Article

Steric Clash in the SET Domain of Histone Methyltransferase NSD1 as a Cause of Sotos Syndrome and Its Genetic Heterogeneity in a Brazilian Cohort

Kyungsoo Ha 1,2, Priya Anand 3, Jennifer A. Lee 4, Julie R. Jones 4, Chong Ae Kim 5, Debora Romeo Bertola 5, Jonathan D. J. Labonne 1, Lawrence C. Layman 4,6, Wolfgang Wenzel 3 and Hyung-Goo Kim 1,6,*

1 Section of Reproductive Endocrinology, Infertility & Genetics, Department of Obstetrics and Gynecology, Department of Neurosciences and Regenerative Medicine, Medical College of Georgia, Augusta University, 1120 15th Street, Augusta, GA 30912, USA; kyungsooha1@gmail.com (K.H.); molgenetics_and_epigenetics@hotmail.com (J.D.J.L.); lalayman@augusta.edu (L.C.L.)
2 New drug development center, Osong Medical Innovation Foundation, 28160 Cheongju, Korea
3 Institute of Nanotechnology (INT), Karlsruhe Institute of Technology (KIT), 76344 Karlsruhe, Germany; priya.anand@kit.edu (P.A.); wolfgang.wenzel@kit.edu (W.W.)
4 Molecular Diagnostic Laboratory, Greenwood Genetic Center, Greenwood, SC 29646, USA; jalee@ggc.org (J.A.L.); julie.jones@ggc.org (J.R.J.)
5 Unit of Clinical Genetics, Instituto da Criança, FMUSP, São Paulo 05403-000, Brazil; chong.kim@hc.fm.usp.br (C.A.K.); debora.bertola@hc.fm.usp.br (D.R.B.)
6 Neuroscience Program, Medical College of Georgia, Augusta University, 1120 15th Street, Augusta, GA 30912, USA
* Correspondence: hkim@augusta.edu; Tel.: +1-706-721-5764; Fax: +1-706-721-0340

Academic Editor: Roel Ophoff
Received: 1 August 2016; Accepted: 21 October 2016; Published: 9 November 2016

Abstract: Most histone methyltransferases (HMTase) harbor a predicted Su(var)3–9, Enhancer-of-zeste, Trithorax (SET) domain, which transfers a methyl group to a lysine residue in their substrates. Mutations of the SET domains were reported to cause intellectual disability syndromes such as Sotos, Weaver, or Kabuki syndromes. Sotos syndrome is an overgrowth syndrome with intellectual disability caused by haploinsufficiency of the nuclear receptor binding SET domain protein 1 (NSD1) gene, an HMTase at 5q35.2–35.3. Here, we analyzed NSD1 in 34 Brazilian Sotos patients and identified three novel and eight known mutations. Using protein modeling and bioinformatic approaches, we evaluated the effects of one novel (I2007F) and 21 previously reported missense mutations in the SET domain. For the I2007F mutation, we observed conformational change and loss of structural stability in Molecular Dynamics (MD) simulations which may lead to loss-of-function of the SET domain. For six mutations near the ligand-binding site we observed in simulations steric clashes with neighboring side chains near the substrate S-Adenosyl methionine (SAM) binding site, which may disrupt the enzymatic activity of NSD1. These results point to a structural mechanism underlying the pathology of the NSD1 missense mutations in the SET domain in Sotos syndrome. NSD1 mutations were identified in only 32% of the Brazilian Sotos patients in our study cohort suggesting other genes (including unknown disease genes) underlie the molecular etiology for the majority of these patients. Our studies also found NSD1 expression to be profound in human fetal brain and cerebellum, accounting for prenatal onset and hypoplasia of cerebellar vermis seen in Sotos syndrome.

Keywords: Sotos syndrome; NSD1; SET domain; histone methyltransferase; intellectual disability
1. Introduction

Sotos syndrome (MIM 117550), also known as cerebral gigantism with intellectual disability and delayed development of motor skills, was initially described in 1964, and hundreds of cases have been subsequently reported [1–5]. Most reported cases have been sporadic, but occasional familial cases have demonstrated that Sotos syndrome is also autosomal dominant [6,7]. Because of the phenotypic overlap, Sotos syndrome is sometimes difficult to distinguish from several other clinical disorders such as Weaver syndrome (MIM 277590), Bannayan–Riley–Ruvalcaba syndrome (MIM 153480) and Beckwith–Wiedemann syndrome (MIM 130650) [8]. Therefore, identification of a causative gene was necessary for the precise genetic diagnosis and understanding of the molecular and physiological basis of Sotos syndrome.

In 2002, positional cloning in a Sotos syndrome individual with a balanced translocation, t(5;8)(q35;q24.1), resulted in the identification of a nuclear receptor binding SET domain protein 1 (NSD1) located at the 5q35 breakpoint as a causative gene, and early studies have shown that haploinsufficiency of NSD1 is the underlying cause of this syndrome [9,10]. To date, over 400 different mutations in NSD1 associated with Sotos syndrome have been reported. Various mutations abrogating NSD1 function include missense mutations, partial gene deletions, microdeletions and truncating mutations resulting from small nucleotide insertions, deletions or splice-site mutations [3,4,11,12]. More than 80% of patients with Sotos syndrome carry NSD1 mutations, whereas for 20% of phenotypically characterized cases with Sotos syndrome underlying aberrations in the NSD1 gene have not been detected [5,13]. In particular, partial or whole NSD1 gene 5q35 microdeletions are the most common cause of Sotos syndrome in Japanese patients. In non-Japanese patients, however, 5q35 microdeletions are uncommon, accounting for only 10% of affected individuals [4,11,14,15].

NSD1 encodes a protein containing multiple functional domains including an SET [Su(var)3–9, Enhancer-of-zeste, Trithorax] domain, which is required for histone methyltransferase (HMTase) activity and thus present in most HMTases [16–18]. NSD1 also apparently contains five zinc finger-like plant homeodomain (PHD) domains and two proline-tryptophan-tryptophan-proline (PWWP) motifs, both of which are involved in protein–protein interactions [19,20]. However, the number of functional domains in NSD1, especially the PHD domain, has not been clearly defined yet [13,19,21,22]. NSD1 is a member of mammalian histone lysine methyltransferases that play important roles in multiple aspects of development and disease by acting as a transcriptional intermediary factor capable of both negatively and positively influencing transcription of nuclear receptors, such as the estrogen, retinoic acid and thyroid hormone receptors, depending on the cellular context [22–25].

Most NSD1 missense mutations are present in the functional domains of NSD1, and to date a total of 21 missense mutations have been identified in the SET domain [4,5,11,13,26–29]. Although the SET domain has a crucial role in NSD1 function, the mechanisms by which these mutations result in loss-of-function have been poorly understood. In this study, we performed a systematic screening for NSD1 mutations to evaluate the range and contribution of different NSD1 aberrations to the spectrum of associated clinical features in Brazilian Sotos patients. In addition, using protein modeling and bioinformatic approaches, we investigated the impact of all reported missense mutations in the SET domain of the NSD1 protein. This study suggests that mutations in the SET domain of NSD1 may disrupt its HMTase activity by changing protein stability and interfering with its ability to bind a ligand.

2. Materials and Methods

2.1. Patients

A total of 34 Brazilian patients with Sotos syndrome were analyzed in this study. Each patient had been examined by a clinical geneticist or experienced pediatrician and satisfied strict criteria for a diagnosis of Sotos syndrome including facial features, macrosomia and neurological findings. We also checked the internal whole exome sequencing database of 600 healthy Brazilian controls to confirm
that the mutations we found are not polymorphisms. For the screening of familial inheritance, parental and sibling DNA samples from seven families were also analyzed to see whether the novel mutations in four patients are de novo or inherited. Written informed consent forms were obtained from all involved patients. This study was approved by the Institutional Review Board of Augusta University and Hospital das Clínicas-São Paulo (CAPPesq number 0371/2010).

2.2. Genomic DNA Extraction

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods of phenol/chloroform extraction and ethanol precipitation [30].

2.3. NSD1 Mutation Analysis

For each patient, exons 2 through 23 of the *NSD1* gene were amplified by polymerase chain reaction (PCR) using approximately 100 ng of genomic DNA and 2× AmpliTaq Gold® PCR Mastermix (Life Technologies, Carlsbad, CA, USA) in a 25 µL reaction volume. PCR conditions were as follows: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s; 72 °C for 5 min. Primer sequences are listed in Table S1 in Supplementary Materials. PCR products were purified using USB® ExoSAP-IT® reagent (Affymetrix, Santa Clara, CA, USA) following the manufacturer’s protocol, and sequenced using M13 primers and BigDye® Terminator chemistry version 3.1 (Life Technologies) in a 10 µL reaction volume. DNA sequencing reaction conditions were as follows: 96 °C for 1 min; 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 1 min 15 s. Each product was then purified using either the Agencourt® CleanSEQ® dye terminator removal protocol on a Biomek® FXP workstation (Beckman Coulter, Brea, CA, USA) or the EdgeBio Performa® DTR V3 96-Well Short Plates method of dye terminator removal. Products were sequenced by the Sanger dideoxynucleotide method using an Applied Biosystems 3730XL DNA Analyzer instrument (Life Technologies). Sequence reads were aligned to reference sequence NM_022455.4 and analyzed for DNA sequence changes using Sequencher® software version 4.9 DNA analysis software (Gene Codes Corporation, AnnArbor, MI, USA) and Sequence Pilot version 4.2.2 (JSI Medical Systems, Ettenheim, Germany) software. Any identified sequence alterations were queried against the Single Nucleotide Polymorphism database (dbSNP; NCBI; http://www.ncbi.nlm.nih.gov/SNP/) and the Human Gene Mutation Database (HGMD® Professional; BIOBASE Biological Databases; https://portal.biobase-international.com).

2.4. Reverse Transcription-quantitative PCR (RT-qPCR)

Primers targeting *NSD1* exons 5 were designed for RT-qPCR (Table S2. Total RNA from whole human brain, fetal brain and from an additional 11 different central nervous system (CNS) regions (Clontech, Mountain View, CA, USA) were used to investigate *NSD1* transcripts levels. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the RevertAid First cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). RT-qPCR assays were performed using 2 µL cDNA, 2.5 µM primer and 10 µL FastStart DNA Green Master (Roche, Indianapolis, IN, USA) in a 20 µL reaction volume.

2.5. Protein Modeling and Bioinformatics Analysis

Molecular Dynamics (MD) simulations were carried out using the program package GROMACS 4.5.4. [31]. The force field OPLS-AA [32] was used in all MD simulations. The crystal structure of the native SET domain (PDB ID: 3OOI) and the I2007F model (generated using MOE (Molecular Operating Environment, 2013.08; Chemical Computing Group Inc., Montreal, QC, Canada) without S-Adenosyl methionine (SAM) and histone peptide were used as a starting point for MD simulations. The native and mutant proteins were solvated with Simple Point Charge (SPC) water molecules [33]. Periodic boundary conditions were applied in an isothermal–isobaric (NPT) ensemble simulation. The system was neutralized by adding sodium ions and the temperature was controlled using a Berendsen thermostat [34] with a coupling time of 0.2 ps. The minimized system was equilibrated.
for 1000 ps each at 300 K in a position restrained molecular dynamics simulation in order to solvate the proteins. The equilibrated systems were then subjected to molecular dynamics simulations for 150 ns each at 300 K. From the trajectory we computed the distance between selected amino acids, which we plotted using the GRaphing, Advanced Computation and Exploration (GRACE) program (http://plasma-gate.weizmann.ac.il/Grace).

Some information regarding the effect of point mutations on the NSD1 protein function could already be obtained at the sequence level using silico approaches. We used in silico tools including I-Mutant 2.0 [35], SPPIDER [36], Polyphen [37], PANTHER [38] and SIFT [39] (on all the mutations in Table 4) to calculate the relative residue stability constants for the wild-type (WT) protein and its mutant forms. These methods are trained on existing sets of mutations/phenotype association data and use sequence homologs, structural information, such as solvent accessible surface area and changes in amino acid properties, to provide information as input to machine learning methods for phenotype prediction.

3. Results

We first investigated the domain structure of NSD1 using bioinformatic approaches. The NSD1 gene encodes a protein containing three PHD domains at amino acid positions 1543–1589, 1707–1751 and 2120–2160, predicted with high confidence using Pfam [40] and National Center for Biotechnology Information Basic Local Alignment Search tool (NCBI–BLAST) [41]. The SMART [42] server also predicts putative PHD domains at positions 1590–1639 and 1640–1693. However, a putative PHD domain at position 2164–2205 reported previously [19] was not found with this approach. The bioinformatics approach confirmed the existence of other domains, including one SET domain at amino acid position 1942–2065, two PWWP domains at positions 323–388 and 1756–1818, one associated with SET (AWS) domain at position 1890–1940 and one Post–SET domain at position 2066–2082, as previously reported (Figure 1A,B and Table 1) [19]. Three nuclear localization signals (NLSs) are located at amino acid positions 512–529, 1157–1174 and 1471–1488 [21].

Assaying the expression levels of NSD1 mRNA in various regions of the brain by RT-qPCR revealed that the gene is highly expressed throughout the brain. Approximately 60-fold higher expression was detected in the cerebellum, while ~30-fold higher transcript levels were recorded in the insula, parietal lobe and hippocampus compared to lymphocytes. Fetal brain showed ~75-fold higher transcript levels relative to lymphocytes. The lowest levels of NSD1 transcripts were detected in the dorsal root ganglion and spinal cord (Figure 2).

| Domains | Amino acid Positions |
|---------|---------------------|
| PWWP1   | 323–388             |
| NLS1    | 512–529             |
| NLS2    | 1157–1174           |
| NLS3    | 1471–1488           |
| PHD1    | 1543–1589           |
| PHD2    | 1590–1639           |
| PHD3    | 1640–1693           |
| PHD4    | 1707–1751           |
| PWWP2   | 1756–1818           |
| AWS     | 1890–1940           |
| SET     | 1942–2065           |
| Post-SET| 2066–2082           |
| PHD5    | 2120–2160           |

PWWP: proline-tryptophan-tryptophan-proline domain; NLS: nuclear localization signal; PHD: plant homeodomain domain; AWS: associated with SET domains; SET: Su(var)3–9, Enhancer-of-zeste, Trithorax domain.
**Figure 1.** The location of three novel mutations in nuclear receptor binding SET domain protein 1 (NSD1). (A) Exonic structure of NSD1 (NM_022455.4) with three novel mutations identified in Brazilian Sotos syndrome patients. Open and gray boxes indicate coding exons as well as the 5’ and 3’ untranslated regions, respectively. Specific functional domains are indicated by colored boxes, and positions of nuclear localization signals were from a previous report [21]. Figure is not to scale.

**Figure 2.** NSD1 expression pattern in various regions of the brain. Transcripts of NSD1 were detected in many regions of the brain including the hippocampus. The highest transcript levels were detected in fetal brain (~75-fold), while the cerebellum also showed high levels of transcript (~60-fold) relative to lymphocytes. NSD1 transcripts in the dorsal root ganglion and spinal cord were much lower compared to other regions assayed.
Genomic DNAs samples from 34 Brazilian patients with phenotypes of Sotos or Sotos-like syndrome as well as DNA samples from unaffected members of seven unrelated families were screened for intragenic NSD1 mutations by Sanger sequencing analysis. We identified NSD1 mutations in 11 out of 34 patients, including three novel mutations (Table 2). In our patient DGDP186, one novel nonsense mutation (c.5004C>A; NM_022455.4) was identified in exon 14 resulting in the substitution of a tyrosine by a premature stop codon TAA at amino acid position 1668 in the third PHD domain (p.Y1668X; NP_071900.2, Figure 1A, Table 2). This substitution results in the premature truncation of the NSD1 protein with the deletion of C-terminal functional domains of NSD1 including the PWWP2, AWS, SET and PHD domains 3–5 (Figure 1B). Parental DNA analysis demonstrated that the c.5004C>A (p.Y1668X) mutation in this patient is de novo.

| Patient | DGDP186 (Male) | DGDP 291 (Female) | DGDP306 (Female) |
|---------|----------------|-------------------|-----------------|
| Age at diagnosis | 9 months | 2 years 7 months | 1 year 5 months |
| Current age | 10 years | 23 year | 5 years |
| Nucleotide change (NM_022455.4) | c.5004C>A | c.6019A>T | c.2699delC |
| Exon | 14 | 20 | 5 |
| Amino acid change (NP_071900.2) | p.Y1668X | p.I2007F | p.P900LfsX12 |
| Familial analysis | de novo | Both mother and brother with no variant, father unavailable | de novo |
| Brazilian control analysis | not found in 600 controls | not found in 600 controls | not found in 600 controls |

**Developmental Milestone**

| Milestone | DGDP186 (Male) | DGDP 291 (Female) | DGDP306 (Female) |
|-----------|----------------|-------------------|-----------------|
| Prenatal overgrowth | − | − | − |
| Excessive growth velocity | + | + | + |
| Advanced bone age | − | + | + |
| Large hands and feet | − | + | + |
| Developmental delay | Mild | Mild | Mild |
| Lack of fine motor control | − | − | − |
| Walking at | 22 months | 24 months | 23 months |
| Speaking | Delayed | Delayed | Delayed |
| Delayed learning ability | − | + | N/A |

**Facial Dysmorphism**

| Dysmorphism | DGDP186 (Male) | DGDP 291 (Female) | DGDP306 (Female) |
|-------------|----------------|-------------------|-----------------|
| Macrocephaly | + | + | + |
| Prominent forehead | + | + | + |
| High-arched palate | + | − | + |
| Pointed chin | + | + | + |

**Other Features**

| Feature | DGDP186 (Male) | DGDP 291 (Female) | DGDP306 (Female) |
|---------|----------------|-------------------|-----------------|
| Hypotonia | + | + | + |
| Seizures | − | − | − |
| Scoliosis | + | + | − |
| Enlarged ventricles | + | N/A | + |
| Artrial septal defect | − | + | − |
| Pulmonary stenosis | − | + | − |

N/A, not applicable.
One missense mutation (c.6019A>T, p.I2007F) was identified in patient DGDP291 (Figure 1A, Table 2). This alteration occurs within the highly conserved SET domain (Figure 1B), where it may have critical effects on the histone methyltransferase activity of NSD1. Paternal DNA was unavailable for analysis; however, the familial genetic analysis including the healthy mother and unaffected brother revealed that this mutation was likely de novo, and this mutation was not present in the other 34 patients or the more than 600 Brazilian control individuals. This change is also not present in the Exome Aggregation Consortium (ExAC) database [43]. Analysis of its position within NSD1 provides further evidence supporting a pathogenic role for this missense mutation. This alteration occurs at amino acid residue 2007 within the functional domain that is conserved not only in other species such as mice, rat and chicken, but also in the known human paralogs of NSD1, NSD2 and NSD3 (Figure 3).

In another patient, DGDP306, a one base-pair deletion in exon 5 of the NSD1 gene (c.2699delC) was identified which leads to a frameshift mutation resulting in the premature termination of protein translation at amino acid 911 (p.P900LfsX12; Figure 1A, Table 2). The remaining protein fragment contains only the N-terminal region of the NSD1 protein, including one functional PWWP domain (Figure 1B). This may lead to loss-of-function of the protein causing Soto syndrome. Analysis of parental samples indicated that the mutation had occurred de novo. Since two of the novel mutations were predicted to result in premature truncation of the NSD1 protein because of either nonsense or frameshift mutations, these two mutations are considered to be pathogenic.

Additionally, eight previously-reported mutations including three frameshift mutations (p.S985CfsX25 [13], p.K1002EfsX8 [44] and p.K1580NfsX62 [45]), two nonsense mutations (p.R632X [1] and p.Q1989X [4]), one splice-site mutation (c.5892+1G>T, exon 19 skipping [5]) and two missense mutations (p.L1917P [46] and p.R2017Q [11]) were identified in our Sotos patients. Among them, two mutations, c.4740delA and c.5892+1G>T, were de novo in patients DGDP173 and DGDP176, respectively, whose parental samples were available. The nomenclature of the previously reported frameshift mutation p.K1002EfsX8 [44] has been corrected herein to p.K1002EfsX8. We also observed 16 polymorphisms (Table S3). These were considered nonpathogenic, since they were already identified in multiple individuals including those in healthy populations. Since exons 5 (2560 bp) and 23 (1628 bp without 3’-untranslated region (UTR)) are very large, a number of polymorphisms have been identified in these two exons. The polymorphisms we identified in our patients were also located only in exons 5 and 23, except for one single nucleotide polymorphism (SNP) rs79098301 in intron 17 (Table S2).
Interestingly, one synonymous polymorphism rs28580074 in exon 23 (c.6829T>C, p.L2277L) was observed in all of our 34 Sotos patients, of which 26 patients were homozygous for this change.

The clinical features of the patients with novel mutations are summarized in Table 2. Our patient DGDP186 with a novel nonsense mutation has a typical facial gestalt in association with a variable number of additional major criteria including overgrowth, developmental delay and facial dysmorphism (Figure 4A,B). Although this patient did not show advanced bone age, one of the major diagnostic criteria for Sotos syndrome, his other phenotypic features satisfy other major diagnostic criteria such as macrocephaly, post-natal overgrowth and delayed milestones. Another patient DGDP306 with a novel frameshift mutation also has the typical facial dysmorphisms observed in other Sotos patients. The neurologic involvement seems to be predominant in this patient characterized by hypotonia, seizures and increased subarachnoid spaces. This girl had a markedly advanced bone age (her bone age was three years six months at one year eight months of age). This patient shows excessive growth velocity compared to other control populations at the same age. In addition, her developmental milestones are delayed. The third Sotos patient DGDP291 with a novel NSD1 mutation also shows a typical Sotos phenotype including post-natal overgrowth, developmental delay and facial gestalt (Figure 4C,D). She has advanced bone age and macrocephaly as well as delayed learning ability. The phenotypic features of other Sotos patients with reported NSD1 mutations are also summarized in Table 3.

![Figure 4](image_url)

**Figure 4.** General features in Brazilian Sotos syndrome patients with NSD1 mutations. (A,B) Facial features of DGDP186 at age eight years with macrocephaly, prominent forehead, ocular hypertelorism, downsloping palpebral fissures and pointed chin; and (C,D) facial features of DGDP291 at age 21 years with macrocephaly, prominent forehead, ocular hypertelorism, downsloping palpebral fissures and pointed chin.
Table 3. Clinical features in Brazilian Sotos patients with known NSD1 mutations.

| Patient | DGDP168 | DGDP171 | DGDP173 | DGDP174 | DGDP176 | DGDP180 | DGDP183 | DGDP305 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Age at diagnosis | 7 years | 14 years 3 months | 8 years 3 months | 9 months | 1 years 6 months | 1 years 9 months | 7 years | 2 years 8 months |
| Current age | 25 years | 27 years | 18 years | 13 years | 20 years | 15 years | 18 years | 7 years |
| Nucleotide change (NM_022455.4) | 3004_3005 delAA | 5750T>A | 4740delA | 6050G>A | 5892+1G>T | 2954_2955 delCT | 1894C>T | 5905C>T |
| Exon or intron | 5 | 18 | 12 | 20 | Intron 19 | 5 | 5 | 19 |
| Amino acid change (NP_071900.2) | p.K1002EfsX8 | p.L1917P | p.K1580NfsX62 | p.R2017Q | Exon 19 skipping | p.5985CfsX25 | p.R632X | p.Q1989X |

Developmental Milestone

- Prenatal overgrowth: + + + + + + + +
- Excessive growth velocity: + + + + + + + +
- Advanced bone age: + + + + + + + +
- Large hands and feet: + + + + + + + +
- Developmental delay: Mild Mild Mild Mild Mild Mild Mild Mild
- Lack of fine motor control: + + + + + + + +
- Walking at: 15 months 24 months 18 months 20 months 17 months 20 months Delayed 20 months
- Speaking: Delayed Delayed Normal Delayed Delayed Delayed Delayed Normal
- Delayed learning ability: + + + + + + + +

Facial Dysmorphism

- Macrocephaly: + + + + + + + +
- Prominent forehead: + + + + + + + +
- High-arched palate: + + + + + + + +
- Pointed chin: + + + + + + + +

Other features

- Hypotonia: + + + + + + + +
- Seizures: − − − − − − − −
- Scoliosis: − − − − − − − −
- Enlarged ventricles: + + + + + + + +
To explore the effect of I2007F point mutation in the SET domain of the protein encoded by NSD1, we generated a mutant structural model using sequence alignment (ClustalW) [47] based on the homology to the human histone-lysine N-methyltransferase NSD1 SET domain (PDB ID: 3OOI) [48] (116 amino acids; spanning the amino acid sequence 1942–2063) as template protein. Figure 5A shows the model for the SET domain which is composed of three β-sheets (yellow) arranged in a triangular fashion and one long helix (red) exposed to the outside. The novel mutation p.I2007F is predicted to be non-tolerable using SIFT server [39,49–52] and is predicted to decrease the stability of the protein with a negative DDG (delta delta G/free energy change) value of −1.65. This mutation is located in the evolutionarily fully conserved SET domain, with both WT and mutant residues interacting with surrounding amino acids. Mutation of this invariant residue to phenylalanine likely causes steric clashes with neighboring side chains, as phenylalanine is bulkier than isoleucine, and can affect the SAM ligand-binding pocket. To verify this assumption, we performed MD simulations on the wild type SET domain of the NSD1 protein and the protein with the p.I2007F alteration to predict the conformational change induced by the mutation in and around the ligand binding site. Figure 5B (black line) illustrates the distance D1 between native p.R1952 and p.I1993 residues in the ligand binding pocket and Figure 5C (black line) illustrates the distance D2 between native p.D2002 and p.T2038 residues around the ligand-binding pocket. The WT protein maintains a distance in the range of 10.3–16.9 Å for D1 (D1 (average) = 10.48 ± 1.88 Å) and distance in the range of 10.8–15.3 Å (D2 (average) = 12.60 ± 0.82 Å) for D2. We also measured the distances between these positions in the crystal structure of the human histone-lysine N-methyltransferase NSD1 SET domain (PDB ID: 3OOI) which was D1 = 13.8 Å and D2 = 8.2 Å. In the p.I2007F mutant the distance D1 shrinks to a range of 3.4–13.7 Å (D1 (average) = 9.79 ± 1.97 Å) and the D2 to a range of 3–7 Å (D2 (average) = 6.67 ± 1.22 Å). Substitution of isoleucine to phenylalanine reduces the size of the binding pocket that can strongly affect the ligand/histone peptide-binding properties. We also performed principal component analysis (PCA) on the trajectories. The PCA scatter plot for the WT and I2007F mutant in Figure 6A,B shows a significant difference in the motion of both systems as evident from the characteristic structures plotted along the direction of two principal components. From the PCA scatter plot it is clear that the eigenvectors computed from the MD trajectories for both the systems, the WT and p.I2007F mutant (Figure 6), differ significantly. This indicates the difference in protein motion between the WT and the mutant protein. The most pronounced flexibility observed in the WT protein was around residues I2007–M2020 (loop), which flips the residues K2013 and Y2016 towards the SAM binding pocket. The residues R2018 and M2020 in this loop region are known to interact with K20 of H4 peptide through hydrogen bonding [53] thus stabilizing the complex. A similar conserved hydrogen bonding is observed amongst all lysine-HMTases (H4K20-SETD8, H3K4-SETD7, H3K27-vSET, GLP and Dim-5). However, this motion is not observed in the pI2007F mutant, the significant structural motion observed in the mutant occurs in the vicinity of the K2031-G2041 loop. This is also supported by the difference in the distance between residues D2003–T2038 in the Figure 5C. From the analysis on WT and I2007F mutant, it is clear that the p.I2007F mutation causes changes in the conformational state of the protein and the motion observed during the simulation, suggest change in and around the binding pocket in the mutant state (closed state) as compared to the open state in WT protein. It is likely that the loop flipping around residues I2007–R2017 (loop) might participate in the H4 peptide binding to the SET domain. However, in our simulations, the SAM co-factor and H4 peptide are not included in the complex, so these interactions are not possible to observe.
Figure 5. Inhibitory effect of the novel mutation, p.I2007F on ligand-binding properties. (A) Structural model for the human NSD1 SET domain protein (1946–2063 amino acids) in complex with S-Adenosyl methionine (SAM) (PDB ID: 3O0I). Color code: red indicates helix; yellow specifies strands; green signifies loops; and blue represents positions of the missense mutation sites including our novel mutation p.I2007F in Table 4. Residues p.R1952, p.Y1997, p.R2017, p.N2020, and p.H2021 interact with SAM, which is bound in a pocket formed by the SET domain. The SAM binding site is also labeled. Six mutations (p.I2007F, p.R2017Q, p.R2017W, p.H2021R, p.C2027R and p.G2041D) which might cause steric clashes with neighboring amino acid side chains are also shown in blue and underlined; (B,C) Molecular Dynamics (MD) Trajectory analysis of the wild-type (WT) (in black) and p.I2007F mutant (in red); where X-axis is the length of the simulation in nanoseconds (ns) and Y-axis is the distance in nano-meter (nm); (B) Distance D1 between residues p.R1952 and p.I1993 in the ligand-binding site for native type (black line) and mutant model (red line). Substitution of phenylalanine shrinks the distance D1 from a range of 10.3–16.9 Å (D1 = 10.48 ± 1.88 Å) to a range of 3.4–13.7 Å (D1 = 9.79 ± 1.97 Å); (C) Distance D2 between residues p.D2002 and p.T2038 in the ligand-binding site for native type (black line) and mutant model (red line). Substitution of phenylalanine shrinks the distance D2 from a range of 10.8–15.3 Å (D2 = 12.60 ± 0.82 Å) to a range of 3.0–7.0 Å (D2 = 6.67 ± 1.22 Å). Therefore, the mutation from isoleucine to phenylalanine at amino acid position 2007 significantly reduces the size of the binding pocket that strongly affects ligand-binding properties.
A number of point mutations have been previously reported in the SET domain, including p.R1952W, p.G1955D, p.Y1997S, p.C2027R, p.G2041D, p.Y2058C [4], p.I1962I, p.R1984G [26], p.R1984Q, p.Y1997C, p.R2017W [27], p.Y1997H, p.N2020S [13], p.R2005Q, p.R2017Q [11], p.A2009V, p.H2021R, p.C2027Y, p.W2032L [5], p.R2039C [29] and p.T2055I [28]. Figure 5A shows the positions of the point mutations in the SET domain model. We performed a bioinformatics-based analysis using SPPIDER [36], PolyPhen2 [37] and SIFT [49] on the effect of all of these mutations (Table 4). We also used the HMM based evolutionary approach PANTHER to investigate the impact of point mutations on protein function [38]. Using the HMM based evolutionary approach, 18 out of 22 point mutations were designated as deleterious with subPSEC (substitution position-specific evolutionary conservation) scores in the range of −9.24 to −3.04. Four other mutations exhibited a subPSEC score in the range of −1.96 to −2.97. The structural model of the human SET domain (PDB ID: 3OOI) allowed us to observe the interactions of residues p.R1952, p.Y1997, p.R2017, p.N2020, p.N2065 and p.H2021 with the SAM [48], which is bound in a pocket formed by the SET domain (Figure 5A). SAM is a common co-substrate involved in methyl group transfers in a number of biological processes, and changes in the structural stability due to mutations near the ligand-binding site could affect the interaction of the NSD1 protein with the ligand SAM [48]. In order to investigate this possibility, we generated homology models of the SET domain with the 22 missense mutations listed in Table 4 (shown in Figure 5A, labeled as blue). Mutations p.I2007F, p.R2017W, p.R2017Q, p.H2021R, p.C2027R and p.G2041D may cause steric clashes with neighboring amino acid side chains or may affect the water-mediated hydrogen bond [54,55] with SAM, thus likely affecting the structural stability of the protein. The activity of four NSD1 mutations p.R1952W, p.R1984Q, p.R2017W and p.Y1997C in the SET domain was investigated [56]. For all four mutations, the results showed complete loss of the protein’s ability to methylate histone for all the four mutations, which is in accordance with the findings published for p.R1952W, p.R1984Q, and p.R2017Q [48]. Using SIFT analysis we also analyzed whether these missense mutations in the SET domain may affect the protein function based on sequence homology and the physical properties of amino acids. This tool calculates a score for every substitution and predicts the functional effect. All SET domain mutations were predicted to be non-tolerable and to affect protein function with scores < 0.05 (Table 4).
Table 4. Summary of the predictions of the bioinformatics tools PolyPhen 2, SPPIDER, SIFT, I-MUTANT 2.0 and PANTHER, with regard to the effect of point mutations in the NSD1 gene.

| AA Position | Polyphen2 (Predicted Impact) | PolyPhen2 (PSIC Score) | SPPIDER | SIFT Predicted Impact (Tolerance Index) | DDG Value Kcal/Mol | I-Mutant 2.0 Predicted Impact | PANTHER (subPSEC) |
|-------------|------------------------------|------------------------|---------|----------------------------------------|--------------------|------------------------------|-------------------|
| p.R1952W    | Probably damaging            | 0.999                  | Non-interacting | Intolerant (0.00)                      | 0.18               | Increase Stability            | −3.04478          |
| p.G1955D    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −1.47              | Decrease Stability            | −7.27103          |
| p.I1962T    | Possibly damaging            | 0.934                  | Non-interacting | Intolerant (0.00)                      | −0.95              | Decrease Stability            | −2.82147          |
| p.R1984G    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −0.93              | Decrease Stability            | −6.53802          |
| p.R1984Q    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −0.53              | Decrease Stability            | −6.59809          |
| p.Y1997H    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −0.95              | Decrease Stability            | −3.31488          |
| p.Y1997S    | Probably damaging            | 0.999                  | Non-interacting | Intolerant (0.00)                      | −0.70              | Decrease Stability            | −3.43065          |
| p.Y1997C    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −1.22              | Decrease Stability            | −4.51444          |
| p.R2005Q    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.01)                      | −1.13              | Decrease Stability            | −1.95697          |
| p.I2007F    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −1.65              | Decrease Stability            | −2.97211          |
| p.A2009V    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −1.25              | Decrease Stability            | −2.74529          |
| p.R2017Q    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −0.27              | Decrease Stability            | −6.4321           |
| p.R2017W    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | 0.02               | Increase Stability            | −8.09732          |
| p.N2020S    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −0.81              | Decrease Stability            | −6.32023          |
| p.H2021R    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.01)                      | 0.16               | Increase Stability            | −9.24832          |
| p.C2027R    | Possibly damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | 0.31               | Increase Stability            | −3.94723          |
| p.C2027Y    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −0.52              | Decrease Stability            | −4.23261          |
| p.W2032L    | Probably damaging            | 0.999                  | Non-interacting | Intolerant (0.001)                     | −1.03              | Decrease Stability            | −6.15612          |
| p.R2039C    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −1.58              | Decrease Stability            | −3.90311          |
| p.G2041D    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | −2.27              | Decrease Stability            | −3.7109           |
| p.T2055I    | Probably damaging            | 0.999                  | Non-interacting | Intolerant (0.00)                      | −0.97              | Decrease Stability            | −3.05772          |
| p.Y2058C    | Probably damaging            | 1.000                  | Non-interacting | Intolerant (0.00)                      | 0.27               | Increase Stability            | −7.73506          |

AA: amino acid, DDG: delta delta G/free energy change.
We further analyzed these mutations using PolyPhen2 [37], which uses information about the structure of the protein (hydrophobicity, charge effects and charges in molecular contacts) and multiple sequence alignment to predict the effect of mutations based on the Position Specific Independent Count Score difference. Based on the calculated alignment score and difference in structural parameters, PolyPhen2 designated all 22 missense variants as being “probably damaging” with a score of 1 or very close to it. In order to improve prediction accuracy of structure based tools, we also used the support vector machine based tool I-Mutant 2.0 [35], which predicts the effect of point mutations on protein stability. Seventeen missense mutations including p.I2007F are predicted to decrease stability with negative scores among 22 variants (Table 4). In addition we used SPPIDER [36] analysis to determine whether sites affected by these mutations are possible binding sites in the NSD1 sequence, as such sites are likely to affect protein function and signaling. Although SPPIDER did not predict any residue to be an interaction site (Table 4), I-Mutant 2.0 suggested that these mutations might increase or decrease the stability of the protein, indicating the deleterious effects of these mutations.

4. Discussion

Because of the phenotypic overlap with other groups of overgrowth syndromes, such as Weaver, Bannayan–Riley–Ruvalcaba and Beckwith–Wiedemann syndromes, Sotos syndrome may be challenging to diagnose [8]. Although the presence of the three major criteria and several other conditions associated with Sotos syndrome allow us to infer a clinical diagnosis of typical Sotos syndrome, the genetic analysis of the NSD1 gene is considered another criterion for the confirmation of the molecular diagnosis. NSD1 mutations are found in 70%–93% of classical cases of Sotos syndrome, but the molecular basis is unknown for remaining patients [11,14,15,57]. In our study, we have identified 11 different NSD1 mutations including three novel ones in 11 Brazilian Sotos patients. The prevalence of NSD1 mutations in our Brazilian Sotos patients was lower (32%, 11 out of 34 patients) compared to the frequency reported for other populations. Although the whole genome microarray did not identify any large 5q35 microdeletions involving the NSD1 gene in the 23 patients that were negative by NSD1 sequencing analysis, additional studies such as Multiplex Ligation-dependent Probe Amplification (MLPA) or targeted high density SNP array need to be performed to rule out the presence of smaller NSD1 deletions. Therefore, this low prevalence of NSD1 intragenic mutations in Brazilian Sotos patients is probably due to ethnic differences, a different frequency of the microdeletion, mutations in non-coding regions or mutations in other genes.

The mutations abrogating NSD1 function include missense mutations, nonsense mutations, small intragenic insertions/deletions, splicing defects and microdeletions, which are distributed over the entire gene including both coding and non-coding sequences [1,4,11]. Chromosomal microdeletions involving the NSD1 gene have been described as a major cause of Sotos syndrome in Japanese patients (prevalence >45%) [14], but are less frequently found in non-Japanese patients (approximately 10% of prevalence), where intragenic point mutations are highly prevalent [4,11,57]. The reason for this discrepancy is not known, but the presence of low-copy repeats (LCRs), highly homologous sequences, in regions flanking proximal and distal breakpoints of the common deletion suggests that LCRs may mediate the deletion [14]. A previous study identified one patient with total deletion of NSD1 and two patients with partial deletion of NSD1 among 30 Brazilian patients with the clinical diagnosis of Sotos syndrome [58], suggesting that the prevalence of microdeletions involving NSD1 is likely to be low in Brazilian Sotos patients. Among the 11 patients with 11 different NSD1 mutations in our study, three patients had NSD1 mutations, including one missense, one nonsense and one-frameshift mutation.

In agreement with previous studies in other populations, the phenotypic characterization of Brazilian Sotos patients with a mutant NSD1 genotype suggests that the most consistent diagnostic criterion in Sotos syndrome is represented by facial dysmorphism, in association with a variable number of additional major criteria [1,11,12,59]. As summarized in Tables 2 and 3, most NSD1 mutant Sotos patients exhibited a typical facial gestalt including macrocephaly (10/11, 91%), prominent forehead (11/11, 100%), ocular hypertelorism (11/11, 100%) and pointed chin (11/11, 100%). Other
frequent features among our patients with intragenic mutations were the presence of hypotonia (11/11, 100%), excessive growth velocity (10/11, 91%) and delayed learning ability (8/9, 89%). Advanced bone age, one of the major diagnostic criteria for Sotos syndrome, was not significant in five patients. In contrast, other criteria including seizures, heart defects and intellectual disability were inconsistent features, although most patients displayed delayed developmental milestones. We also tried to compare phenotypes in our Sotos patients to other reported cases with the same mutations. Due to the unavailability of the detailed phenotypic information of the reported cases with the same mutations identified by our study, however, it was not possible to establish a correlation between clinical features and the type of mutations. More data should be accumulated to explain appropriate genotype-phenotype correlations and ethnic difference in the prevalence of different NSD1 mutations.

NSD1 contains multiple functional domains including a zinc finger-like PHD domain, a PWWP motif and a SET domain. In an earlier study, NSD1 had been reported to contain six functional domains (PHD-1, PHD-2, PHD-3, PWWP-1, PWWP-2 and SET) [22]. However, a recent study proposed the existence of additional PHD domains (two canonical and one putative) in NSD1 [19]. Therefore, the number of functional domains in NSD1, especially the PHD domains, has not been clearly defined yet. Our bioinformatics analyses suggested that NSD1 contains five PHD domains (Table 1, Figure 1B). However, a putative PHD domain (amino acid positions 2164–2205) reported previously [19] could not be identified. Other functional domains including two PWWP motifs, one SET domain and one post-SET domain also appeared present in NSD1, and their locations highly overlap with previous reports [19,22].

NSD1 is a family member of histone methyltransferases and regulates the expression of target genes through the post-translational modification of histones. Thus, disruption of HMTase activity in NSD1 could have severe phenotypic consequences [60]. Studies have shown that NSD1 methylates on histone H3 lysine 36 (H3K36), H4 lysine 44 (H4K44) and H1.5 lysine 168 (H1.5K168) [24,61]. NSD1 has also been shown to activate nuclear factor-κB (NF-κB)-p65, a subunit of the NF-κB transcription complex through K218/K221 methylation. NF-κB is a central coordinator of immune and inflammatory responses [62]. Furthermore, translocations involving NSD1 have been found in patients with acute myeloid leukemia (AML) and breast cancer, indicating that NSD1 may function in the regulation of multiple downstream genes involved in various processes, such as bone morphogenesis, development and cancer [61,63]. A recent in vitro study showed that PHD domains in NSD1 bind mono-, di- or tri-methylated histones, H3K4 and H3K9, and point mutations in PHD domains would disrupt the transcriptional regulation of NSD1 by compromising the binding to these methylated lysines and to the transcription cofactor Nizp1 [19].

Twenty-one missense mutations have been previously reported in the SET domain [5,11,13,26–29,50] and one of the novel missense mutations we identified, c.6019A>T (p.I2007F), also occurred at a highly conserved amino acid in the SET domain (Figure 3). The SET domain is required for methyltransferase activity in all HMTases that are critical for genetic regulation at the epigenetic level [64,65]. Several different mechanisms have been proposed, but there has been no consensus regarding the understanding of the catalytic mechanism upon the transfer of the methyl group by the SET domain. However, the recently determined three-dimensional structures of the SET domains from chromosomal proteins support the notion that SAM and the lysine residue of the substrate histone tail must first be bound with a proper orientation in the catalytic pocket of the SET domain. Then, methyl transfer from SAM to the ε-amino of the target lysine likely proceeds by a direct in-line S_N2 nucleophilic attack through a nearby tyrosine residue [66–68]. Several different bioinformatics analyses suggested that all 22 missense mutations in the SET domain would have deleterious effects, which may be induced by changes in the functional activity of this domain, by changing protein stability or by interfering with the ability to bind to a ligand, among other factors. These data are in agreement with published results [48,56]. Specifically, we have investigated p.I2007F, where the side chain of phenylalanine is significantly larger than that of isoleucine, pushing neighboring side chains outwards, possibly into the binding pocket. As a result, the ligand may either completely or partially lose its
affinity with the protein, which would correspond to a significant loss-of-function of this domain. This computational result has yet to be confirmed by experimental data. However, SIFT analysis suggested that this mutation would affect the protein function, supported by molecular dynamics simulation results which revealed that it would significantly reduce the size of the binding pocket that strongly affects ligand-binding properties. This result is consistent with the previous observation that the Sotos mutation involving five arginine residues in the SET domain had greatly reduced H3K36 methyltransferase activities in vitro; some of them showed almost undetectable enzymatic activities [48]. Therefore, it may have significant deleterious effects on the methyltransferase activity of NSD1 resulting in altered gene expression patterns by the deregulation of histone modifications at H3K36 and H4K44 [48]. Moreover, uncontrolled gene expression resulting from mutations in genes encoding histone modifiers including histone acetyltransferases, deacetylases, methyltransferases and kinases causes developmental defects, in particular those involving the heart and nervous system, as well as cancers [69–71]. In fact, some types of human congenital diseases have been shown to involve the abnormal expression or activity of a family of histone methyltransferases. These include NSD2/ Wolf-Hirschhorn Syndrome Candidate 1 (WHSC1) in Wolf-Hirschhorn syndrome, NSD3 in cancer, euchromatin histone methyl transferase1 (EHMT1) in the 9q subtelomeric deletion syndrome and enhancer of zeste, drosophilahologomolog 2 (EZH2) in Weaver syndrome [72–75]. More recently, mutations DNMT3A, another DNA methyltransferase involved in the de novo methylation of DNA were also identified in patients with overgrowth syndromes [76]. Our data combined with two previous published data sets [48] suggest that point mutations in the NSD1 SET domain disrupt its transcriptional activity by interfering with its ability to bind a ligand required for methyl group transfer and by changing protein stability. Therefore, highly conserved amino acids in the SET domain are critical for the transcriptional activity of NSD1 required for the regulation of gene expression of a number of downstream targets. Abrogation of this NSD1 transcriptional regulation would result in the multiple phenotypes observed in Sotos patients.

NSD1 was reported to be expressed strongly in fetal brain, kidney, skeletal muscle, spleen and the thymus in Northern blot analysis [22]. However, little is known about its expression in various brain regions. Using RT-qPCR, we examined the NSD1 expression pattern in human brain, fetal brain, and additional 11 CNS human tissues compared to lymphocyte (Figure 2). Among the tissues examined, fetal brain displayed the highest NSD1 expression, suggesting a fetal onset of the neurological phenotype of this congenital disorder. It also showed high expression in cerebellum, which is commensurate with the hypoplasia of cerebellar vermis in Sotos syndrome [13].

Since NSD1 mutations have not been detected in 7%–30% of Sotos patients, other genes were also screened in some cases of unexplained Sotos syndrome. Because of their high sequence similarities with NSD1, both the NSD2 and NSD3 genes were tested previously as potential candidates for NSD1-negative Sotos syndrome cases [77]. However, no deletions were identified in 78 overgrowth syndrome cases without NSD1-mutation. Two missense alterations in NSD2 were identified in two non-Sotos overgrowth cases but neither was within a functional domain [77]. This group also identified three synonymous and two intronic variants in NSD2 and two synonymous substitutions in NSD3. Intragenic deletion of glypican 3 (GPC3) involved in Simpson–Golabi–Behmel syndrome was identified in a patient who was originally diagnosed with Sotos syndrome [78,79], and two 11p15 anomalies including paternal uniparental disomy and epigenetic defects (abnormal methylation status) were also identified among 20 patients with Sotos syndrome [80]. However, the molecular defect underlying a significant proportion of sporadic Sotos cases still remains unknown. In particular, in our Brazilian Sotos cohort, 68% of Sotos syndrome patients represented in 23 families have an unknown etiology. Therefore, these NSD1 mutation-negative families will surely contribute to the identification of new genes involved in Sotos syndrome in the future.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/7/11/96/s1. Table S1: PCR primers used for NSD1 mutational analysis. Table S2: Primers used for RT-qPCR. Table S3: SNPs of NSD1 found in our 34 Sotos patients.
Acknowledgments: The authors would like to thank all Brazilian Sotos family members who kindly agreed to participate in this study. K.H. is supported by a postdoctoral training grant from NIH (T32HL07676); P.A. and W.W. also thank funding from Federal Ministry of Education and Research in the Molecular Interaction Engineering Program (031A095C, IM-1). We thankfully acknowledge the funding support provided by Caroline Jones-Carrick and Collin Carrick.

Author Contributions: K.H. was responsible for data collection and drafted the manuscript; P.A. performed protein modeling and bioinformatics analysis; J.A.L. and J.R.J. performed the SNP analyses; C.A.K. collected patient samples and characterized clinical phenotypes; D.R.B. contributed to SNP analyses; J.D.L. performed NSD1 expression analysis; W.W. advised on molecular simulation analyses; L.C.L. reviewed and improved the draft; and H.K. conceived the project and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Cecconi, M.; Forzano, F.; Milani, D.; Cavani, S.; Baldo, C.; Selicorni, A.; Pantaleoni, C.; Silengo, M.; Ferrero, G.B.; Scarano, G.; et al. Mutation analysis of the NSD1 gene in a group of 59 patients with congenital overgrowth. Am. J. Med. Genet. A 2005, 134, 247–253. [CrossRef] [PubMed]
2. Cole, T.R.; Hughes, H.E. Sotos syndrome: A study of the diagnostic criteria and natural history. J. Med. Genet. 1994, 31, 20–32. [CrossRef] [PubMed]
3. Sotos, J.F.; Dodge, P.R.; Muirhead, D.; Crawford, J.D.; Talbot, N.B. Cerebral Gigantism in Childhood. A Syndrome of Excessively Rapid Growth and Acromegalic Features and a Nonprogressive Neurologic Disorder. N. Engl. J. Med. 1964, 271, 109–116. [CrossRef] [PubMed]
4. Tatton-Brown, K.; Douglas, J.; Coleman, K.; Bajut, G.; Cole, T.R.; Das, S.; Horn, D.; Hughes, H.E.; Temple, I.K.; Faravelli, F.; et al. Genotype-phenotype associations in Sotos syndrome: An analysis of 266 individuals with NSD1 aberrations. Am. J. Med. Genet. 2005, 77, 193–204. [CrossRef] [PubMed]
5. Saugier-Veber, P.; Bonnet, C.; Afenjar, A.; Drouin-Garraud, V.; Coubes, C.; Fehrenbach, S.; Holder-Espinasse, M.; Roume, J.; Malan, V.; Portnoi, M.F.; et al. Heterogeneity of NSD1 alterations in 116 patients with Sotos syndrome. Hum. Mutat. 2007, 28, 1098–1107. [CrossRef] [PubMed]
6. Goldstein, D.J.; Ward, R.E.; Moore, E.; Fremion, A.S.; Wappner, R.S. Overgrowth, congenital hypotonia, nystagmus, strabismus, and mental retardation: Variant of dominantly inherited Sotos sequence? Am. J. Med. Genet. 1988, 29, 783–792. [CrossRef] [PubMed]
7. Zonana, J.; Sotos, J.F.; Romshe, C.A.; Fisher, D.A.; Elders, M.J.; Rimoin, D.L. Dominant inheritance of cerebral gigantism. J. Pediatr. 1977, 91, 251–256. [CrossRef] [PubMed]
8. Tatton-Brown, K.; Rahman, N. Sotos syndrome. Eur. J. Hum. Genet. 2007, 15, 264–271. [CrossRef] [PubMed]
9. Imaizumi, K.; Kimura, J.; Matsuo, M.; Kurosawa, K.; Masuno, M.; Niikawa, N.; Kuroki, Y. Sotos syndrome associated with a de novo balanced reciprocal translocation t(5;8)(q35;q24.1). Am. J. Med. Genet. 2002, 107, 58–60. [CrossRef] [PubMed]
10. Kurotaki, N.; Imaizumi, K.; Harada, N.; Masuno, M.; Kondoh, T.; Nagai, T.; Ohashi, H.; Naritomi, K.; Tsukahara, M.; Makita, Y.; et al. Haploinsufficiency of NSD1 causes Sotos syndrome. Nat. Genet. 2002, 30, 365–366. [CrossRef] [PubMed]
11. Douglas, J.; Hanks, S.; Temple, I.K.; Davies, S.; Murray, A.; Upadhyaya, M.; Tomkins, S.; Hughes, H.E.; Cole, T.R.P.; Rahman, N. NSD1 mutations are the major cause of Sotos syndrome and occur in some cases of Weaver syndrome but are rare in other overgrowth phenotypes. Am. J. Hum. Genet. 2003, 72, 132–143. [CrossRef] [PubMed]
12. Faravelli, F. NSD1 mutations in Sotos syndrome. Am. J. Med. Genet. C Semin. Med. Genet. 2005, 137C, 24–31. [CrossRef] [PubMed]
13. Waggoner, D.J.; Raca, G.; Welch, K.; Dempsey, M.; Anderes, E.; Ostrovnaya, I.; Alkhateeb, A.; Kamimura, J.; Matsumoto, N.; Schaeffer, G.B.; et al. NSD1 analysis for Sotos syndrome: Insights and perspectives from the clinical laboratory. Genet. Med. 2005, 7, 524–533. [CrossRef] [PubMed]
14. Kurotaki, N.; Harada, N.; Shimokawa, O.; Miyake, N.; Kawame, H.; Uetake, K.; Makita, Y.; Kondoh, T.; Ogata, T.; Hasegawa, T.; et al. Fifty microdeletions among 112 cases of Sotos syndrome: Low copy repeats possibly mediate the common deletion. Hum. Mutat. 2003, 22, 378–387. [CrossRef] [PubMed]
15. Nagai, T.; Matsumoto, N.; Kurotaki, N.; Harada, N.; Niikawa, N.; Ogata, T.; Imaizumi, K.; Kurosawa, K.; Kondoh, T.; Ohashi, H.; et al. Sotos syndrome and haploinsufficiency of NSD1: Clinical features of intragenic mutations and submicroscopic deletions. J. Med. Genet. 2003, 40, 285–289. [CrossRef] [PubMed]
16. Zhang, X.; Yang, Z.; Khan, S.I.; Horton, J.R.; Tamaru, H.; Selker, E.U.; Cheng, X. Structural basis for the product specificity of histone lysine methyltransferases. Mol. Cell 2003, 12, 177–185. [CrossRef]
17. Zhang, X.; Tamaru, H.; Khan, S.I.; Horton, J.R.; Keeffe, L.J.; Selker, E.U.; Cheng, X. Structure of the Neurospora SET domain protein DIM-5, a histone H3 lysine methyltransferase. Cell 2002, 111, 117–127. [CrossRef]
18. Allali-Hassani, A.; Kuznetsova, E.; Hajian, T.; Wu, H.; Dombrovski, L.; Li, Y.; Gräslund, S.; Arrowsmith, C.H.; Schapira, M.; Vedadi, M. A Basic Post-SET Extension of NSDs Is Essential for Nucleosome Binding In Vitro. J. Biomol. Screen. 2014, 19, 928–935. [CrossRef] [PubMed]
19. Pasillas, M.P.; Shah, M.; Kamps, M.P. NSD1 PHD domains bind methylated H3K4 and H3K9 using interactions disrupted by point mutations in human sotos syndrome. Hum. Mutat. 2011, 32, 292–298. [CrossRef] [PubMed]
20. Stec, I.; Nagl, S.B.; van Ommen, G.J.; den Dunnen, J.T. The PWWP domain: A potential protein-protein interaction domain in nuclear proteins influencing differentiation? FEBS Lett. 2000, 473, 1–5. [CrossRef]
21. Wang, X.; Yeh, S.; Wu, G.; Hsu, C.; Wang, L.; Chiang, T.; Yang, Y.; Guo, Y.; Chang, C. Identification and characterization of a novel androgen receptor coregulator ARA267-alpha in prostate cancer cells. J. Biol. Chem. 2001, 276, 40417–40423. [CrossRef] [PubMed]
22. Kurotaki, N.; Harada, N.; Yoshiura, K.; Sugano, S.; Niikawa, N.; Matsumoto, N. Molecular characterization of NSD1, a human homologue of the mouse Nsd1 gene. Gene 2001, 279, 197–204. [CrossRef]
23. Huang, N.; vom Baur, E.; Garnier, J.M.; Lerouge, T.; Vonesch, J.L.; Lutz, Y.; Chambon, P.; Losson, R. Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. EMBO J. 1998, 17, 3398–3412. [CrossRef] [PubMed]
24. Berdasco, M.; Ropero, S.; Setien, F.; Fragà, M.F.; Lapunzina, P.; Losson, R.; Alaminos, M.; Cheung, N.K.; Rahman, N.; Esteller, M. Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma. Proc. Natl. Acad. Sci. USA 2009, 106, 21830–21835. [CrossRef] [PubMed]
25. Rayasam, G.V.; Wendling, O.; Angrand, P.O.; Mark, M.; Niederrreither, K.; Song, L.; Lerouge, T.; Hager, G.L.; Chambon, P.; Losson, R. NSD1 is essential for early post-implantation development and has a catalytically active SET domain. EMBO J. 2003, 22, 3153–3163. [CrossRef] [PubMed]
26. De Boer, L.; Kant, S.; Karperien, M.; van Beers, L.; Tjon, J.; Vink, G.R.; van Tol, D.; Dauwerse, H.; le Cessie, S.; Beemer, F.A.; et al. Genotype-phenotype correlation in patients suspected of having Sotos syndrome. Clin. Dysmorphol. 2007, 16, 175–181. [CrossRef] [PubMed]
27. Kutzner, C.; van der Spoel, D.; Fechner, M.; Lindahl, E.; Schmitt, U.W.; de Groot, B.L.; Grubmüller, H. Speeding up parallel GROMACS on high-latency networks. J. Comput. Chem. 2007, 28, 2075–2084. [CrossRef] [PubMed]
28. Kaminski, G.A.; Friesner, R.A. Evaluation and Reparametrization of the OPLS-AA Force Field for Proteins via Comparison with Accurate Quantum Chemical Calculations on Peptides. J. Phys. Chem. 2001, 105, 6474–6487. [CrossRef]
29. Jorgensen, W.L.; Chandrasekhar, J.; Madura, J.D.; Impey, R.W.; Klein, M.L. Comparison of Simple Potential Functions for Simulating Liquid Water. J. Chem. Phys. 1983, 79, 926–935. [CrossRef]
30. Berendsen, H.J.C.; Postma, J.P.M.; van Gunsteren, W.F.; DiNola, A.; Haak, J.R. Molecular-Dynamics with Coupling to an External Bath. J. Chem. Phys. 1984, 81. [CrossRef]
35. Capriotti, E.; Fariselli, P.; Casadio, R. I-Mutant2.0: Predicting stability changes upon mutation from the protein sequence or structure. *Nucleic Acids Res.* 2005, 33, W306–W310. [CrossRef] [PubMed]

36. Porollo, A.; Meller, J. Prediction-based fingerprints of protein-protein interactions. *Proteins* 2007, 66, 630–645. [CrossRef] [PubMed]

37. Adzhubei, I.A.; Schmidt, S.; Peshkin, L.; Ramensky, V.E.; Gerasimova, A.; Bork, P.; Kondrashov, A.S.; Sunyaev, S.R. A method and server for predicting damaging missense mutations. *Nat. Methods* 2010, 7, 248–249. [CrossRef] [PubMed]

38. Mi, H.; Poudel, S.; Muruganujan, A.; Casagrande, J.T.; Thomas, P.D. PANTHER version 10: Expanded protein families and functions, and analysis tools. *Nucleic Acids Res.* 2016, 44, D336–D342. [CrossRef] [PubMed]

39. Ng, P.C.; Henikoff, S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res.* 2003, 31, 3812–3814. [CrossRef] [PubMed]

40. Punta, M.; Coggill, P.C.; Eberhardt, R.Y.; Mistry, J.; Tate, J.; Boursnell, C.; Pang, N.; Forslund, K.; Ceric, G.; Clements, J.; et al. The Pfam protein families database. *Nucleic Acids Res.* 2012, 40, D290–D301. [CrossRef] [PubMed]

41. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* 1990, 215, 403–410. [CrossRef]

42. Letunic, I.; Doerks, T.; Bork, P. SMART 7: Recent updates to the protein domain annotation resource. *Nucleic Acids Res.* 2012, 40, D302–D305. [CrossRef] [PubMed]

43. Lek, M.; Karczewski, K.J.; Minikel, E.V.; Samocha, K.E.; Banks, E.; Fennell, T.; O’Donnell-Luria, A.H.; Ware, J.S.; Hill, A.J.; Cummings, B.B.; et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 2016, 536, 285–291. [CrossRef] [PubMed]

44. Kotilainen, J.; Pohjola, P.; Pirinen, S.; Arte, S.; Nieminen, P. Premolar hypodontia is a common feature in Sotos syndrome with a mutation in the *NSD1* gene. *Am. J. Med. Genet. A* 2009, 149A, 2409–2414. [CrossRef] [PubMed]

45. Tong, T.M.; Hau, E.W.; Lo, I.F.; Chan, D.H.; Lam, S.T. Spectrum of *NSD1* gene mutations in southern Chinese patients with Sotos syndrome. *Chin. Med. J. (Engl.)* 2005, 118, 1499–1506. [PubMed]

46. Nicita, F.; Tarani, L.; Spalice, A.; Grasso, M.; Papetti, L.; Cecconi, M.; di Biasi, C.; Ursitti, F.; Iannetti, P. Novel missense mutation (L1917P) involving sac-domain of *NSD1* gene in a patient with Sotos syndrome. *J. Genet.* 2011, 90, 147–150. [CrossRef] [PubMed]

47. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007, 23, 2947–2948. [CrossRef] [PubMed]

48. Qiao, Q.; Li, Y.; Chen, Z.; Wang, M.; Reinberg, D.; Xu, R.M. The structure of NSD1 reveals an autoregulatory mechanism underlying histone H3K36 methylation. *J. Biol. Chem.* 2011, 286, 8361–8368. [CrossRef] [PubMed]

49. Kumar, P.; Henikoff, S.; Ng, P.C. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat. Protoc.* 2009, 4, 1073–1081. [CrossRef] [PubMed]

50. Ng, P.C.; Henikoff, S. Predicting the Effects of Amino Acid Substitutions on Protein Function. *Annu. Rev. Genom. Hum. Genet.* 2006, 7, 61–80. [CrossRef] [PubMed]

51. Ng, P.C.; Henikoff, S. Accounting for Human Polymorphisms Predicted to Affect Protein Function. *Genome Res.* 2002, 12, 436–446. [CrossRef] [PubMed]

52. Ng, P.C.; Henikoff, S. Predicting Deleterious Amino Acid Substitutions. *Genome Res.* 2001, 11, 863–874. [PubMed]

53. Morishita, M.; di Luccio, E. Structural insights into the regulation and the recognition of histone marks by the SET domain of NSD1. *Biochem. Biophys. Res. Commun.* 2011, 412, 214–219. [CrossRef] [PubMed]

54. Kozbial, P.Z.; Mushegian, A.R. Natural history of S-adenosylmethionine-binding proteins. *BMC Struct. Biol.* 2005, 5. [CrossRef] [PubMed]

55. McCarthy, A.A.; McCarthy, J.G. The structure of two N-methyltransferases from the caffeine biosynthetic pathway. *Plant Physiol.* 2007, 144, 879–889. [CrossRef] [PubMed]

56. Kudithipudi, S.; Lungu, C.; Rathert, P.; Happel, N.; Jeltsch, A. Substrate specificity analysis and novel substrates of the protein lysine methyltransferase NSD1. *Chem. Biol.* 2014, 21, 226–237. [CrossRef] [PubMed]
57. Turkmen, S.; Gillessen-Kaesbach, G.; Meinecke, P.; Albrecht, B.; Neumann, L.M.; Hesse, V.; Palanduz, S.; Balg, S.; Majewski, F.; Fuchs, S.; et al. Mutations in NSD1 are responsible for Sotos syndrome, but are not a frequent finding in other overgrowth phenotypes. *Eur. J. Hum. Genet.* 2003, 11, 858–865. [CrossRef] [PubMed]

58. Fagali, C.; Kok, F.; Nicola, P.; Kim, C.; Bertola, D.; Albano, L.; Knaiffmann, C.P. MLPA analysis in 30 Sotos syndrome patients revealed one total NSD1 deletion and two partial deletions not previously reported. *Eur. J. Med. Genet.* 2009, 52, 333–336. [CrossRef] [PubMed]

59. Tatton-Brown, K.; Rahman, N. Clinical features of NSD1-positive Sotos syndrome. *Clin. Dysmorphol.* 2004, 13, 199–204. [CrossRef] [PubMed]

60. Bhaumik, S.R.; Smith, E.; Shilatifard, A. Covalent modifications of histones during development and disease pathogenesis. *Nat. Struct. Mol. Biol.* 2007, 14, 1008–1016. [CrossRef] [PubMed]

61. Wang, G.G.; Cai, L.; Pasillas, M.P.; Kamps, M.P. NUP98-NSD1 links H3K36 methylation to *Hox-A* gene activation and leukaeomogenesis. *Nat. Cell Biol.* 2007, 9, 804–812. [CrossRef] [PubMed]

62. Lu, T.; Jackson, M.W.; Wang, B.; Yang, M.; Chance, M.R.; Miyagi, M.; Gudkov, A.V.; Stark, G.R. Regulation of NF-kappaB by NSD1/FBXL11-dependent reversible lysine methylation of p65. *Proc. Natl. Acad. Sci. USA* 2010, 107, 46–51. [CrossRef] [PubMed]

63. Zhao, Q.; Caballero, O.L.; Levy, S.; Stevenson, B.J.; Iseli, C.; de Souza, S.J.; Galante, P.A.; Busam, D.; Leversha, M.A.; Chaladavada, K.; et al. Transcriptome-guided characterization of genomic rearrangements in a breast cancer cell line. *Proc. Natl. Acad. Sci. USA* 2009, 106, 1886–1891. [CrossRef] [PubMed]

64. Kouzarides, T. Histone methylation in transcriptional control. *Curr. Opin. Genet. Dev.* 2002, 12, 198–209. [CrossRef]

65. Xiao, B.; Wilson, J.R.; Gamblin, S.J. SET domains and histone methylation. *Curr. Opin. Struct. Biol.* 2003, 13, 699–705. [CrossRef] [PubMed]

66. Trievel, R.C.; Beach, B.M.; Dirk, L.M.; Houtz, R.L.; Hurley, J.H. Structure and catalytic mechanism of a SET domain protein methyltransferase. *Cell* 2002, 111, 91–103. [CrossRef]

67. Hu, P.; Zhang, Y. Catalytic mechanism and product specificity of the histone lysine methyltransferase SET7/9: An ab initio QM/MM-FE study with multiple initial structures. *J. Am. Chem. Soc.* 2006, 128, 1272–1278. [CrossRef] [PubMed]

68. Xiao, B.; Jing, C.; Wilson, J.R.; Walker, P.A.; Vasisht, N.; Kelly, G.; Howell, S.; Taylor, I.A.; Blackburn, G.M.; Gamblin, S.J. Structure and catalytic mechanism of the human histone methyltransferase SET7/9. *Nature* 2003, 421, 652–656. [CrossRef] [PubMed]

69. Zaidi, S.; Choi, M.; Wakimoto, H.; Ma, L.; Jiang, J.; Overton, J.D.; Romano-Adesman, A.; Bjornson, R.D.; Breitbart, R.E.; Brown, K.K.; et al. De novo mutations in histone-modifying genes in congenital heart disease. *Nature* 2013, 498, 220–223. [CrossRef] [PubMed]

70. Pedersen, M.T.; Helin, K. Histone demethylases in development and disease. *Trends Cell Biol.* 2010, 20, 662–671. [CrossRef] [PubMed]

71. Portela, A.; Esteller, M. Epigenetic modifications and human disease. *Nat. Biotechnol.* 2010, 28, 1057–1068. [CrossRef] [PubMed]

72. Stec, I.; Wright, T.J.; van Ommen, G.J.; de Boer, P.A.; van Haeringen, A.; Moorman, A.F.; Altherr, M.R.; den Dunnen, J.T. WHSCL, a 90 kb SET domain-containing gene, expressed in early development and homologous to a Drosophila dysmorph gene maps in the Wolf-Hirschhorn syndrome critical region and is fused to IGH in t(4;14) multiple myeloma. *Hum. Mol. Genet.* 1998, 7, 1071–1082. [CrossRef] [PubMed]

73. Tonon, G.; Wong, K.K.; Maulik, G.; Brennan, C.; Feng, B.; Zhang, Y.; Khatry, D.B.; Protopopov, A.; You, M.J.; Aguirre, A.J.; et al. High-resolution genomic profiles of human lung cancer. *Proc. Natl. Acad. Sci. USA* 2005, 102, 9625–9630. [CrossRef] [PubMed]

74. Kleefstra, T.; Smidt, M.; Banning, M.J.; Oudakker, A.R.; Van Esch, H.; de Brouwer, A.P.; Nillesen, W.; Sistermans, E.A.; Hamel, B.C.; de Bruijn, D.; et al. Disruption of the gene *Euchromatin Histone Methyl Transferase1* (Eu-HMTase1) is associated with the 9q34 subtelomeric deletion syndrome. *J. Med. Genet.* 2005, 42, 299–306. [CrossRef] [PubMed]

75. Gibson, W.T.; Hood, R.L.; Zhan, S.H.; Bulman, D.E.; Fejes, A.P.; Moore, R.; Mungall, A.J.; Eydoux, P.; Babul-Hirji, R.; An, J.; et al. Mutations in EZH2 cause Weaver syndrome. *Am. J. Hum. Genet.* 2012, 90, 110–118. [CrossRef] [PubMed]
76. Tatton-Brown, K.; Seal, S.; Ruark, E.; Harmer, J.; Ramsay, E.; Del Vecchio Duarte, S.; Zachariou, A.; Hanks, S.; O’Brien, E.; Aksglaede, L.; et al. Mutations in the DNA methyltransferase gene DNMT3A cause an overgrowth syndrome with intellectual disability. Nat. Genet. 2014, 46, 385–388. [CrossRef] [PubMed]

77. Douglas, J.; Coleman, K.; Tatton-Brown, K.; Hughes, H.E.; Temple, I.K.; Cole, T.R.; Rahman, N. Childhood Overgrowth Collaboration. Evaluation of NSD2 and NSD3 in overgrowth syndromes. Eur. J. Hum. Genet. 2005, 13, 150–153. [CrossRef] [PubMed]

78. Ginter, D.N.; Scott, C.I. Cerebral gigantism. Birth Defects Orig. Artic. Ser. 1975, 11, 415–422. [PubMed]

79. Li, M.; Shuman, C.; Fei, Y.L.; Cutiongco, E.; Bender, H.A.; Stevens, C.; Wilkins-Haug, L.; Day-Salvatore, D.; Yong, S.L.; Geraghty, M.T.; et al. GPC3 mutation analysis in a spectrum of patients with overgrowth expands the phenotype of Simpson-Golabi-Behmel syndrome. Am. J. Med. Genet. 2001, 102, 161–168. [CrossRef]

80. Baujat, G.; Rio, M.; Rossignol, S.; Sanlaville, D.; Lyonnet, S.; le Merrer, M.; Munnich, A.; Gicquel, C.; Cormier-Daire, V.; Colleaux, L. Paradoxical NSD1 mutations in Beckwith-Wiedemann syndrome and 11p15 anomalies in Sotos syndrome. Am. J. Hum. Genet. 2004, 74, 715–720. [CrossRef] [PubMed]

© 2016 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).