Sotos syndrome is associated with deregulation of the MAPK/ERK-signaling pathway

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Submitted

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Abstract

Sotos syndrome is characterized by tall stature, characteristic craniofacial features and mental retardation. It is caused by haploinsufficiency of the NSD1 gene, a SET-domain histone lysine methyltransferase which interacts with nuclear hormone receptors such as the retinoic acid receptor. In this study, genome-wide expression studies were performed on dermal fibroblasts from nine Sotos syndrome patients with a confirmed NSD1 abnormality. A significant association was demonstrated with the Mitogen-Activated Protein Kinase (MAPK) pathway. Members of the fibroblast growth factor family such as FGF4, FGF6, FGF13, FGF18, FGF19 and the FGFR2 contributed strongly to the differential expression of this pathway. In addition, phosphorylation studies of key kinases in fibroblasts demonstrated a possible diminished activity state of the MAPK/ERK pathway in Sotos syndrome. The Ras Interacting Protein 1 (RASIP1), a proposed Ras effector, was identified to be differentially expressed with upregulated expression in Sotos syndrome. siRNA experiments in HEK293 cells confirmed RASIP1 as a target of NSD1. In transfection experiments in HEK293 cells RASIP1 dose-dependently potentiated bFGF induced expression of the MAPK responsive SBE reporter providing further support for a link between NSD1 and the MAPK/ERK signaling pathway. With immunohistochemistry, we demonstrated NSD1 expression in the terminally differentiated hypertrophic chondrocytes of normal human epiphyseal growth plates at different developmental ages. From short stature syndromes such as hypochondroplasia and Noonan syndrome, it is known that the activation level of the FGF-MAPK/ERK-pathway in epiphyseal growth plates is a determining factor for statural growth. Therefore, we propose that deregulation of the MAPK/ERK pathway in SoS results in altered hypertrophic differentiation of NSD1 expressing chondrocytes and may be a determining factor in statural overgrowth and accelerated skeletal maturation in SoS.
Introduction

Sotos syndrome (SoS; OMIM 117550) is characterized by three main features, i.e. overgrowth (height and/or head circumference ≥ 98th percentile), facial dysmorphism and a variable level of mental retardation (1). It is caused by haploinsufficiency of the nuclear receptor binding SET domain protein 1 (NSD1) gene located at 5q35.2-5q35.3 (2). However, the biological processes translating the genetic aberrations into phenotypic features remain unknown.

NSD1 is expressed in various tissues such as fetal and adult brain, skeletal muscle, kidney, spleen, thymus, lung and in fibroblasts (3,4). It encodes a protein which consists of multiple functional domains; two nuclear receptor interaction domains (NID-L and NID+L), one Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain, an associated with SET domain (AWS), two Proline-Tryptophan-Tryptophan-Proline (PWWP) domains, four Plant Homeodomains (PHDs) type zinc finger and one C5HCH domain. Originally, mouse Nsd1 was identified in a two-hybrid screen with the retinoic acid receptor alpha (RARα) as bait (5). It was shown that Nsd1 interacted with a number of nuclear hormone receptors, such as the estrogen receptor α and β and thyroid hormone receptors (5). These interactions occurred either in absence of the ligand through NID-L (retinoic acid receptor and thyroid receptor) or in presence of the ligand through NID+L (retinoic acid receptor, thyroid hormone receptors, retinoid X and estrogen receptors). It was postulated that NSD1 could either act as a co-repressor or a co-activator of these nuclear receptors depending on the cellular context and the presence or absence of the respective hormones (5).

Further in vitro experiments showed that the SET domain possesses methyltransferase activity (6). NSD1 specifically methylated histone H3 at lysine 36 (H3-K36) and histone H4 at lysine 20 (H4-K20). The dual activity of NSD1 in relation to the methyltransferase activity was further exemplified by showing repression of the MEIS1 oncogene in a neuroblastoma model, while acting as a coactivator of the same gene in transfected cells expressing the NUP98-NSD1 fusion protein (7,8). Additionally, an NSD1-interacting zinc-finger protein (ZNF496) has been identified specifically interacting with the C5HCH domain of NSD1 (9). ZNF496 represses gene transcription and its activity is at least partly dependent on its interaction with NSD1. Based on its role in repression of gene transcription, it has been hypothesized that heterozygous inactivation of NSD1 results in loss of repression of growth promoting genes (6). Consequently, increased activity of these genes would lead to the
characteristic overgrowth phenotype of SoS. Unfortunately, experimental evidence for this hypothesis is lacking due to the fact that heterozygous knock out mice of Nsd1 did not exhibit a SoS phenotype. Homozygous Nsd1-/ mice died in utero before E10.5 showing the indispensable role of Nsd1 in embryogenesis (6). To date no patients have been described with homozygous loss of NSD1.

Our group previously studied the expression of members of the Growth Hormone (GH)/ Insuline like growth factor 1 (IGF1) axis in SoS patients with a confirmed NSD1 mutation (10). Modestly increased plasma levels of IGFBP-2 and IGFBP-6 and reduced levels of IGF-I, IGF-II, IGFBP-3 and IGFBP-4 were detected in plasma of these patients. Reduced levels of IGF-I and IGF-II in particular are, however, more reminiscent of short rather than tall stature such as observed in SoS. The relationship between NSD1 and the GH/IGF1 axis remains elusive. Thus, although a number of functions of NSD1 have been identified, the molecular mechanisms leading to SoS in patients with NSD1 mutations are still largely unclear.

The aim of the current study was therefore to identify downstream effectors of NSD1 and to map these effectors in signaling pathways associated with growth. Genome-wide mRNA expression profiles of dermal fibroblasts from SoS patients with a confirmed NSD1 abnormality were compared to expression patterns in age and sex-matched controls. With regard to the NSD1 interaction with RARα, we hypothesized that differences in gene expression between fibroblasts of SoS patients and controls would be more pronounced in the presence of all trans-retinoic acid (RA).

**Material and Methods**

**Subjects**
This study was approved by the Medical Ethical Committee of the Leiden University Medical Center and informed consent was given by the patients and/or their parents or legal guardians. The study included cell lines of skin fibroblasts which were obtained from 9 SoS patients with a confirmed NSD1 abnormality and 9 sex- and age matched normal donors as described previously by De Boer et al. (10). Details per individual are presented in Tables 1 and 2.
### Table 1. Characteristics of Sotos syndrome patients

| No. | Age at biopsy | Sex | Nucleotide change | Predicted protein change | Identifier in our previous study (11) |
|-----|---------------|-----|-------------------|--------------------------|---------------------------------------|
| 1   | 3.2           | M   | c.6463+1G>A       | p.?                      | Patient 10                            |
| 2   | 4.5           | F   | c.6241T>G         | p.L2081V                 | Patient 28                            |
| 3   | 5.7           | F   | c.5950C>G         | p.R1984G                 | Patient 11                            |
| 4   | 7.4           | M   | c.4548_4549delGGinsC | p.E1516fsX            | Patient 2                             |
| 5   | 12.5          | M   | Deletion NSD1 exon 1-3 and FGFR4 |                  | Patient 4                             |
| 6   | 15.2          | M   | c.5435T>A         | p.V1812D                 | Patient 14                            |
| 7   | 17.8          | M   | c.2809delCGinsT   | p.R937X                  | Patient 3                             |
| 8   | 18.9          | F   | c.3531delT        | p.F1177fsX              | Patient 8                             |
| 9   | 33.5          | M   | c.4108C>T         | p.Q1370X                 | Patient 34                            |

1 M: Male; F: Female

### Table 2. Characteristics of controls

| No. | Age at biopsy | Sex |
|-----|---------------|-----|
| 1   | 3.7           | F   |
| 2   | 4             | F   |
| 3   | 5.4           | M   |
| 4   | 7             | M   |
| 5   | 11.6          | M   |
| 6   | 15.9          | M   |
| 7   | 16.1          | M   |
| 8   | 17.1          | M   |
| 9   | 32            | M   |

1 M: Male; F: Female
Cell culture
Skin fibroblasts were cultured in 25cm² flasks (Corning Inc. Life sciences, Schiphol-Rijk, The Netherlands) in Dulbecco’s modified Eagle’s Medium supplemented with glutamax (DMEM 31966; Gibco, Breda, The Netherlands) and containing 10% fetal calf serum (FCS; Lonza, Verviers, Belgium), 50IU/ml penicillin and 50μg/ml streptomycin (Gibco). Medium was changed twice a week and cells were split at a ratio of 1:6 upon reaching monolayer confluence. The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. In all experiments cell passages were between 2 and 10.

All trans-retinoic acid stimulation
At reaching 80-90% monolayer confluency cells were stimulated with RA (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 10⁻⁶M for 48h. Because of the light-sensitivity of RA, cell cultures were kept in the dark.

Total RNA extraction, labeling and microarray hybridization
Total RNA extraction was performed with the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). RNA concentrations were determined using a spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Quality and integrity were checked using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) by ServiceXS (www.servicexs.nl, Leiden, The Netherlands). Generation of cDNA, cRNA, and biotin-labeling were performed by ServiceXS according to manufacturer’s guidelines (www.affymetrix.com). Hybridization to GeneChip HG-U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) and scanning was performed by the Leiden Genome Technology Center (www.lgtc.nl) following manufacturer’s protocols (www.affymetrix.com). The whole genome HG-U133 Plus 2.0 Array contains 54120 probe sets covering 38572 UniGene clusters.

Statistical analysis
Analyses of the microarray expression data were made in R (version 2.7.0; www.r-project.org). Probe level data was preprocessed with the Robust Multi-array Average (RMA) algorithm (12) in the Bioconductor (www.bioconductor.org) (13) affy package (version 1.18.2) (14). This algorithm includes background correction, quantile normalization of corrected perfect match probes (15) and calculation of the level of expression using median polish. Analyses of differentially expressed genes were performed using the Linear Models for Microarray Data (limma, version 2.14.5) package (16). A correction for multiple testing was performed using
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the Benjamin-Hochberg method. Genes with an adjusted p.value of <0.05 were considered to be statistically significant.

Prior to analysis, a selection was made of 10 Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) signaling pathways and 26 Gene Ontology (GO; www.geneontology.org) terms associated with signal transduction and chromatin regulation. A complete list of selected GO-terms can be found in the supplementary data (Table 1S). The Global Test package (version 4.10.0) was used for the pathway analysis (17). The permutation method was employed and correction for multiple testing was performed. Results with an adjusted p.value <0.05 were considered statistically significant.

Real-Time quantitative PCR
Real-Time quantitative PCR (qPCR) was performed to validate differentially expressed genes identified in the microarray analysis. The following genes were selected: RASIP1, RBM47, COMP, and FGF13. For FGF13 qPCR was performed for both splice-forms separately (FGF13A and B) and with a primer set detecting both splice-forms (FGF13C). QuantiTect primers were purchased from Qiagen (Qiagen Benelux B.V., Venlo, The Netherlands). RNA from all samples after RA stimulation was reverse transcribed into cDNA using the Superscript First-Strand kit (Invitrogen, Breda, The Netherlands). Subsequently 25ng of cDNA was amplified on the Bio-Rad iCycler (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) in a mixture containing a final concentration of 1x iQ SYBR Green mix (Bio-Rad) and 1x Quantitect primer. qPCR conditions included a denaturing step of 15 minutes at 95°C, followed by 40 cycles of 15 s at 95°C, 30s at 55°C and 30s at 72°C. Fold changes were adjusted for the expression of the housekeeping gene β2 microglobulin following the 2^{ΔΔCt} method (18).

NSD1 siRNA study
For the NSD1 siRNA experiment, HEK293t cells were seeded at 200.000 cells per well in a 96-wells plate (Becton Dickinson, Breda, The Netherlands). After 24 hours, cells were trypsinized and seeded in a 96-wells plate at 7500 cells per well. Then, 24 hours later, cells were transfected with siRNA for NSD1 or non-targeting siRNA and incubated for 48 hours before collecting samples for RNA isolation. Expression levels were determined with qPCR following procedures described above.
**RASIP1 transfection study**

HEK293t cells were seeded at 10,000 cells/well in a 96 wells plate. After 24 hours, cells were co-transfected with the MAPK-responsive SBE promoter reporter construct in combination with increasing amounts of a RASIP1 expression vector and pRL-CMV Renilla control, using Fugene HD (Roche Applied Science, Almere, The Netherlands). pUC19 DNA was used to adjust DNA concentrations in the transfection experiment. Twenty-four hours after transfection, cells were stimulated with 10 ng/ml bFGF for 24 hours. Then cells were lysed by adding 20 µl Cell Culture Lysis Reagent (Promega Benelux B.V., Leiden, The Netherlands) per well, after which the Dual-Glo Luciferase Assay (Promega) for Firefly and Renilla luciferase was performed and measured using Victor Multilabel Counter 1420 (PerkinElmer, Groningen, The Netherlands). Values were normalized for Renilla and expressed as fold induction compared to control which was set to 1 +/- standard error of mean. For statistical analysis, OneWay ANOVA was performed using the SPSS Statistics 17.0 software.

**Protein phosphorylation studies**

Cells were seeded in 6 well dishes (Corning Inc.) at $1.5 \times 10^5$ cells per well and cultured at 37°C for 48h in full medium (see above). Subsequently RA was added with a final concentration of $10^{-6}$M and cells were cultured for another 48h. This was followed by incubation in serum free DMEM containing 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, USA) for 14 hrs after which cells were stimulated for 10 minutes with basic Fibroblast Growth Factor (bFGF; Sigma-Aldrich) with a final concentration of 50 ng/ml. Cells were lysed with the Bio-Plex Cell Lysis Kit (Bio-Rad), protein concentrations were determined using the BCA protein assay Kit (Perbio Science, Etten-Leur, The Netherlands) and were subsequently diluted to 250 μg/ml. Phosphorylated and total ATF2, cJun, ERK1/ERK2, ERK2, HSP27, JNK, MEK1, p38MAPK and p90RSK were detected using the Bio-Plex suspension array system following manufacturer’s protocol (www.bio-rad.com). Briefly, 6.25 μg protein was incubated in 96 wells filter plates with coupled beads recognizing either the phosphorylated or total protein. Incubation was at room temperature and lasted 15-18 h. Subsequently, incubation with detection antibodies was performed for 30 min, followed by 10 min incubation with streptavidin-phycoerythrin. The fluorescence levels of the phosphorylated proteins and total proteins were analyzed with the Bio-Plex suspension array system. Differences in phosphorylation were examined with a correction for total protein levels by univariate analysis using the SPSS Statistics 16.0 software. P-values of < 0.05 were considered significant.
**Immunohistochemistry**

Monoclonal anti-NSD1 antibodies (1NW1A10 and 3NW3F8) were obtained with kind permission from prof. dr. R. Losson at IGBMC/GIE-CERBM, France (5). Immunohistochemistry was performed as previously described (19). The study protocol was approved by the Local Medical Ethics Committee of the Leiden University Center, Leiden, The Netherlands. Informed consent was obtained from the patients and/or their parents. Human growth plates included were of different developmental ages: fetal (17 weeks), 1 year and 13 years of age. The fetal growth plate was obtained from the tibia of a normally developed aborted fetus. The growth plate from a one year old patient was obtained from a surgically removed 6th toe in an otherwise healthy, normally growing and developing infant. The growth plate of a 13 year old patient was obtained from the femur head after surgery because of epiphyseolysis. This patient exhibited overgrowth, without a specific diagnosis.

**Results**

**Differentially expressed genes in Sotos syndrome**

To study differences in gene expression profiles in SoS, RNA obtained from dermal fibroblasts from nine SoS patients with confirmed NSD1 alterations was compared with dermal fibroblast RNA from nine age and sex matched controls using genome wide expression profiling. Differentially expressed probe sets were identified using Limma analysis. The significant probe sets and their corresponding genes are shown in Table 3 A and B. In basal situation, five probe sets were differentially expressed after correction for multiple testing. These probes sets corresponded to 4 genes: *Ras interacting protein 1 (RASIP1)*, Plakophilin 3 (*PKP3*), RNA binding motif 47 (*RBMM47*), and *KIAA0895*. After RA stimulation, the differential expression of *RASIP1* and *RBMM47* was preserved and 2 new genes (Mucolipin 3 (*MCOLN3*) and *KIAA1128*) were identified to be differentially expressed. Significance of the PKP3 and KIAA0895 probe sets was lost. Expression of *RASIP1* and *PKP3* was up regulated in SoS, while expression of the other identified genes was down regulated.

To validate the microarray results, qPCR was performed for *RASIP1* and *RBMM47* in samples which had been stimulated with RA. Two additional controls were incorporated: *Fibroblast Growth Factor 13 (FGF13)* as an example of the most down regulated gene in SoS with a fold change of 11.1 (p=0.24) in basal situation and 13.5 (p=0.18) after RA stimulation and...
Table 3A. Differentially expressed probe sets in SoS

| No. | Probe set ID | Gene symbol | Gene name                  | Entrez ID | Log Fold Change | p.value | p.value adjusted for multiple testing |
|-----|--------------|-------------|----------------------------|-----------|----------------|---------|--------------------------------------|
| 1   | 220027_s_at  | RASIP1      | Ras interacting protein 1  | 54922     | 2.1            | 1.75E-08 | 0.001                                |
| 2   | 209873_s_at  | PKP3        | plakophilin 3              | 11187     | 0.4            | 4.87E-07 | 0.013                                |
| 3   | 222496_s_at  | RBM47       | RNA binding motif protein 47| 54502     | -0.5           | 1.43E-06 | 0.026                                |
| 4   | 213424_at    | KIAA0895    | KIAA0895 protein           | 23366     | -0.4           | 2.87E-06 | 0.033                                |
| 5   | 218035_s_at  | RBM47       | RNA binding motif protein 47| 54502     | -0.5           | 3.00E-06 | 0.033                                |

Table 3B. Differentially expressed probe sets in SoS after stimulation with RA

| No. | Probe set ID | Gene symbol | Gene name                  | Entrez ID | Log Fold Change | p.value | p.value adjusted for multiple testing |
|-----|--------------|-------------|----------------------------|-----------|----------------|---------|--------------------------------------|
| 1   | 220027_s_at  | RASIP1      | Ras interacting protein 1  | 54922     | 2.0            | 2.36E-07 | 0.013                                |
| 2   | 229797_at    | MCOLN3      | mucolipin 3                | 55283     | -0.7           | 6.79E-07 | 0.019                                |
| 3   | 222496_s_at  | RBM47       | RNA-binding motif protein 47| 54502     | -0.8           | 1.44E-06 | 0.026                                |
| 4   | 240499_at    | KIAA1128    | KIAA1128                   | 54462     | -0.7           | 2.93E-06 | 0.040                                |
COMP (fold change 4.8; p=0.15) gene as an example of an upregulated gene, although these differences did not reach statistical significance after correction for multiple testing. For all genes, the qPCR results confirmed the gene expression profiles detected with the microarray (Figure 1), although for RASIP1 considerably higher fold changes (16.1 vs 4.1) were found with qPCR. For FGF13 qPCR confirmed down regulation of the two known splice variants.

**Differentially expressed signaling pathways in Sotos syndrome**

To study the association of NSD1 with signal transduction pathways, 10 pathways were a priori selected from the KEGG-database. In addition 26 GO-terms were selected which overlapped with the KEGG signaling pathways or were involved in chromatin transcription. The signaling pathways and GO-terms were selected based on a previously established role in growth regulation and/or NSD1 function (5,6). A global test (17) was performed independently from the Limma-analysis and included all probe sets of the whole genome.

![Figure 1](image_url)  
**Figure 1. qPCR validation of differentially expressed genes**

Differential gene expression of RASIP1, RBM47, FGF13 (splice variants A and B; C represents a primer set detecting both splice-forms) and COMP was studied using qPCR. Total RNA of the 9 SoS and 9 control fibroblasts cultures stimulated with RA for 48 hours was reversed transcribed into cDNA and used as input. Fold change represents the average difference in expression level of the respective gene after stimulation with RA between the SoS-fibroblasts and controls. They were adjusted for the expression of the housekeeping gene β2 microglobulin using the $2^{-\Delta\Delta C_{t}}$ method. Black bars depict the fold changes detected with microarray and white bars show the average fold change of triplicate qPCR experiments. Fold changes indicating downregulated expression are represented with negative values. Error bars represent the standard error of the mean.
expression microarrays. The KEGG-signaling pathways and GO-terms were tested in SoS versus control either unstimulated or stimulated with RA (Table 4). In the basal situation none of the signaling pathways was significantly associated with SoS. After stimulation with RA, a significant association was detected with the mitogen activated protein kinase (MAPK) pathway (adjusted p.value = 0.023). This was further supported by the significant association (adjusted p-value = 0.003) with the MAPK kinase kinase cascade GO-term, which partially overlaps in gene content with the KEGG MAPK pathway. Furthermore, although no significant associations with other KEGG signaling pathways were found after correction for multiple testing, we noted that stimulation with RA improved adjusted p-values for all pathways compared to the basal situation (Table 4).

Table 4. Signaling pathways analysis results

| KEGG no. | Name                      | Basal situation | RA stimulation |
|----------|----------------------------|-----------------|----------------|
|          | p.value    | FDR adj. p.value | p.value    | FDR adj. p.value |
| 4010     | MAPK signaling pathway    | 0.062           | 0.341         | 0.002           | 0.023         |
| 4012     | ErbB signaling pathway    | 0.090           | 0.341         | 0.020           | 0.087         |
| 4310     | Wnt signaling pathway     | 0.102           | 0.341         | 0.026           | 0.087         |
| 4330     | Notch signaling pathway   | 0.218           | 0.546         | 0.036           | 0.089         |
| 4350     | TGF-beta signaling pathway| 0.324           | 0.648         | 0.074           | 0.148         |
| 4370     | VEGF signaling pathway    | 0.516           | 0.677         | 0.138           | 0.230         |
| 4630     | Jak-STAT signaling pathway| 0.550           | 0.677         | 0.192           | 0.274         |
| 4020     | Calcium signaling pathway | 0.556           | 0.677         | 0.247           | 0.309         |
| 4070     | Phosphatidylinositol signaling system | 0.610 | 0.677 | 0.299 | 0.332 |
| 4150     | mTOR signaling pathway    | 0.870           | 0.870         | 0.758           | 0.758         |

| GO-term  | Name                     | Basal situation | RA stimulation |
|----------|--------------------------|-----------------|----------------|
|          | p.value    | FDR adj. p.value | p.value    | FDR adj. p.value |
| 0000165  | MAPKKK cascade           | 0.003           | 0.082         | 1.23E-04        | 0.003         |
The KEGG MAPK pathway consists of 781 probe sets and the GO MAPKKK cascade contains 433 probe sets. We determined the contribution of each of these probe sets to the differential expression of the MAPK pathway in SoS versus controls after RA treatment. The 50 most influential probe sets are shown in Figure 2. The probe set for FGF13 contributed strongly to the differential expression of the MAPK pathway and MAPKKK cascade. FGF13 was the most down regulated gene in SoS after RA-treatment (fold change 13.5) as detected with the Limma-analysis. This down regulation did not reach significance after correction for multiple testing (p = 0.18) but was confirmed by qPCR analysis. Besides FGF13 also FGF4, FGF6, FGF18, FGF19 and the FGFR2 contributed strongly to the differential expression of the MAPK pathway. Three distinct probe sets for the TRAF2 and NCK interacting kinase (TNIK) gene were highly influential on the difference between Sotos and controls in the MAPKKK cascade. Like FGF13, these probes were also down regulated. In the Limma-analysis the three probes were found to have fold changes of 2.0 (p = 0.17), 1.3 (p = 0.20) and 2.0 (p = 0.26), respectively. The signature of the FGF-signaling pathway as observed in the KEGG pathway analysis was less clear in the GO MAPKKK pathway analysis.

The most differentially expressed gene between SoS and controls is RASIP1
RASIP1 specifically interacts with GTP-bound Ras and acts as an effector of endomembrane localized Ras (20), which plays an important role in MAPK-signaling. To study the role of RASIP1 in MAPK signaling in more detail we first performed siRNA experiments in HEK293 cells. As shown in Figure 3 knock down of NSD1 results in up regulation of RASIP1 mRNA expression in line with the microarray data. This confirms that RASIP1 expression is regulated by the expression levels of NSD1. As shown in Figure 4, transient transfection of a RASIP1 expression vector in HEK293 cells increased MAPK-luciferase reporter activity dose-dependently, particularly in the presence of bFGF. As RASIP1 is not included in the KEGG and GO-terms, these results provide an independent line of evidence of altered MAPK-signaling in SoS.

Figure 2. Geneplots of the probe sets influencing the MAPK pathway
Geneplots are shown for the 50 most influential probe sets from the KEGG MAPK pathway (A) and for the GO-term MAPKKK cascade (B) after stimulation with RA that contribute to the differential pathway expression in SoS and control. Probe sets are scaled to unit standard deviations and the height of the bars are the number of standard deviations above the cut-off level of 0.7. Higher bars indicate higher influence on the pathway. Probe sets with the highest influence on the pathway (i.e. FGF13 in Figure 2A and TNIK in Figure 2B) are depicted on the left. Corresponding gene names are written below each bar.
Influence
table.

A

- Decreased in SoS compared to control after RA stimulation
- Increased in SoS compared to control after RA stimulation

B

- Decreased in SoS compared to control after RA stimulation
- Increased in SoS compared to control after RA stimulation
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Differential activation of the MAPK pathway in SoS

We next determined whether the differential expression of the MAPK pathway in SoS cell lines after RA stimulation was corroborated with differential activation of this pathway at the protein level. We focused on determining the activation status of key kinases (ERK1/ERK2, ERK2, JNK, MEK1, p38MAPK and p90RSK) of this pathway and downstream effectors like the transcription factors (ATF2, cJUN) and the heat shock protein HSP27. Phosphorylated and total ERK1/ERK2, ERK2, HSP27, JNK, MEK1, p38MAPK and p90RSK were quantitatively assessed using the multiplex Bio-Plex suspension array in the 9 SoS and control fibroblasts and were corrected for total protein. In a univariate analysis including all samples, a trend was observed for a lower activation state of the kinases MEK1, ERK1/ERK2, and ERK2 in basal conditions, but this did not reach significance (p=0.06 – p=0.11) (Figure 5A). A borderline

Figure 3. *NSD1* knock down increases *RASIP1* expression

Expression of *NSD1* was downregulated with siRNA (black bar on the left), which resulted in up regulation of *RASIP1* expression (black bar on the right), while no difference in expression was detected for the controls (white and grey bars). Fold change represents the average difference in expression level of the respective gene. Fold changes were adjusted for the expression of the housekeeping gene β2 microglobulin using the 2^-ΔΔCt method.
significant association with a higher activation state in SoS was found for cJUN (p=0.04). For the other proteins no differences were observed between SoS and controls. Furthermore, no differences were observed between SoS and controls after stimulation with RA.

Critical assessment of all data points of phosphorylated proteins revealed, however, that the phosphorylation level of a number of proteins in fibroblasts of SoS patient no. 2 were remarkably higher than the values observed in other SoS fibroblasts as well as in the control group. These differences could not be explained by experimental variation in e.g. protein loading. Principal component analysis including all data points of phosphorylated proteins, clearly demonstrated that the response in this cell line was distinct from other SoS and control fibroblast cultures (Supplementary Figure 1S). This was further confirmed using the outlier detection method implemented in the R package mvoutlier (available from cran.r-

Figure 4. bFGF induced SBE reporter activation is potentiated by RASIP1
Values are expressed as fold induction compared to control. Control was not stimulated with bFGF and no RASIP1 was co-transfected. Co-tranfection of RASIP1 did not affect basal reporter activity. bFGF (10 ng/ml) significantly stimulated SBE reporter activity (indicated with #; p < 0.05). RASIP1 enhanced bFGF induced reporter dose-dependently (indicated with *; p < 0.05).
Figure 5. Results protein phosphorylation
The results are shown for the phosphorylation levels for the MEK1 (A), ERK1/ERK2 (B), ERK2 (C), p38MAPK (D), cJUN (E), ATF2 (F), JNK (G), HSP27 (H) and RSK90 (I). Bar heights depict the mean fluorescence intensity levels measured (MFI) and the p-values for the difference between SoS and control (after correction for total protein levels) are shown above the bars.
Figure 5. Results protein phosphorylation (continued)

E  
Phosphorylated cJUN

F  
Phosphorylated ATF2

G  
Phosphorylated JNK

H  
Phosphorylated HSP27
Both the simple and the sophisticated sign method indicated the patient as an outlier at a critical value of 0.99 (21).

Analysis was repeated after removal of the fibroblasts of patient no. 2 from the SoS group. Now the activation state of MEK1, ERK1/ERK2 and p38MAPK in basal conditions was significantly reduced in SoS (Figure 5B). The basal activity of ERK2 was also reduced but this did not reach significance (p=0.06). The borderline significant association with a higher activation state of cJUN (p=0.04) did hardly change (p=0.05). Removal of the outlier did not change the activation state observed for the other proteins. In RA stimulated cells, the activation state of MEK1 (p=0.02) was lower in SoS, while no differences were observed between the other proteins.

**Stimulation with bFGF**

Given the suggestive FGF-signaling pathway signature in the MAPK pathway analysis (Figure 2A), we subsequently determined the activation of the MAPK pathway after stimulation with bFGF. The phosphorylation state of all proteins increased after stimulation with bFGF, both in basal and the RA condition, in concordance with the appreciated role of this growth factor in MAPK-signaling (22). Differences in mean phosphorylation levels which were (close to) significant were found in the bFGF condition for MEK1 (without SoS no. 2; p=0.05) and for p90RSK (p=0.04 and p=0.05 with and without SoS no. 2, respectively), although in the latter overall fluorescence intensity levels were in the low-detection range. In the RA+ bFGF condition, no differences were detected. For the ERK1/ERK2 and ERK2 proteins,
fluorescence levels after stimulation with bFGF showed saturated values and differences in mean intensity levels could not be calculated.

**NSD1 is expressed in the human growth plate**

Longitudinal growth is regulated by a complex interplay of multiple growth factors and their receptors in the epiphyseal growth plate (23). We employed immunohistochemistry to study the expression of NSD1 in human growth plate specimens. As shown in Figure 6, NSD1 was expressed in the terminally differentiated hypertrophic chondrocytes at different developmental ages. Similar results were found using two distinct monoclonal antibodies.

**Figure 6. NSD1 expression in the human growth plate**

Expression of NSD1 is shown in the femoral growth plate of a fetus at the age of 17 weeks (A), in a toe of a 1 year old subject (B) and in the tibial growth plate of a 13 year old subject (C). NSD1 is expressed in the terminally differentiated hypertrophic chondrocytes.

**Discussion**

In order to elucidate biological pathways explaining how NSD1 haploinsufficiency results in phenotypic features such as overgrowth in SoS a comprehensive study of dermal fibroblasts from SoS patients was performed. We obtained evidence that SoS syndrome is associated with a deregulation of the MAPK/ERK signaling pathway. The MAPK/ERK signaling pathway is an important regulator of cell differentiation, proliferation and apoptosis and has been implicated in many human diseases such as Alzheimer disease and cancer (24). More recently, activating mutations in this pathway have been identified as the causative factor
in a number of short stature syndromes, such as Noonan syndrome, Costello syndrome and LEOPARD syndrome (25).

Deregulated MAPK/ERK signaling pathway in SoS is based on the following observations. First, a significant association of NSD1 expression and the MAPK/ERK signaling pathway was shown in the fibroblast microarray study. Second, RASIP1, a downstream Ras effector and hence interfering with the RAS/MAPK/ERK signaling cascade, was observed to be upregulated in SoS. Knock down experiments confirmed RASIP1 as a NSD1 target. Third, lower phosphorylation levels in SoS of several key MAP kinases of the MAPK/ERK pathway were detected. Fourth, in a transfection model, RASIP1 dose-dependently potentiated bFGF induced expression of the MAPK- responsive SBE reporter construct. One may argue that the associations in some of the individual experiments were weak or revealed a more complex picture (e.g. the results of the phosphorylation experiments). However the analysis of each independently performed experiment points into the same direction and when taken together they provide strong evidence for a deregulation of the MAPK/ERK signaling pathway in SoS.

Microarray analysis identified a relatively small number of significantly differentially expressed genes. This is likely explained by the relatively small number of samples, due to the limited number of SoS patients available, in combination with a considerable level of biological variation, hence reducing the power of the experiment to obtain significance (26). In addition, dermal fibroblasts may not be the most optimal model for studying the effects of NSD1 haploinsufficiency in relation to overgrowth. However, 2-15% of the SoS patients exhibit nail hypoplasia (27) with possible overgrowth of the surrounding skin (28). Furthermore, dermal fibroblasts have successfully been used to elucidate molecular mechanisms underlying growth disorders (29-32) and, in marked contrast to growth plate chondrocytes are easily available for analysis. Furthermore, dermal fibroblasts express NSD1 (4). Based on these considerations, we believe that dermal fibroblasts are suited for studying the molecular mechanisms underlying the overgrowth in SoS.

The first line of evidence linking NSD1 to deregulated MAPK/ERK signalling was derived from the KEGG and GO-term pathway analysis. Given the established role of NSD1 in retinoic acid signalling, we hypothesized that the effects on gene expression and pathway analysis between SoS and control would be more pronounced after treatment with RA. Indeed, differences in gene expression and pathway analysis became more pronounced after RA
treatment in support of our hypothesis. This might be due to an increase in fold change of the genes in the MAPK pathway after RA treatment (data not shown). However such an effect was not seen in the phosphorylation studies. An explanation for this difference might be the 48 hour period of stimulation with RA, which is sufficient to detect differences on an mRNA level, but may be too short to reflect differences at a protein level.

The second line of evidence for an association with the MAPK/ERK kinase signaling pathway was derived from the Limma analysis, which identified \textit{RASIP1} as the number 1 differentially expressed gene between SoS and control. Knock down experiments confirmed RASIP1 as a direct target of NSD1. RASIP1 interacts in a GTP-dependent manner with endomembrane-associated Ras and is recruited to the Golgi by activated Ras (Ras-GTP) (20). RASIP1 binds to several members of the Ras family, for example to the activated H-Ras and Rap1A proteins and is proposed to be a downstream Ras-effector, which is a central player in the MAPK/ERK pathway (20). The physiological significance of such compartmentalized signaling of Ras, i.e. Ras signaling on endosomes such as endoplasmic reticulum, the Golgi apparatus and mitochondria, has not been elucidated yet (33). Since it was shown that Golgi-associated Ras was able to activate the MAPK signaling cascade in a timely and quantitatively different manner than plasma membrane Ras, it was proposed that compartmentalized Ras signaling would increase the complexity of possibilities for downstream signaling (34). This is an independent line of evidence linking SoS to deregulated MAPK/ERK signaling, since \textit{RASIP1} was not included in the GO-terms en KEGG MAPK pathway. Therefore, increased RASIP1 expression in interference with activated Ras, might be related to the differences in cell growth and differentiation observed in SoS.

Third line of evidence is the trend of lower phosphorylation levels of MEK1, ERK1/ERK2, ERK2 in SoS in basal conditions and for MEK1 after stimulation with RA. This trend became significant after exclusion of SoS no. 2 as an outlier. Lower phosphorylation levels of key kinases would indicate a decreased activation of the MAPK/ERK pathway in SoS. In contrast, a fourth line of evidence shows that in our transfection experiments RASIP1 dose-dependently potentiated bFGF induced expression of the SBE reporter in HEK293 cells, suggesting a possible increased activation state of the MAPK/ERK pathway, since RASIP1 was found to be upregulated in SoS. These findings apparently contradict each other and require a critical evaluation.
First, RASIP1 interacts with endomembrane-associated Ras and such compartmentalized signaling of Ras/MAPK might be more complex than can be simulated in our transfection model (34). Second, the effect of NSD1 on the MAPK/ERK pathway may be cell type dependent. Such cell type dependency has been described for many cofactors of transcription regulation and may depend on the cell type specific expression of key transcription factors (reviewed in (35). Third, NSD1 was shown to influence many genes involved in the MAPK pathway (Figure 2 A and B) and the net effect of these changes might be more important for the MAPK/ERK activity state than a single up regulation of RASIP1 in a transient transfection assay. Furthermore, yet unknown feedback mechanisms in the promotion and attenuation of FGF signaling through the Ras/MAPK/ERK pathway might play a role (22) as we observed a strong FGF-signature in the differentially expressed MAPK/ERK KEGG signaling pathway in fibroblasts.

Although we cannot resolve the activity state of the MAPK/ERK pathway in SoS based solely on our data, lower activity rather than increased activity of the pathway in SoS would be more in line with literature since there is a clear association of an increased activity state of the MAPK/ERK pathway with short stature (25,36). In relation with this, in our microarray data, members of the FGF family such as FGF4, FGF6, FGF13, FGF18, FGF19 and FGFR2 contributed strongly to the differential expression of the MAPK pathway. FGF13 was even found to be the most influential gene in the KEGG MAPK signaling pathway and the second most influential in the GO MAPKKK pathway (Figure 2A and B). FGF13 belongs to a family of four fibroblast growth factor homologous factors which is conserved among vertebrates (37). These genes show structural sequence similarity with fibroblast growth factors (FGFs) but lack the secretion signal sequences and can therefore not interact with FGF receptors (37). FGF13 is expressed in the developing and adult mice nervous system (38), in human fetal and infant brain, in fetal muscle (39) and in chicken neural tissue (40). It is thought to play a role in patterning and growth of skeletal elements during chicken limb development (40). Unfortunately, no Fgfr13 -/- mutant mice are known today. A patient with an 46,Y,dup(X)(q26q28) including FGF13 has been described who exhibited short stature, severe developmental delay, hypotonia and several dysmorphic features (39). With regards to the expression patterns and available data it is tempting to speculate that reduced expression of FGF13 in SoS patients might be related to the abnormal neural development or increased longitudinal growth.
With regards to the “FGF imprint” in our data, FGF signaling through the MAPK/ERK pathway in the epiphyseal growth plate is known to play an important role in skeletal development (41). Gain-of-function alterations of the \textit{FGFR3} gene are the cause in several well-known growth failure disorders due to impaired endochondral bone formation such as achondroplasia and hypochondroplasia (reviewed in (36)). Mutant mice with a constitutive active \textit{FGFR3} mutation were dwarfed with shortened, disorganized epiphyseal growth plates containing few proliferating and hypertrophic chondrocytes (42). As an opposite phenotype, \textit{Fgfr3}\textsuperscript{-/-} mice showed skeletal overgrowth with increased long bones and vertebrae (43,44). The epiphyseal growth plates of these mice had an increased height caused by expansion of the zone of proliferating and hypertrophic chondrocytes (43,44). In general, FGFR3 is proposed to act as a negative regulator of bone growth by two downstream mechanisms: i.e. inhibiting chondrocyte proliferation through the STAT1-pathway (45) and by inhibiting hypertrophic chondrocyte differentiation through the MAPK-pathway (46). Not only upstream but also downstream components of the FGF-MAPK/ERK pathway are involved in growth regulation. Constitutive active mutations in more downstream genes such as \textit{KRAS} and \textit{BRAF} result in an increased activation of the MAPK/ERK pathway and hence in short stature syndromes as for example Noonan or cardio-facio-cutaneous syndrome (25,36).

Further evidence for the importance of the activity of the MAPK/ERK pathway can be derived from studies concerning the C-type natriuretic peptide (CNP). In the cartilage of transgenic mice with an increased expression of CNP a decrease of ERK1/ERK2 phosphorylation was detected showing that CNP inhibits the MAPK/ERK pathway (47). Similar results were found in the murine chondrogenic ATDC5 cell line (48). Furthermore, impaired growth in mutant mice with an activated \textit{Fgfr3} in their cartilage was rescued by an increased expression of CNP (47). Recently, increased expression of \textit{NPPC}, a precursor of C-type natriuretic peptide, has been shown to result in a tall stature phenotype (49,50).

All these observations suggest that the level of activation of the MAPK/ERK pathway is a determining factor for longitudinal growth and that this is regulated at the hypertrophic chondrocytes of the epiphyseal growth plate (46). This is especially interesting, since we have shown \textit{NSD1} expression in the terminally differentiated chondrocytes of normal human epiphyseal growth plates during different developmental ages (Figure 6). It is therefore tempting to speculate that deregulated MAPK/ERK signaling in SoS results in altered hypertrophic differentiation of \textit{NSD1} expressing chondrocytes and that this may be
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a determining factor in statural overgrowth and accelerated skeletal maturation in SoS.

In conclusion, for the first time SoS is shown to be associated with deregulation of the MAPK/ERK pathway. An altered activity of this pathway may be an important contributor to the longitudinal overgrowth.

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**Supplementary data**

**Table 1S. Analyzed KEGG Signaling pathways**

| KEGG pathway number | Description                                      |
|---------------------|--------------------------------------------------|
| 4010                | MAPK signaling pathway                           |
| 4012                | ErbB signaling pathway                           |
| 4310                | Wnt signaling pathway                            |
| 4330                | Notch signaling pathway                          |
| 4350                | TGF-beta signaling pathway                       |
| 4370                | VEGF signaling pathway                           |
| 4020                | Calcium signaling pathway                        |
| 4070                | Phosphatidylinositol signaling system             |
| 4150                | mTOR signaling pathway                           |

| GO-term              | Description                                      |
|---------------------|--------------------------------------------------|
| GO:0000165          | MAPKKK cascade                                   |
| GO:0003713          | Transcription coactivator activity               |
| GO:0004707          | MAPK activity                                    |
| GO:0004879          | Ligand-dependent nuclear receptor activity        |
| GO:0007173          | Epidermal growth factor receptor signaling pathway|
| GO:0007179          | Transforming growth factor beta receptor signaling pathway|
| GO:0007219          | Notch signaling pathway                          |
| GO:0007259          | JAK-STAT cascade                                 |
| GO:0008063          | Toll signaling pathway                           |
| GO:0008277          | Regulation of G-protein coupled receptor protein signaling|
| GO:0016570          | Histone modification                             |
| GO:0016922          | Ligand dependent nuclear receptor binding         |
| GO:0019722          | Calcium-mediated signaling                       |
| GO:0019933          | cAMP-mediated signaling                          |
| GO:0030111          | Regulation of Wnt receptor signaling pathway      |
| GO:0030296          | Protein tyrosine kinase activator activity        |
| GO:0030509          | BMP signaling pathway                            |
| GO:0030522          | Intracellular receptor-mediated signaling pathway  |
| GO:0031929          | TOR signaling pathway                            |
| GO:0035814          | Negative regulation of gene expression, epigenetic|
| GO:0045815          | Positive regulation of gene expression, epigenetic|
| GO:0045892          | Negative regulation of transcription, DNA dependent|
| GO:0045893          | Positive regulation of transcription, DNA dependent|
| GO:0048010          | Vascular endothelial growth factor receptor signaling pathway|
| GO:0048011          | Nerve growth factor receptor signaling pathway    |
Supplementary data

Figure 1S. Results of the outlier analysis
Bi-plots of the principal component analysis on the phosphorylation levels of all investigated proteins are shown for basal condition in (A) and after stimulation with RA in (B). Circles correspond with the 9 control samples and triangles with the Sotos samples. The arrow points to the detected outlier.
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