------|------|
| - Thick lips/macrostomia | - | - | + | + | - | - | + | | + | + |
| - Low-set ears | + | + | + | + | - | + | + | + | + | + |
| - Thickened helix | - | - | - | - | + | + | - | + | + | + |
| - Large, thick ear lobe | - | + | + | + | - | - | + | + | + | + |
| - Micrognathia | + | + | - | - | + | - | - | + | + | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS21 | NS22 | NS23 | NS24 | NS25 | NS26 | NS27 | NS28 | NS29 | NS30 |
|-----------------------------------|-------------|---------------|---------|------------|-------------|-----------|----------|--------------|-------|----------|
| Code | N1183 | N118_3_father | 08-0045 | N1157 | N1212 | N1185 | HD227 | HD215_father | HD215 | HD715 |
| Sex | M | M | M | M | M | F | F | M | F | M |
| Age at evaluation | 12 y, 8 m | 40 y | 3 y | 28 y | 11 y, 7 m | 15 y, 3 m | 7 y, 4 m | 37 y | 3 y | 1 y, 5 m |
| Amino acid change | P894R | P894R | G434K | M269T | R552M | C441Y | S548R | L550P | L550P | R552G |
| Polyhydramnios | + | N.R. | - | N.R. | - | + | - | - | + | + |
| Fetal macrosomia | + | - | - | - | - | + | + | - | - | + |
| Neonatal/infantile growth failure | - | - | - | N.R. | + | + | + | - | + | + |
| Poor sucking | - | - | - | - | - | + | - | - | + | + |
| Poor swallowing | - | - | - | - | - | + | - | - | + | + |
| Apneas | - | - | - | - | - | - | - | - | - | + |
| Short stature (<3rd centile) | + | + | - | - | + | + | - | - | - | + |
| Height/length (cm) | 137cm | 160cm | 96cm | 170cm | 131.8cm | 147 cm | 118 | 171 | 93 | 79 |
| Age | 12 y, 8 m | 40 y | 3 y | 28 y | 11 y, 7 m | 15 y, 3 m | 7 y, 4 m | 37 y | 3 y | 1 y, 5 m |
| Centile | 0.4th – 2nd | 0.4th – 2nd | 75th | 9th – 25th | 0.4th – 2nd | < 3rd | 25th | 25th | 50th | 25th |
| Delayed bone age | + | - | - | - | + | + | + | - | + | + |
| GH deficiency | - | - | - | - | - | - | - | - | - | - |
| Craniofacial anomalies | + | + | + | + | + | + | + | + | + | + |
| - Macrocephaly | + | + | - | + | - | - | - | + | + | + |
| - Scaphocephaly | - | - | - | - | - | - | - | - | - | - |
| - High forehead | + | - | - | + | + | - | + | + | + | - |
| - Bitemporal narrowing | - | - | - | + | + | - | + | + | + | - |
| - Prominent supraorbital ridge | - | - | + | + | - | + | + | + | + | - |
| - Downslanting palpebral fissures | - | + | - | + | + | + | + | + | + | + |
| - Hypertelorism | + | + | + | + | - | + | + | + | + | + |
| - Epicanthal folds | - | - | - | - | + | + | + | - | - | - |

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| Case | NS21 | NS22 | NS23 | NS24 | NS25 | NS26 | NS27 | NS28 | NS29 | NS30 |
|--------------------------|------|------|------|------|------|------|------|------|------|------|
| - Palpebral ptosis | + | + | + | + | + | + | + | + | + | + |
| - Flat nasal bridge | - | - | - | + | + | + | + | - | + | + |
| - Broad nasal root | - | - | - | - | - | - | - | - | - | - |
| - Prominent philtrum | - | + | + | - | + | + | - | - | + | + |
| - Thick lips/macrostomia | + | + | + | + | - | + | + | - | + | + |
| - Low-set ears | + | + | + | + | + | + | + | + | + | + |
| - Thickened helix | + | + | + | + | + | + | + | + | + | + |
| - Large, thick ear lobe | + | - | - | - | + | + | - | - | + | + |
| - Micrognathia | - | - | - | - | + | + | - | - | - | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS31 | NS32 | NS33 | NS34 | NS35 | NS36 | NS37 | NS38 | NS39 | Total |
|-----------------------------------|----------|----------|----------|-----------|-----------|-----------|-------|----------|-------|------------------------|
| Code | HD303 | 04-0400 | HD099 | S1073 | S1089 | HD1090 | S1108 | S1121 | S1135 | |
| Sex | M | M | F | M | M | F | M | F | F | 25 M, 14F |
| Age at evaluation | 3 y, 5 m | 8 y, 2 m | 5 y, 0 m | 18 y | 34 y | 13 y, 7 m | 17 y | 6 y | 3 y | 8 y, 10 m ⁺ |
| Amino acid change | R552G | R552G | W729L | R552G | R552G | R552S | R552S | R552K | I733F | |
| Polyhydramnios | - | + | + | - | + | + | - | - | + | 18/37 (49%) |
| Fetal macrosomia | + | + | - | + | + | - | + | + | - | 14/38 (37%) |
| Neonatal/infantile growth failure | + | - | + | - | - | - | - | - | - | 17/35 (49%) |
| Poor sucking | + | - | + | - | - | - | - | - | - | 9/38 (24%) |
| Poor swallowing | + | - | + | - | - | - | - | - | - | 8/37 (22%) |
| Apneas | - | - | + | - | + | - | - | - | - | 5/37 (12%) |
| Short stature (<3rd centile) | - | - | - | - | - | - | - | - | - | 11/39 (28%) |
| Height/length (cm) | 100 | 115 | 102 | 73.5 | 165 | 154 | | 118 | | |
| Age | 3 y, 5 m | 8 y, 2 m | 5 y, 0 m | 20 y | 34 y, 7 m | 13 y, 7 m | | 8 y, 1 m | | |
| Centile | 75th | <3rd | 10th | 25th-50th | 5th | 25th | 5th | 5th | 10th | |
| Delayed bone age | + | + | - | - | - | + | - | - | - | 15/26 (58%) |
| GH deficiency | - | - | - | - | - | - | - | - | - | 3/26 (12%) |
| Craniofacial anomalies | + | + | + | + | + | + | + | + | + | 39/39 (100%) |
| - Macrocephaly | + | + | + | - | + | - | + | - | + | 24/39 (61%) |
| - Scaphocephaly | - | - | - | - | - | - | - | - | - | 0/39 (0%) |
| - High forehead | - | - | + | - | - | - | - | - | + | 25/39 (64%) |
| - Bitemporal narrowing | - | - | + | - | - | - | - | - | - | 17/39 (44%) |

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| Case | NS31 | NS32 | NS33 | NS34 | NS35 | NS36 | NS37 | NS38 | NS39 | Total |
|-----------------------------------|------|------|------|------|------|------|------|------|------|-------------|
| - Prominent supraorbital ridge | - | - | + | - | + | - | + | + | + | 22/39 (56%) |
| - Downslanting palpebral fissures | + | + | + | + | + | + | + | + | + | 35/39 (90%) |
| - Hypertelorism | + | + | + | + | + | + | + | + | + | 34/39 (87%) |
| - Epicanthal folds | + | - | + | + | + | + | + | + | + | 26/39 (67%) |
| - Palpebral ptosis | + | + | + | + | + | + | + | + | + | 34/39 (87%) |
| - Flat nasal bridge | + | + | + | + | + | + | + | + | + | 27/39 (69%) |
| - Broad nasal root | - | - | - | - | + | - | - | + | + | 14/38 (37%) |
| - Prominent philtrum | - | + | - | - | + | - | + | - | + | 22/39 (56%) |
| - Thick lips/macrostomia | + | + | + | + | + | + | + | + | + | 25/38 (66%) |
| - Low-set ears | + | + | + | + | + | + | + | + | + | 38/39 (97%) |
| - Thickened helix | + | + | + | + | + | + | - | + | + | 30/39 (77%) |
| - Large, thick ear lobe | - | - | + | + | + | + | + | + | + | 25/38 (66%) |
| - Micrognathia | - | - | - | - | - | - | + | - | - | 12/38 (32%) |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS1 | NS2 | NS3 | NS4 | NS5 | NS6 | NS7 | NS8 | NS9 | NS10 |
|-------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| Congenital heart defects | + | + | + | + | + | + | + | - | + | + |
| - Pulmonic stenosis (PS) | + | + | + | + | - | + | + | - | + | + |
| - Pulmonary valve dysplasia | - | - | - | - | - | - | - | - | - | - |
| - Mitralic valve dysplasia | - | - | - | - | - | - | - | - | - | - |
| - Supravalvular PS | - | - | - | - | - | - | - | - | - | - |
| - Atrial septal defect | - | - | + | - | - | - | - | - | - | - |
| - Hypertrophic cardiomyopathy | - | - | - | - | - | - | - | - | - | - |
| - Atrioventricular canal | - | - | - | - | - | - | - | - | - | - |
| - Tetralogy of Fallot | - | - | - | - | - | - | - | - | - | - |
| - Ventricular septal defect | - | - | - | - | + | - | - | - | - | + |
| - others | - | - | - | - | - | - | - | + | - | - |
| Skin features | - | - | + | - | - | - | + | - | + | + |
| - Dark skin | - | - | - | - | - | - | - | - | + | + |
| - palmo/plantar crease | - | - | + | - | - | - | - | - | - | - |
| - Pruritus | - | - | - | - | - | - | + | - | - | - |
| - others | - | - | - | - | - | - | - | - | - | - |
| Ectodermal features | + | - | + | - | + | + | + | + | + | + |

| Case | NS1 | NS2 | NS3 | NS4 | NS5 | NS6 | NS7 | NS8 | NS9 | NS10 |
|-------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| - Sparse/absent scalp hair | - | - | - | - | - | - | + | - | - | - |
| - Thin hair | - | - | - | - | - | + | + | - | - | - |
| - Curly hair | + | - | - | - | + | - | - | + | - | + |
| - Sparse eyebrows | + | - | + | - | + | + | + | + | + | + |
| - Keratosis pilaris faciei | + | - | - | - | - | - | - | - | - | - |
| - Thin dystrophic nails | - | - | - | - | - | - | - | - | - | - |
| Musculoskeletal abnormalities | + | - | + | + | + | + | + | + | + | + |
| - Short webbed neck | + | - | + | + | + | + | + | + | + | + |
| - Cubitus valgus | - | - | - | - | + | + | + | + | + | + |
| - Hyperextensible joints | - | - | - | - | - | - | - | - | - | + |
| - Pectus anomalies | - | - | + | + | + | + | + | + | + | + |
| Undescended testes | + | - | - | + | + | + | + | + | + | + |
| CNS involvement | - | + | - | + | - | - | - | - | - | + |
| - Mental retardation | - | + | - | - | - | - | - | - | - | + |
| - Hyperactivity | - | + | - | - | - | - | - | - | - | + |
| - CNS abnormalities | - | - | - | - | - | - | - | - | - | + |
| - Seizures/EEG abnormalities | - | - | - | - | - | - | - | - | - | + |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS11 | NS12 | NS13 | NS14 | NS15 | NS16 | NS17 | NS18 | NS19 | NS20 |
|-------------------------------|------|------|------|------|------|------|------|------|------|------|
| Congenital heart defects | + | + | + | + | + | + | + | + | + | + |
| - Pulmonic stenosis (PS) | - | - | + | + | + | + | + | + | + | + |
| - Pulmonary valve dysplasia | - | - | - | - | + | - | - | - | + | - |
| - Mitralic valve dysplasia | - | - | - | - | + | - | - | - | - | - |
| - Supravalvular PS | - | - | - | - | - | - | - | - | - | - |
| - Atrial septal defect | - | - | + | - | - | - | - | + | + | + |
| - Hypertrophic cardiomyopathy | - | - | - | - | - | - | - | - | + | - |
| - Atrioventricular canal | - | - | - | - | - | - | - | - | - | - |
| - Tetralogy of Fallot | - | - | - | - | - | - | - | - | - | - |
| - Ventricular septal defect | - | + | - | - | - | - | - | - | + | - |
| - others | + | + | - | - | - | - | - | - | - | - |
| Skin features | - | - | + | + | + | + | - | + | - | - |
| - Dark skin | - | - | + | - | - | - | - | - | - | - |

| Case | NS11 | NS12 | NS13 | NS14 | NS15 | NS16 | NS17 | NS18 | NS19 | NS20 |
|-------------------------------|------|------|-------|------|---------|------|---------|-------|----------|-------|
| - palmo/plantar crease | - | - | + | + | - | - | - | + | - | - |
| - Pruritus | - | - | - | + | - | - | - | - | - | - |
| - others | - | - | +(SK) | - | +(DrSk) | +(K) | - | - | - | - |
| Ectodermal features | + | + | + | + | + | + | + | + | + | + |
| - Sparse/absent scalp hair | + | - | + | + | + | - | - | - | - | - |
| - Thin hair | + | + | + | + | + | - | - | + | + | + |
| - Curly hair | - | + | + | + | + | + | - | - | - | + |
| - Sparse eyebrows | + | + | + | + | + | + | + | + | + | + |
| - Keratosis pilaris faciei | - | - | + | - | - | + | - | - | - | - |
| - Thin dystrophic nails | - | - | - | - | + | - | - | - | - | - |
| Musculoskeletal abnormalities | + | - | + | + | + | + | + | + | + | + |
| - Short webbed neck | - | - | + | + | + | + | - | + | + | + |
| - Cubitus valgus | - | - | - | - | - | - | - | - | - | - |
| - Hyperextensible joints | - | - | + | + | + | - | - | + | - | - |
| - Pectus anomalies | + | - | + | + | - | + | +(PE) | +(PC) | +(PE) | - |
| Undescended testes | - | - | - | - | - | + | - | - | - | - |
| CNS involvement | - | - | - | - | - | - | - | + | - | - |
| - Mental retardation | - | - | - | - | - | - | - (***) | + | - (****) | - (^) |
| - Hyperactivity | - | - | - | - | - | - | - | - | - | - |
| - CNS abnormalities | - | - | - | - | - | - | - | - | - | - |
| Seizures/EEG abnormalities | - | - | - | - | - | - | - | - | - | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS21 | NS22 | NS23 | NS24 | NS25 | NS26 | NS27 | NS28 | NS29 | NS30 |
|-------------------------------|------|------|------|------|------|------|------|------|------|------|
| Congenital heart defects | - | - | + | + | + | + | + | - | + | + |
| - Pulmonic stenosis (PS) | - | - | + | + | - | + | - | - | + | + |
| - Pulmonary valve dysplasia | - | - | + | - | - | + | - | - | + | - |
| - Mitralic valve dysplasia | + | - | - | - | + | - | - | - | - | + |
| - Supravalvular PS | - | - | - | - | - | - | - | - | + | - |
| - Atrial septal defect | - | - | + | - | - | - | + | - | - | - |
| - Hypertrophic cardiomyopathy | - | - | - | - | - | - | - | - | - | - |
| - Atrioventricular canal | - | - | - | - | - | - | - | - | - | - |
| - Tetralogy of Fallot | - | - | - | - | - | - | - | - | - | - |

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| Case | NS21 | NS22 | NS23 | NS24 | NS25 | NS26 | NS27 | NS28 | NS29 | NS30 |
|-------------------------------|------|------|------|------|------|--------------|------|------|------|-------|
| - Ventricular septal defect | - | - | - | - | - | - | - | - | - | - |
| - others | - | - | - | - | AVD | MVP,AVDRCoTA | - | - | - | - |
| Skin features | + | + | + | - | - | - | + | + | + | + |
| - Dark skin | + | + | + | - | - | - | + | + | + | + |
| - Palmo/plantar crease | - | - | - | - | - | - | - | - | - | + |
| - Pruritus | + | - | - | - | - | - | - | - | - | + |
| - others | - | - | - | - | - | - | - | - | - | +(CA) |
| Ectodermal features | + | + | + | + | - | + | + | + | + | + |
| - Sparse/absent scalp hair | - | - | - | - | - | - | - | - | - | - |
| - Thin hair | - | - | - | - | - | - | - | - | - | - |
| - Curly hair | + | + | + | + | - | + | + | + | + | + |
| - Sparse eyebrows | + | - | + | - | - | + | + | - | + | + |
| - Keratosis pilaris faciei | + | - | + | - | - | + | - | - | - | - |
| - Thin dystrophic nails | - | - | - | - | - | - | - | + | - | - |
| Musculoskeletal abnormalities | + | + | + | + | + | + | + | + | + | + |
| - Short/webbed neck | - | + | - | - | + | + | + | - | + | + |
| - Cubitus valgus | - | - | - | - | - | + | - | + | + | - |
| - Hyperextensible joints | + | + | + | - | - | + | + | + | + | + |
| - Pectus anomalies | + | - | - | + | + | + | + | + | + | + |
| Undescended testes | + | - | + | + | + | - | - | + | - | + |
| CNS involvement | - | - | - | - | - | + | - | - | - | - |
| - Mental retardation | - | - | - | - | - | +(*****) | - | - | - | - |
| - Hyperactivity | - | - | - | - | - | + | - | - | - | - |
| - CNS abnormalities | - | - | - | - | - | - | - | - | - | - |
| - Seizures/EEG abnormalities | - | - | - | - | - | - | - | - | - | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS31 | NS32 | NS33 | NS34 | NS35 | NS36 | NS37 | NS38 | NS39 | Total |
|-----------------------------|------|------|------|------|------|------|------|------|------|-------------|
| Congenital heart defects | + | + | + | + | + | + | + | + | + | 35/39 (90%) |
| - Pulmonic stenosis (PS) | - | + | + | + | - | + | + | + | - | 27/39 (69%) |
| - Pulmonary valve dysplasia | + | + | + | - | - | + | + | + | - | 11/39 (28%) |
| - Mitralic valve dysplasia | + | - | - | - | - | - | - | - | + | 6/39 (15%) |
| - Supravalvular PS | - | + | + | - | - | + | - | - | - | 4/39 (10%) |

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| Case | NS31 | NS32 | NS33 | NS34 | NS35 | NS36 | NS37 | NS38 | NS39 | Total |
|-------------------------------|------|------|------|------|------|------|------|------|------|-------------|
| - Atrial septal defect | - | + | + | + | - | - | - | - | - | 10/39 (26%) |
| - Hypertrophic cardiomyopathy | + | - | - | - | + | - | - | - | - | 3/39 (8%) |
| - Atrioventricular canal | - | - | - | - | - | - | - | - | - | 0/39 (0%) |
| - Tetralogy of Fallot | - | - | - | - | - | - | - | - | - | 0/39 (0%) |
| - Ventricular septal defect | - | - | - | - | - | + | - | - | - | 5/39 (13%) |
| - others | - | - | - | - | - | - | - | - | - | |
| Skin features | + | + | + | - | + | + | + | - | - | 22/39 (57%) |
| - Dark skin | + | + | + | - | + | + | + | + | + | 18/39 (46%) |
| - palmo/plantar crease | - | - | - | - | + | - | - | + | - | 7/38 (18%) |
| - Pruritus | + | + | + | - | - | - | - | + | - | 8/36 (22%) |
| - others | | | | | | | | | + | (CA) |
| Ectodermal features | + | + | + | + | - | + | + | + | + | 35/39 (90%) |
| - Sparse/absent scalp hair | - | - | - | - | - | - | - | + | + | 7/39 (18%) |
| - Thin hair | - | - | - | - | - | - | - | + | + | 12/39 (31%) |
| - Curly hair | + | + | + | + | - | + | + | + | + | 27/39 (69%) |
| - Sparse eyebrows | - | + | + | - | - | + | + | + | + | 30/39 (77%) |
| - Keratosis pilaris faciei | + | + | + | - | + | + | + | + | - | 13/39 (33%) |
| - Thin dystrophic nails | - | - | - | - | + | - | - | + | - | 4/38 (10%) |
| Musculoskeletal abnormalities | + | + | + | + | + | + | + | + | + | 37/39 (95%) |
| - Short webbed neck | + | + | + | + | + | + | + | + | + | 31/39 (79%) |
| - Cubitus valgus | - | - | + | + | + | + | + | + | + | 16/37 (43%) |
| - Hyperextensible joints | + | + | + | - | - | - | - | + | - | 17/37 (46%) |
| - Pectus anomalies | + | + | + | + | + | + | + | + | + | 32/39 (82%) |
| Undescended testes | - | + | | + | + | | | | | 18/26 (69%) |
| CNS involvement | - | - | - | - | - | - | - | - | - | 5/38 (13%) |
| - Mental retardation | - | - | - | - | - | - | - | - | - | 4/39 (13%) |
| - Hyperactivity | - | - | - | - | - | - | - | - | - | 1/37 (3%) |
| - CNS abnormalities | - | - | - | - | AC | - | - | - | - | 2/37 (5%) |
| - Seizures/EEG abnormalities | - | - | - | - | - | - | - | - | - | 1/39 (3%) |

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Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS1 | NS2 | NS3 | NS4 | NS5 | NS6 | NS7 | NS8 | NS9 | NS10 |
|--------------------------------|-----|-----|---------|---------|---------|--------|-----|--------|-----|------|
| Ocular abnormalities | - | - | + (Sb) | + (Ast) | - | + (Sb) | - | + (My) | - | + |
| Gastrointestinal abnormalities | - | - | + (GER) | - | - | - | - | - | - | - |
| Renal abnormalities | - | - | - | - | - | - | - | - | - | - |
| Coagulation defects | - | - | - | - | + (VWD) | - | - | - | - | - |
| Mandibular giant cell lesions | - | - | - | - | - | - | - | - | - | - |
| Other tumors | - | - | - | - | - | - | - | - | - | - |
| Others | - | - | - | - | - | - | - | IH | - | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS11 | NS12 | NS13 | NS14 | NS15 | NS16 | NS17 | NS18 | NS19 | NS20 |
|--------------------------------|------|--------|------------|-----------|------|------|---------|----------------|-------------------|---------------|
| Ocular abnormalities | - | - | + (IC, My) | - | - | - | - | + (My, Ast,Sb) | - | - |
| Gastrointestinal abnormalities | - | - | - | - | - | - | - | - | - | + (GER solv.) |
| Renal abnormalities | - | + (BH) | + (IN) | - | - | - | + (ERP) | - | - | - |
| Coagulation defects | - | - | - | - | - | + | - | + (CPD) | + ^(##) | - |
| Mandibular giant cell lesions | - | - | - | - | - | - | - | - | - | + |
| Other tumors | - | - | - | - | - | - | - | - | - | - |
| Others | - | - | V, CoHipD | luDy, AbU | SFF | LTT | - | - | - | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS21 | NS22 | NS23 | NS24 | NS25 | NS26 | NS27 | NS28 | NS29 | NS30 |
|--------------------------------|-----------------|------|-------|------|------|------|------|------|------|------|
| Ocular abnormalities | - | - | - | - | - | - | + | - | - | - |
| Gastrointestinal abnormalities | - | - | - | - | - | - | - | - | - | - |
| Renal abnormalities | - | - | - | - | - | - | - | + | - | - |
| Coagulation defects | - | - | - | - | - | - | - | + | - | - |
| Mandibular giant cell lesions | + | - | - | - | - | - | - | - | - | - |
| Other tumors | abRMS, cGI, GCC | - | - | - | - | - | - | - | - | - |
| Other | N, L, HypThy | - | HTGum | - | - | - | - | - | - | - |

Supp. Table S3. Clinical features of *SOS1* mutation-positive subjects (continued)

| Case | NS31 | NS32 | NS33 | NS34 | NS35 | NS36 | NS37 | NS38 | NS39 | Total |
|--------------------------------|------|------|--------|------|------|------|------|------|------|-------------|
| Ocular abnormalities | - | - | + (Sb) | + | + | - | + | + | + | 13/39 (33%) |
| Gastrointestinal abnormalities | - | - | - | - | + | - | - | - | - | 3/38 (8%) |
| Renal abnormalities | - | - | - | - | - | - | - | - | - | 4/38 (10%) |
| Coagulation defects | - | + | - | - | + | + | - | + | - | 9/34 (26%) |
| Mandibular giant cell lesion | - | - | - | - | - | + | - | - | - | 3/39 (8%) |
| Other tumors | - | - | - | - | - | - | - | - | - | 1/39 (3%) |
| Others | - | - | - | - | - | ArS | - | - | - | |

abRMS= abdominal rhabdomyosarcoma; AbU= absence of uvula; AC= Arnold-Chiari malformation; Ar= arrhythmia; ArCy= arachnoid cyst; ArS= articular synovitis; Ast= astigmatism; AVD= aortic valve dysplasia; BH= bilateral hydronephrosis; CoHipD= congenital hip dysplasia; CA= Cavernous angiomata; cGl= cerebral glioma; CPD= coagulation-platelet dysfunction; DrSk= dry skin; DUT= Double urinary tract; ERP= enlarged renal pelvis; F= female; GCC= granular cell tumors; GER= gastroesophageal reflux; HypoGly= Hypoglycemia; HypThy= hypothyroidism; HTGum= Hypertrophic gums; IH= inguinal hernia; K= keloid; L= lentigines; lC= left cataract; lN= left nephrolithiasis; luDy= 1st lumbar and 4th cervical vertebral dysmorphism; LV= left ventricle; LTT= large thumbs and toes; M= male; MVI= mitral valve insufficiency; MVP= mitral valve prolapse; My= Myopia; N= nevi; PC= pectus carenatum; PE= pectus excavatum; PP= pes planovalgus; RCoTA= right cor triatriatum; s= severe; Sb= strabismus; solv.= solved; SK= skin keloids; SFF= severe flat feet; TI= tricuspid insufficiency; V= valgism; VWD= von willebrand disease.

⁺Median value (range: 5 months to 40 years), [#]More prominent on the left eye; ^{##}Coagulation factors VII, IX, and XI deficiency, [^]First steps at 16-17 months of age; *The subject had hypoxia as a complication of the heart surgery; ** Developmental delay was documented, with unsupported walking reached at 18-20 months and first spoken words at 30 months. Cognitive development was impaired, with requirement of a full time special education setting. Poor fine motor skill was also reported; ***Speech delay; ****Wechsler Preschool and Primary Scale of Intelligence Total Intellectual Quotient = 81; at 4 years, according to the Griffith's scale, the General Quotient was border-line to the inferior normal limits, with impairment of linguistic skills and oculo-manual coordination; *****Cognitive deficits were secondary to surgery complication during the neonatal period (IQ score= 44, WISC-R scale).