# From NSD1 to Sotos syndrome : a genetic and functional analysis

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# Chapter 11

## General discussion

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The aim of this study was to perform a comprehensive study of Sotos syndrome and it's molecular background. Therefore, the following three yet unresolved topics were addressed:

1. The molecular basis and mechanisms of the microdeletions in Sotos syndrome.
2. The causative molecular alterations in patients with features of Sotos syndrome but without *NSD1* abnormalities. With regard to this, a comparable study was performed focussing on recently discovered *TGFBR* mutations in Marfan syndrome.
3. The signaling pathways and downstream effectors of NSD1.

In this chapter our main findings will be discussed, as well as future perspectives.

## 1. The molecular basis and mechanisms of the microdeletions in Sotos syndrome

After the discovery of *NSD1* being the responsible gene for Sotos syndrome, a remarkable ethnic difference was found in prevalence of microdeletions and intragenic point mutations. In approximately 50% of the Japanese Sotos syndrome patients a commonly sized microdeletion (~2.2 Mb) was found (Chapter 3, Table 1). In contrast, in non-Japanese Sotos syndrome patients, microdeletions are detected in approximately 6 to 10% of the patients. Previously it was shown that the breakpoints of the recurrent microdeletion clustered in blocks of highly homologous sequences (1). However, a detailed molecular structure of these regions and the mechanism through which they mediate the formation of the microdeletion remained unknown.

In chapter 4 we have shown that these blocks consist of a 394.0-kb sized proximal low copy repeat (PLCR) and a 429.8-kb sized distal low copy repeat (DLCR), which have an overall sequence homology of ~98.5%. Additionally, we refined the deletion size to 1.9 Mb. The LCRs flank a ~1.3 Mb region encompassing multiple genes, including *NSD1*. A detailed analysis of the region revealed that the LCRs can be divided in different parts which are mainly in an inverted orientation, except for the PLCR-B and DLCR-2B parts which are in direct

orientation. By mapping at a nucleotide level the junction fragments of 37 out of 47 patients with a common deletion, we showed that the breakpoints cluster in a 3-kb recombination hotspot and that non-allelic homologous recombination (NAHR) between the directly orientated regions is the responsible mechanism underlying the typical microdeletions. This mechanism was, although in fewer Sotos syndrome patients, confirmed by others (2). Also in other genomic disorders such as Charcot-Marie-Tooth disease and Smith Magenis syndrome, NAHR was shown to be the predominant cause of microdeletions and reciprocal duplications (3).

Further evidence for NAHR as an important mechanism in microdeletions in Sotos syndrome was derived from the genomic analysis of four additional patients, described in chapter 5. Although these patients harboured deletion breakpoints outside the identified 3-kb recombination hotspot, we showed that their deletions were still caused by NAHR between the directly orientated LCRs. Recently, we have also mapped the breakpoints in three Sotos syndrome patients with non-recurrent, atypical sized deletions at the sequence level (4). Alu-repeat mediated recombination in two and a non-homologous end-joining mechanism in a third patient suggest that the molecular mechanisms causing the recurrent and non-recurrent deletions in Sotos syndrome are distinct.

It remains unknown why in approximately 80% of the patients the breakpoints are clustered in a 3-kb recombination hotspot that has been identified in chapter 4. For instance, its size is only 6% of the whole region in direct orientation (i.e. DLCR-2B). Contributing factors for increased susceptibility might be the increased sequence similarity (~99.4%) or a 10-fold increase of the translin motif. This motif has been found to be significantly more prevalent in regions in which translocations or deletions have occurred (5). However, when looking at analysis of similar hotspots in other genomic disorders, no consistent common recombination motif has been discovered yet (6,7). In chapter 5 we have shown that these hotspot regions consist of stabilised DNA duplexes which are flanked by destabilised DNA regions containing scaffold/matrix attachment regions. This suggests that a spatial chromatin configuration could predispose for a hotspot location during NAHR. However, since our data was derived from *in silico* analysis, *in vitro* and *in vivo* experiments are necessary for confirmation.

In six patients we were not able to identify the deletion breakpoints. This could be due to the technical limitations of our long-range PCR assay including possible nucleotide

polymorphisms at the primer-sites or the inability to develop completely overlapping primer-sets for PLCR-B and DLCR-B. Also more complex genomic rearrangements in these patients might have occurred.

By unravelling the molecular basis of the recurrent microdeletions we also sought to find an answer to the question why these microdeletions are more prevalent in the Japanese Sotos syndrome population. In 100% (18/18) of the fathers and in 85% (11/13) of the mothers we detected a heterozygous inversion of the segment between the flanking LCRs. In healthy controls these inversions were detected in ~67% of the males and in ~75% of the females. Similar inversion polymorphism have been known to predispose for deletions in genomic disorders such as Angelman syndrome and Williams-Beuren syndrome (8,9). Furthermore, studies of the 17q21.31 locus have shown that inversion polymorphism might result in different ethnic prevalence and hence in disease susceptibility (10,11).

However, several issues have to be solved before a causative relation between the inversion polymorphism and the higher prevalence of the recurrent microdeletions in Japanese Sotos syndrome patients can be drawn as a definite conclusion. At first, there is the issue of possible selection bias. The number of parents and especially also the controls are very small due to the limited availability of lymphoblastoid cell lines. In addition, cell lines from different ethnic populations should preferably be included. Secondly, there are technical limitations of a three-coloured FISH assay in interphases. Alternative techniques such as fiber-FISH should be considered to determine the spatial configuration of the probes and to exclude possible genomic rearrangements. An alternative technique to overcome this problem could be large-scale sequencing. Taking into account that one has to overcome the hurdle of sequencing in highly homologous low-copy-repeats, this technique might provide not only information about the frequency but also about the location of the inversion breakpoints. Recently, a sperm-based assay was used to determine the rates of *de novo* meiotic deletions and duplications causing Charcot-Marie-Tooth type disease type 1A/hereditary neuropathy with liability to pressure palsies, Williams-Beuren syndrome/dup7(q11.23), AZFa deletions/duplications and LCR17p deletions/duplications (12). Similar analyses performed in Japanese and non-Japanese males might also shed light on the ethnic difference in the occurrence of the typical microdeletions.

## 2. Causative molecular abnormalities in patients with features of Sotos or Marfan syndrome without detected genetic alterations in *NSD1* or *FBN1*

Overall abnormalities of the *NSD1* gene are found in 60-90% of the Sotos syndrome patients as we have shown in chapter 3 (Table 1). However this percentage can vary from as low as 13% in the setting of a diagnostic laboratory (13) to as high as 93% using strict clinical inclusion criteria (14). In a previous study, our group showed a detection rate of 81%, 36% and 0% in Dutch patients diagnosed respectively as typical, dubious and atypical Sotos syndrome (15). Therefore, there are a considerable number of patients either with a typical Sotos syndrome phenotype or with a Sotos syndrome-like phenotype without a confirmed molecular defect of *NSD1*.

We hypothesized that alterations of the *NSD1* promoter region could be the molecular cause in these patients (Chapter 6). In 18 typical Japanese Sotos syndrome patients, the promoter region was analyzed for both genomic sequence abnormalities as for epigenetic alterations such as hemizygous hypermethylation, which may result in haploinsufficiency of *NSD1* and hence in Sotos syndrome. However, we did not detect any abnormalities in the promoter region of these patients. Since upstream regulatory elements of *NSD1* are not known in detail, we cannot exclude that our analysis is incomplete since relevant regions may not have been included in this analysis. Recently, hypermethylation of the *NSD1* promoter region was indeed found in neuroblastoma and glioblastoma cell lines and resulted in a greatly diminished expression of the *NSD1* RNA transcript and protein expression (16). Admittedly, epigenetic changes are commonly found in cancer cells and are more rare in genomic DNA (17).

Recently, mutations of the *RNF135* gene were found to cause a phenotype which shows overlapping features with Sotos syndrome including overgrowth, learning disability and facial dysmorphism (18). We hypothesized that this gene might be responsible for a subset of patients with a Sotos-like phenotype. We analyzed 160 patients referred for *NSD1* analysis on suspicion of Sotos syndrome, but did not detect any pathogenic mutation rendering it unlikely that *RNF135* abnormalities are responsible for patients with features of Sotos syndrome (Chapter 7). In a previous study by others, the *NSD1* gene family members *NSD2* and *NSD3* were analyzed, but no mutations were identified either (19). Also screening of *NIZP1*, an *NSD1* interacting protein, in 17 characteristic Sotos syndrome patients

without detectable NSD1 mutations did not yield pathogenic mutations (20). Recently, abnormalities of the *NFIX* gene were identified in three Sotos syndrome-like patients but the frequency of involvement of this gene in other Sotos syndrome patient cohorts has yet to be established (21). Imprinting anomalies of 11p15, which are the cause of Beckwith-Wiedemann syndrome, were detected in 2 patients in a series of 20 Sotos syndrome patients without *NSD1* abnormalities (22). However, it remains unclear whether this is the result of an overlapping phenotype in these two patients or whether there is indeed an underlying molecular mechanism connecting *NSD1* and 11p15 anomalies. To date, no additional patients have been reported.

These studies exemplify the difficulties in finding molecular explanations in patients without abnormalities in *NSD1*. In characteristic patients with Sotos syndrome, *NSD1* abnormalities, even though not detected with the commonly employed techniques, still appear a likely culprit. New techniques, for example large-scale genomic sequencing for the detection of deep intronic mutations, will possibly contribute to the elucidation of these *NSD1* aberrations. An important limitation of the previous and our studies focussing on individual target genes is probably the heterogeneity of the patients in the groups studied. This, in combination with the low frequency of the genetic abnormalities searched for, constitutes an *a priori* low detection probability and hence a low detection rate. Furthermore, individual candidate genes are usually selected based on few available data. For example, since no other patients have been reported yet, the associated *RNF135* phenotype itself has yet to be confirmed by identification of additional patients. At the same time, only by investigating candidate genes in different patient populations, knowledge about these genes is gathered.

Despite the above mentioned low detection probability of a single candidate gene, this situation changes when there would be a functional relationship, i.e. belonging to the same functional pathway, of *NSD1.* The identification of *TGFBR2* mutations in a large family with a Marfan-like phenotype in combination with experimental data have shown that FBN1 regulates TGFβ activity by sequestering TGFβ (23). Successive identification of *TGFBR1* mutations in patients with manifestations of Marfan syndrome have enhanced the possibilities of genetic screening in these patients (24). In a panel of 49 patients with Marfan syndrome or a Marfan-related phenotype, we have identified *FBN1* mutations in 27 patients (55%), a *TGFBR1* mutation in one patient (2%) and *TGFBR2* mutations in two patients (4%) (Chapter 10). This was in accordance with other studies detecting *TGFBR2* mutations in “non-

FBN1 probands" in 5-10% and a lower detection rate for *TGFBR1* mutations (25-27). Although low in comparison to *FBN1* mutation detection rates, this still means that in nearly one in ten patients without an *FBN1* aberration a genetic cause will be found. Because FBN1 and TGFß belong to the same functional pathway, differentially expressed genes under influence of NSD1 haploinsufficiency, for example *RASIP1* (see further), become new candidate genes for patients with a Sotos syndrome-like phenotype but without *NSD1* abnormalities.

We then used an alternative approach, by turning to a broad technique, i.e. a genome-wide high resolution SNP-array. We analyzed 26 patients previously categorized as "typical" Sotos syndrome patients (n=3), "dubious" (n=15) and "atypical" (n=8). In 4 patients (15%) we detected novel abnormalities which are likely to explain the clinical manifestations found in these patients (Chapter 8). No recurrent abnormalities within our study population were identified, probably due to the heterogeneity of the patient group. This is best exemplified by patient 39 and patient 49 who both show features of Sotos syndrome with clinical scores of 6 and 5, respectively, while the genomic aberrations found differ significantly, i.e. encompassing multiple genes on two different chromosomal locations. A recent similar study using a 1-Mb resolution array-based comparative genomic hybridization array (CGH) investigated 93 patients with an overgrowth condition including several patients with Sotos syndrome (28). Also no recurrent genomic alterations were found, either within that study or when compared to our results. One of the limitations of the results in this kind of studies is that it is sometimes difficult to distinguish between a normal copy number variation or a pathogenic abnormality (29). Parental screening provides additional information, in specific increasing the likelihood of pathogenicity when an abnormality occurs *de novo*. However, the necessity for molecular validation to establish a definite causal relationship between the detected alterations and the exhibited phenotypes remains. On the other hand, because for example the deletion affecting *PLXDC2* in patient 45 has revealed a new candidate gene for overgrowth and/or a Sotos syndrome-like phenotype, screening of *PLXDC2* in patients with such phenotypes seems warranted to prevent delay from waiting for molecular validation. Unfortunately parental DNA for patient 45 was not available for further confirmation.

## 3. Signaling pathways and downstream effectors of NSD1

*NSD1* is expressed in a variety of tissues, including fetal and adult brain, skeletal muscle, spleen, thymus, lung and fibroblasts (30). In chapter 9 we have furthermore shown expression of *NSD1* in cartilage of normal human growth plates of different ages, specifically in the terminally differentiated hypertrophic chondrocytes. The protein was originally identified as both a corepressor as well as a coactivator of nuclear receptors such as the retinoic acid receptor and the thyroid receptor (31). Recent reports have shown that NSD1 exerts a methyltransferase activity through its SET-domain and specifically methylates the histone lysine residues H3-K36 and H4-K20 (32). Methylation of these histones is associated with regulation of chromatin transcription (32). It was described that epigenetic inactivation of NSD1 diminished trimethylation of H3-K36 and that this phenomenon is found in neuroblastoma and glioma cell lines (16). Similar abolishment of histone methylation was detected in Sotos syndrome patients and it can be hypothesized that in Sotos syndrome loss of repression of growth promoting genes results in an increased statural growth (33). However, the molecular signaling cascades through which NSD1 defects are translated into tall stature remain unknown.

In chapter 9 we have investigated these signaling pathways and downstream regulators by performing genome-wide expression arrays on mRNA of dermal fibroblasts from 9 Sotos syndrome patients and 9 age-sex matched controls. We have shown that few genes are differentially expressed with consistent up regulation of the *R*as *i*nteracting *p*rotein *1* (*RASIP1*) both in basal condition as well as after stimulation with retinoic acid (RA). We sought to find an explanation for the limited number of genes found overall to be differentially expressed between Sotos syndrome and control. One explanation could be in the model itself, i.e. that dermal fibroblasts are not the most optimal model for studying *NSD1* expression. On the other hand, Sotos syndrome patients exhibit nail hypoplasia in 2-15% of patients (34) with a possible overgrowth of the surrounding skin (35). In addition, in literature dermal fibroblast have been a successful model to unravel underlying mechanisms in patients with other growth disorders (36-39). A more likely explanation would lie in the reduced power of the experiment to obtain significance due to the relatively small number of samples in combination with a considerable level of biological variation (40).

Furthermore, we investigated possible associations with major signal transduction pathways by using a global test which includes analysis of all expression levels on the microarray chip. In the condition after stimulation with RA a significant association (p-value adjusted for multiple testing was 0.023) was found with the mitogen activated protein kinase (MAPK) pathway from the KEGG database and the MAPK-kinase-kinase GO-term. Within this pathway, *F*ibroblast *G*rowth *F*actor 13 (FGF13) seems to play an important influential role, but also *FGF4*, *FGF6*, *FGF18*, *FGF19* and *FGFR2* contributed strongly to the differential expression of this pathway (Chapter 9, Figure 2A). In addition, Limma-analysis showed that *FGF13* was also the most down regulated gene in Sotos syndrome patients (fold change of 11.1 and 13.5 in basal and in stimulated conditions, respectively). Unfortunately, this down regulation was not statistically significant. Additional protein phosphorylation studies showed a trend for lower phosphorylation levels in Sotos syndrome for MEK1, ERK1/ERK2 and ERK2 in basal conditions and for MEK1 after stimulation with RA suggesting a decreased activity of this pathway. Critical evaluation showed that Sotos syndrome patient nr. 2 behaved statistically distinct for all phosphorylated proteins in comparison to the Sotos and control group. Removal of this patient resulted in a more pronounced decreased activity of the MAPK/ERK pathway. On the other hand, Limma analysis showed differential expression of *RASIP1* with an up regulated expression level of approximately 4 fold in comparison with controls. RASIP1 is proposed to be a downstream Ras-effector which influences signal transduction in the RAS/MAPK/ERK pathway (41). In our transfection experiments RASIP1 dose-dependently potentiated bFGF induced expression of the MAPK-responsive SBE reporter construct. Therefore, because *RASIP1* was found to be up regulated in Sotos syndrome, this would in contrast indicate a possible increased activation state of the MAPK/ERK pathway.

How should these apparently contradicting results be interpreted? In chapter 9 we have discussed this intricate issue in more detail. An explanation might be found in the complexity of the cellular context, which is not mimicked in a transfection experiment of a single gene. For example, signaling of RAS/MAPK through RASIP1 is compartmentalized with complex downstream signaling (41). Furthermore, multiple genes are involved in the differential expression of the MAPK pathway (Chapter 9, Figure 2A and B) and yet unknown feedback mechanisms are thought to influence propagation of FGF signaling through the RAS/MAPK/ERK pathway (42,43).

Although there is need for further investigation, it is clear that all our results point into the direction of deregulated MAPK/ERK pathway in Sotos syndrome. In general, it is for the first time that Sotos syndrome is reported to be associated with a signaling pathway. The MAPK/ERK signaling pathway propagates extracellular signals such as growth factors or stress into intracellular responses and is therefore an important regulator of cellular processes such as differentiation, proliferation and apoptosis (43). It has been implicated in many human diseases such as Alzheimer disease, cancers and interestingly also growth disorders (44,45). Indeed, in the epiphyseal growth plate FGF signaling, especially the Fibroblast Growth Factor Receptor 3 (FGFR3), is known to be a major determinant of skeletal development (46). FGFR3 is thought to negatively influence bone growth by inhibiting chondrocyte proliferation through the STAT1-pathway and to inhibit hypertrophic chondrocyte differentiation through the MAPK-pathway (47,48). For example, gain-of-function alterations of *FGFR3* with an increased activity of the MAPK/ERK pathway are the cause of impaired endochondral bone formation in achondroplasia and hypochondroplasia (49), while *Fgfr3*$^{-/-}$ mice show skeletal overgrowth (50,51). In these mice the long bones and vertebrae were enlarged with an increased height of the epiphyseal growth plate caused by expansion of the proliferating and hypertrophic chondrocytes (50,51). Furthermore, constitutive active mutations in downstream genes of the FGF/RAS/MAPK/ERK pathway such as *KRAS* and *BRAF*, also result in an increased activity and hence in short stature syndromes as for example Noonan or cardio-facio-cutaneous syndrome (45,49).

In summary, longitudinal growth is greatly determined by the activity level of the FGF/RAS/MAPK/ERK pathway. Specifically this seems to take place in the hypertrophic chondrocytes of epiphyseal growth plates, in which we also demonstrated *NSD1* expression. Therefore, we propose that deregulation of this pathway in Sotos syndrome results in altered hypertrophic differentiation of *NSD1* expressing chondrocytes and may be a determining factor in statural overgrowth in Sotos syndrome.

## Concluding remarks and future perspectives

Firstly, from this thesis it can be concluded that NAHR is the underlying mechanism causing commonly sized microdeletions in Sotos syndrome. The location of the inversion breakpoints and its relationship with a higher prevalence of microdeletions in Japanese patients with

Sotos syndrome are interesting topics for future research. New techniques such as next generation sequencing and the recently described sperm-based assay could be useful to shed led on this topic. Secondly, in this thesis it was shown that in patients with features of Sotos syndrome but without *NSD1* abnormalities, genome-wide high resolution SNP array is a powerful method to attain a molecular diagnosis in comparison to a candidate gene approach. Molecular validation to confirm the pathogenicity of the copy number variants detected, should be a focus of future investigation. Furthermore, considering the heterogeneity in the Sotos syndrome-like population, clinically re-assessing the phenotypic features, possibly revisiting the clinical scoring system and including genes with a functional relationship with *NSD1* (e.g. *RASIP1*) might enhance the likelihood to detect genetic abnormalities in these patients. Finally, the conclusion that Sotos syndrome is associated with deregulation of the MAPK/ERK pathway is the first step in establishing a connection between *NSD1* abnormalities and disturbed longitudinal growth regulation resulting in tall stature in Sotos syndrome. Future challenges should focus on elucidating this connection in detail by identifying additionally involved proteins and further clarifying the molecular mechanisms.

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