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Further Evidence of Contrasting Phenotypes Caused by Reciprocal Deletions and Duplications: Duplication of *NSD1* Causes Growth Retardation and Microcephaly

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Key Words
aCGH · Duplication · Growth retardation · Microcephaly · *NSD1* · Sotos syndrome · 5q35

Abstract
Microduplications of the Sotos syndrome region containing *NSD1* on 5q35 have recently been proposed to cause a syndrome of microcephaly, short stature and developmental delay. To further characterize this emerging syndrome, we report the clinical details of 12 individuals from 8 families found to have interstitial duplications involving *NSD1*, ranging in size from 370 kb to 3.7 Mb. All individuals are microcephalic, and height and childhood weight range from below average to severely restricted. Mild-to-moderate learning disabilities and/or developmental delay are present in all individuals, including carrier family members of probands; dysmorphic features and digital anomalies are present in a majority. Craniosynostosis is present in the individual with the largest duplication, though the duplication does not include *MSX2*, mutations of which can cause craniosynostosis, on 5q35.2. A comparison of the smallest duplication in our cohort that includes the entire *NSD1* gene to the individual with the largest duplication that only partially overlaps *NSD1* suggests that whole-gene duplication of *NSD1* in and of itself may be sufficient to cause the abnormal growth parameters seen in these patients. *NSD1* duplications may therefore be added to a growing list of copy number variations for which deletion and duplication of specific genes have contrasting effects on body development.

As our understanding of the phenotypic consequences of genomic copy number variations (CNVs) increases, several examples of opposite phenotypes for deletions and duplications have emerged. Many of these phenotypes involve growth, with deletions and duplications

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having opposite effects on weight (e.g. proximal 16p11.2 microdeletion and microduplication or 17p11.2 in Smith-Magenis and Potocki-Lupski syndromes) [Jacquemont et al., 2011; Lacaria et al., 2012] or head size (e.g. proximal 16p11.2 or distal 1q21.1) [Brunetti-Pierri et al., 2008; Shinawi et al., 2010]. Similar opposite phenotypic effects have been proposed for behavioral phenotypes, such as those seen in Smith-Magenis and Potocki-Lupski syndromes or Williams-Beuren syndrome and its reciprocal duplication [Crespi et al., 2009]. Head growth and behavioral phenotypes have been proposed to be functionally related, with a correlation between the tendencies toward larger head sizes and autism spectrum disorders with some CNVs versus smaller head sizes and schizophrenia with their reciprocal copy state [Crespi et al., 2010]. Further functional support for this model of opposite phenotypes is provided by single-gene disorders in which activating mutations have opposite phenotypic effects from haploinsufficiency or dominant negative mutations. Many of these examples are also related to growth, such as mutations in the *AKT* genes with activating mutations in *AKT1* causing Proteus syndrome [Lindhurst et al., 2011], in *AKT2* causing hypoglycemia and overgrowth [Hussain et al., 2011] and in *AKT3* causing megalencephaly [Poduri et al., 2012; Riviere et al., 2012], whereas loss of gene function causes growth restriction (*Akt1* mouse model) [Chen et al., 2001], hyperglycemia and lipodystrophy *(AKT2)* [George et al., 2004], and microcephaly *(AKT3)* [Ballif et al., 2012]. Similarly, gain of function of *FGFR3* causes achondroplasia and other skeletal dysplasias, whereas loss of function causes tall stature in CATSHL (camptodactyly, tall stature and hearing loss) syndrome [Foldynova-Trantirkova et al., 2012]. In skull development, gain of function of *MSX2* or duplication of *MMP23A/B* causes craniosynostosis, whereas loss of function or deletion causes parietal foramina or late-closing fontanelles, respectively [Jabs et al., 1993; Wilkie et al., 2000; Gajecka et al., 2005].

Sotos syndrome is an autosomal dominant childhood overgrowth syndrome with additional features of characteristic dysmorphisms, mild-to-severe learning disabilities (LD) and advanced bone age. Some individuals may have cardiac or renal defects, seizures and/or scoliosis. The majority of affected individuals have heterozygous loss-of-function mutations within *NSD1* [Tatton-Brown et al., 2005a]. The syndrome can also be caused by heterozygous deletion of *NSD1,* and some of these deletions are caused by recombination between homologous low-copy repeats on 5q35 that mediate recurrent ~2.0-Mb deletions [Mochizuki et al., 2008]. Approximately 15% of Sotos syndrome in individuals of European ancestry are due to these recurrent or other atypical deletions [Douglas et al., 2005]; among individuals of Japanese ancestry, a common polymorphic inversion of the chromosomal region has led to ~50% of Sotos syndrome being caused by *NSD1* deletions [Kurotaki et al., 2003; Visser et al., 2005]. Reciprocal duplications of 5q35 encompassing *NSD1* have been proposed to cause a syndrome opposite to Sotos, characterized by growth retardation, microcephaly, developmental delay, and delayed bone age [Chen et al., 2006; Kirchhoff et al., 2007; Franco et al., 2010; Busse et al., 2011; Zhang et al., 2011]. To better understand and characterize the contribution of *NSD1* duplication to growth retardation and any other developmental phenotypes, we report clinical details of 12 affected individuals from 8 families known to have interstitial duplications involving *NSD1*.

Methods

From May 2004 to February 2012, samples from 53,059 probands were sent to Signature Genomic Laboratories for microarray-based comparative genomic hybridization (aCGH). Samples from 24,736 probands were analyzed on bacterial artificial chromosome (BAC)-based arrays (SignatureChip versions 1–4 and WG, Signature Genomic Laboratories, Spokane, Wash., USA), and 28,323 probands' samples were analyzed using whole-genome oligonucleotide-based arrays (SignatureChipOS version 1, 105K manufactured by Agilent Technologies, Santa Clara, Calif., USA, or versions 2 or 3, 135K manufactured by Roche NimbleGen, Madison, Wisc., USA; all custom designed by Signature Genomics) according to previously described methods [Bejjani et al., 2005; Ballif et al., 2008a, b; Duker et al., 2010]. All versions of the arrays have probe coverage of *NSD1*. Samples that had interstitial duplications involving *NSD1* identified by BAC arrays were rerun on an oligonucleotide-based array to refine the breakpoint locations. Additional individuals were identified following clinical aCGH testing at Seattle Children's Hospital, using a whole-genome, 105K-feature, oligonucleotide-based array (SignatureSelect 1.1, custom-designed by Signature Genomics, manufactured by Agilent Technologies) or a whole-genome, 135K-feature, oligonucleotide-based array (NimbleGen CGX, custom designed by Signature Genomics, manufactured by Roche NimbleGen) or at Cincinnati Children's Hospital, using a whole-genome, single nucleotide polymorphism-based array (610Quad SNP, Illumina, San Diego, Calif., USA), all according to manufacturers' instructions. Subjects identified at Signature Genomics had their duplications visualized through metaphase and interphase fluorescence in situ hybridization (FISH) with BAC clone RP11-99N22 or RP11-15L12, according to previously described methods [Traylor et al., 2009]. When available, parental samples were also analyzed using interphase FISH. Either de-identified clinical information was supplied, or informed consent for publication of clinical information and photographs was obtained according to a protocol approved by the Institutional Review Board-Spokane.

Table 1. Clinical features of individuals with *NSD1* duplications

Subject	1	2	3	4	5a	5b	6	7	8
Sex	female	female	male	male	female	male	male	male	female
Age	13 y	2 y 9 m	21 m	2 y 5 m	4 y	2 y	16 m	8 y	3 y
Duplication coordinates (hg18)	chr5: 175417567–177076245	chr5: 175512657–177360321	chr5: 175512657–177360321	chr5: 175512657–177360321	chr5: 175648881–177316439	chr5: 175512657–177360321	chr5: 175747659–177319010	chr5: 176395876–176761282	chr5: 176534825–180189576
Array platform	Illumina 610 Quad SNP	SignatureChip OS v2.0 (135K)	SignatureChip OS v2.0 (135K)	SignatureChip OS v3.0 (135K)	SignatureSelect 1.1 (105K)	NimbleGen CGX (135K)	SignatureChip OS v2.0 (135K)	SignatureChip OS v2.0 (135K)	SignatureChip OS v1.0 (105K)
Inheritance	unknown	de novo	unknown	maternal	likely maternal (5a and 5b maternal half-sibs)		de novo	maternal	not maternal
Growth parameters	short stature	Ht: –2.2 SD Wt: –3.0 SD OFC: –3.6 SD	Ht: –1 SD Wt <<5th %ile OFC <5th %ile	Ht: –1.5 SD Wt: –2.4 SD OFC: –4.1 SD	Ht: –1.6 SD Wt: –1.4 SD OFC: –2.6 SD	Ht: –1.45 SD Wt: –1.0 SD OFC: –2.0 SD	Ht: –3.5 SD Wt: –3.5 SD OFC: –3.8 SD	Ht: –4.1 SD Wt: –3.5 SD OFC: –4.4 SD	Ht: +0.7 SD Wt: +0.2 SD OFC: –3.9 SD
Bone age	NA	NA	NA	NA	NA	NA	NA	delayed: –3.7 SD	NA
Neuro-development	DD, ADHD	NS	DD, hypotonia	mild global DD, facial hypotonia	borderline DD, attention issues	expressive language delays, nl motor	speech delay, otherwise nl development	DD: special services in school, not doing math	DD: 8 m level at age 20 m
Dysmorphic features	NS	NS	hypertelorism, large PF, pectus excavatum	epicanthal folds, hypotelorism, long philtrum, upturned nose, full lips, micrognathia	upslanting PF, bilateral epicanthal folds, thin upper lip, mild bitemporal narrowing, upturned nose	right class II microtia	epicanthal folds	–	hypertelorism with telecanthus, narrow PF, hypoplastic alae nasi, micrognathia
Skeletal and digital anomalies	NS	NS	–	5th finger clinodactyly	mild 5th finger clinodactyly	right thumb duplication	prominent finger fat pads	short 5th fingers, possibly short ulna	metopic synostosis, left clubfoot
Other features	NS	hypoplastic left heart	tethered cord	strabismus, history of feeding problems	fine tremors, abnormal EEG without seizures, central apnea	normal to mild conductive hearing loss on left, middle ear dysfunction on right	feeding problems, decreased DTRs	small hydrocele (resolved), pituitary cyst (resolved), allergies, asthma, dermatitis	atrial septal defect
Family history	NS	NS	maternal aunt with cerebral palsy	mother's Ht: –0.8 SD, OFC: –3.8 SD, overweight, LD, depression; father's Ht: –2.6 SD, ADHD	mother's Ht: –1.7 SD, microcephalic, IQ 50–60, does not have custody of children		mother's Ht: +0.3 SD; father's Ht: –0.7 SD; brother's Ht: (at 6 y) +2 SD	mother's Ht: –1.7 SD, possible LD; brother (not tested) has LD, behavior problems, nl stature	NS

y = Years; m = months; Ht = height; SD = standard deviation; Wt = weight; OFC = occipitofrontal circumference; NA = not available; DD = developmental delay; ADHD = attention deficit hyperactivity disorder; NS = not specified; nl = normal; PF = palpebral fissures; EEG = electroencephalogram; DTRs = deep tendon reflexes; LD = learning disability; IQ = intelligence quotient; – = feature absent.

Results

Out of 53,059 probands' samples tested at Signature Genomics, 9 (0.017%) had interstitial duplications involving *NSD1*: 6 were whole-gene duplications (3 reciprocal to the recurrent ~2.0-Mb Sotos syndrome deletion), and 3 only partially duplicated *NSD1*. Clinical information was available for 6 of these 9 probands and for 2 probands tested at other laboratories; duplications range in size from 370 kb–3.7 Mb (table 1, fig. 1). None of these individuals (represented in table 1) carry any other clinically significant CNVs. Two of the >1.5-Mb duplications are apparently de

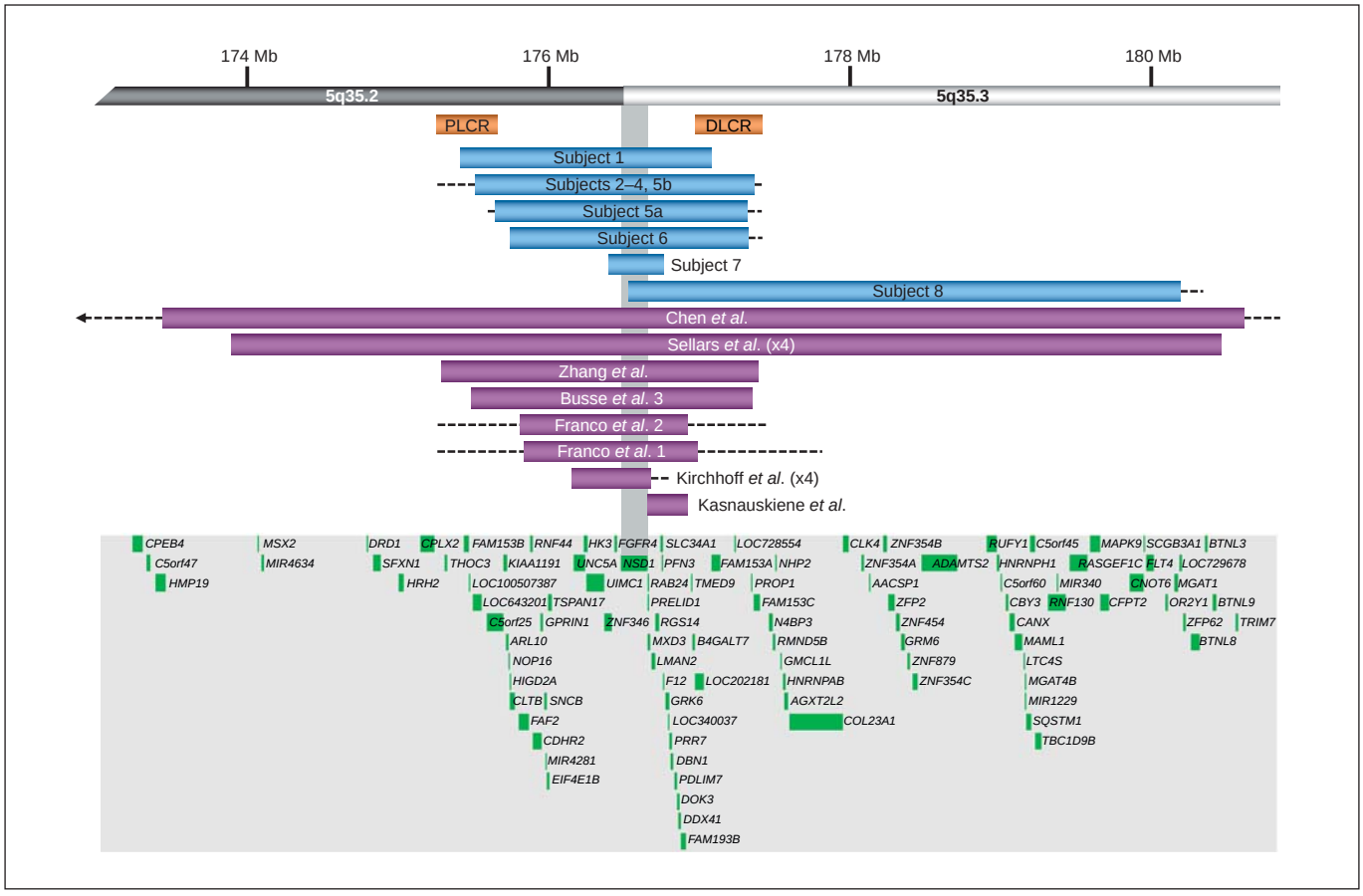

Fig. 1. Duplications in the 5q35 Sotos syndrome region in this cohort and the published literature. At the top of the figure is a partial idiogram showing chromosome bands 5q35.2qter with genomic coordinates corresponding to the hg18 build of the human genome. Orange bars represent the proximal and distal low-copy repeats (PLCR and DLCR) that mediate recurrent deletions and duplications [Mochizuki et al., 2008]. Blue and purple bars represent the duplications in this cohort and those in the literature, respectively. Tetrasomic individuals are indicated by the text 'x4'. Horizontal dashed lines extend through gaps in probe coverage to show the maximum possible size of duplications. Green bars represent the genes in this region, and the vertical, gray-shaded region marks the location of *NSD1*.

novo, whereas one small duplication (in subject 7; 370 kb) and one recurrent reciprocal duplication (in subject 4) were inherited from affected mothers, and another recurrent reciprocal duplication (in subjects 5a and 5b) is inferred to be carried by the half-siblings' affected mother. All individuals with whole-gene duplications of *NSD1* have growth parameters that range from severely restricted to below average, with a mean height of –1.96 standard deviations (SD) and mean head size of –3.5 SD. Children with *NSD1* duplications have a mean weight of –2.47 SD, whereas at least one carrier mother is overweight.

A comparison of the frequency of whole-gene *NSD1* duplications in our clinical aCGH population to that among published controls [Shaikh et al., 2009; Cooper et al., 2011] showed no significant difference (6/53,059 cases vs. 0/10,355 controls; one-tailed p = 0.34, Fisher's exact test). Due to the rarity of the duplication, larger populations are required for a more meaningful comparison.

Discussion

We report 12 individuals from 8 families with interstitial 5q duplications involving *NSD1*; all but one (subject 8) have a whole-gene duplication. The majority of these duplications are reciprocal to the recurrent ~2.0-Mb deletion seen in some individuals with Sotos syndrome. Our cohort also includes a mother and son who carry one of the smallest whole-gene *NSD1* duplications reported to date (subject 7, fig. 1). Similar to previous reports of inter-

Table 2. Summary of phenotypes among individuals with whole-gene *NSD1* duplications

Feature	Frequency (mean *z*-score)[a]
Short stature	9/18 (–2.4)
Underweight	9/13 (–2.9)
Microcephaly	16/16 (–3.6)
Delayed bone age	2/2
Developmental delay	17/17
Hypotonia	2/13
Behavioral abnormalities	3/13
Hearing loss	2/13
Seizures or abnormal EEG	2/13
Brain abnormalities	2/6
Strabismus	2/13
Dysmorphism	9/13
Digital anomalies	7/13
Heart defects, structural and functional	3/14
Hernias	1/13

[a] Includes all 11 subjects (probands and family members) in this report with whole-gene duplications and probands previously reported with molecularly defined duplication [Chen et al., 2006; Franco et al., 2010; Busse et al., 2011; Zhang et al., 2011] or triplication [Kirchhoff et al., 2007; Sellars et al., 2011] of *NSD1*.

stitial 5q35 *NSD1* duplications [Kirchhoff et al., 2007; Franco et al., 2010; Busse et al., 2011; Zhang et al., 2011] and larger, terminal 5q35 duplications in individuals with Hunter-McAlpine syndrome [Hunter et al., 2005; Chen et al., 2006; Sellars et al., 2011], growth retardation (especially microcephaly) and developmental delay (DD) or LD are prominent features in the majority of the individuals in our cohort (tables 1 and 2). While the prevalence of DD/LD in our cohort could be influenced by an ascertainment bias, as these are common indications for referral for clinical microarray-based testing, 3 carrier mothers also have intellectual or learning disabilities, supporting a causative association between *NSD1* duplications and DD/LD. Additional neurological features, including hypotonia, abnormal behaviors and seizures or EEG abnormalities, are reported in a minority of individuals with *NSD1* duplications. Dysmorphic features, frequently mild, as well as digital anomalies, including clinodactyly (subjects 4 and 5a), brachydactyly [Chen et al., 2006; Zhang et al., 2011] (subject 7), syndactyly [Zhang et al., 2011], polydactyly (subject 5b), and absent thumbs [Sellars et al., 2011], are also present in a majority of individuals (table 2). Brachydactyly was also reported in the original family described with Hunter-McAlpine syndrome, though both balanced translocation carriers and individuals with 5q duplications due to unbalanced translocations demonstrated this phenotype [Hunter et al., 2005]. Finally, subject 7 in our cohort has significantly delayed bone age, representing a second report of this feature with *NSD1* duplication and another example of an opposite feature from Sotos syndrome [Franco et al., 2010].

Phenotypes of individuals with *NSD1* duplications may be affected by the presence of additional genes within their duplications, similar to what is seen with deletions in the region. In general, individuals with Sotos syndrome carrying deletions that include genes other than *NSD1* have increased severity of LD, less pronounced overgrowth and, more commonly, cardiac anomalies and seizures [Nagai et al., 2003; Tatton-Brown et al., 2005b; Saugier-Veber et al., 2007]. Specific additional phenotypes are attributable to genes in the region: deletion of *SLC34A1* can lead to nephrocalcinosis and/or infantile hypercalcemia [Kenny et al., 2011], and factor XII deficiency may exist, depending on the genotype of the non-deleted *F12* allele [Kurotaki et al., 2005].

A majority of individuals reported with *NSD1* duplications have had digital anomalies (table 2). This phenotype may be attributable to, or influenced by, the duplication of *PDLIM7*, which encodes a PDZ-LIM scaffold protein that binds both actin and Tbx5, sequestering and repressing this transcription factor that has key roles in heart and limb development [Krause et al., 2004; Camarata et al., 2006]. It is known that haploinsufficiency for *TBX5* causes Holt-Oram syndrome with characteristic thumb anomalies and heart defects [Basson et al., 1997; Li et al., 1997], and knockdown of *Pdlim7* in zebrafish results in heart and pectoral fin (limb) defects [Camarata et al. 2010a, b]. Among individuals reported with 5q35 duplications, the more severe digital anomalies involve the thumbs, preaxial polydactyly in subject 5b and absent thumbs in a previously reported case [Sellars et al., 2011], consistent with *TBX5* defects. The patient with absent thumbs reported by Sellars et al. [2011] had tetrasomy of a 6.6-Mb interstitial 5q35 segment, possibly suggesting more severe effects with 2 extra copies of *PDLIM7*, although the large region of tetrasomy may contain additional genes contributing to the phenotype. Interestingly, subject 7, whose duplication does not involve *PDLIM7*, has short fifth fingers and possibly a short ulna, which may indicate that *NSD1* (which helps regulate proper expression of bone morphogenic protein 4, *BMP4* [Lucio-Eterovic et al., 2010]) or other genes in the duplicated region may also be altering skeletal development. Additionally, the interaction of Pdlim7 with Tbx5 in animal models makes *PDLIM7* a candidate for the heart defects. However, such malformations have

been rarely reported; subject 2 in our cohort has hypoplastic left heart, and subject 8 has an atrial septal defect; the case with tetrasomy of this region reported by Sellars et al. [2011] showed ventricular noncompaction, and a previously reported individual with a recurrent duplication had a ventricular septal defect [Busse et al., 2011].

The small duplication in subject 7 and his mother helps to narrow the critical region for the microcephaly, short stature and LD/DD phenotype associated with these duplications. This duplication includes, at most, 12 genes, including *NSD1* (fig. 1). Subject 8, whose shared duplication region with subject 7 includes 9 whole genes, does not show the below average stature that all other subjects in this cohort display. Also unlike the other subjects in our cohort, subject 8 cannot have a third, intact, functional copy of *NSD1*. Subject 8 does have significant microcephaly, and while this may indicate that microcephaly can be caused by duplication of a gene distal to *NSD1*, this could also be attributable to her craniosynostosis. Furthermore, Kasnauskiene et al. [2011] reported an individual presenting with a Sotos syndrome phenotype with a duplication overlapping the distal end of subject 7's duplication region (fig. 1). The mechanism through which this duplication may be interfering with *NSD1* expression remains to be determined, but combining this report with subject 8's phenotype makes it less likely that duplication of the genes distal to *NSD1* are responsible for growth retardation. This leaves 3 genes uniquely duplicated in our subjects with growth retardation: *ZNF346*, *FGFR4* and *NSD1*. Fibroblast growth factor receptor 4 (FGFR4) is a positive regulator of growth [Lazarus et al., 2007], so it is an unlikely candidate for causing growth retardation. *ZNF346* encodes a zinc finger protein that binds double-stranded RNA, and its overexpression in vitro induces apoptosis [Yang et al., 1999], so this gene could feasibly contribute to growth retardation. However, duplication of the entire *NSD1* gene remains a strong candidate for causing growth restriction. It encodes the nuclear receptor-binding SET domain-containing protein 1, a methyltransferase that works on histones to help regulate proper gene expression [Lucio-Eterovic et al., 2010; Wagner and Carpenter, 2012]. It is feasible that overexpression of *NSD1* could drive expression of its target genes in an opposite pattern from what occurs with *NSD1* haploinsufficiency, resulting in an opposite phenotype. Similar examples are in the literature of genes having opposite effects on growth with gain or loss of function, such as *KCTD13* within 16p11.2 [Golzio et al., 2012], the *AKT* genes [Hussain et al., 2011; Lindhurst et al., 2011; Poduri et al., 2012; Riviere et al., 2012] and *FGFR3* [Foldynova-Trantirkova et al., 2012].

Subject 8 (with a 3.7-Mb duplication) has craniosynostosis, a feature occasionally seen with terminal 5q duplications that has been previously attributed to *MSX2* [Kariminejad et al., 2009]. Our subject represents the first description of a molecularly characterized 5q35 duplication sparing *MSX2* in an individual with craniosynostosis. It is still likely that *MSX2* duplication contributes to craniosynostosis in the previously reported individuals, given that mutations in *MSX2* cause craniosynostosis [Jabs et al., 1993], as does overexpression in mice [Liu et al., 1995]. We cannot rule out that subject 8's duplication interferes with proper *MSX2* expression, particularly as the gene is hypothesized to be regulated by noncoding elements distant from the gene [Ott et al., 2012] and, therefore, may be more sensitive to changes in chromatin conformation. Alternatively, other genes in distal 5q35 have roles in bone development, and overexpression of these may also cause or contribute to the craniosynostosis in subject 8. There are several candidates in the region. *PDLIM7* expression induces bone formation [Boden et al., 1998; Liu et al., 2002]. *ZNF354C* encodes a transcription repressor involved in osteoblastic differentiation and overexpression of which can induce bone formation [Jheon et al., 2009]. *MAPK9* encodes a kinase involved in signal transduction pathways that is required for late-stage differentiation of osteoblasts and overexpression of which causes increased mineral deposition in bone [Matsuguchi et al., 2009]. Additional genes in the region may also play roles in bone formation, including *HNRNPAB* [Fomenkov et al., 2003], *ADAMTS2* [Bar-Yosef et al., 2008] and *SQSTM1* [Chamoux et al., 2009; McManus and Roux, 2012], although there is insufficient literature to support a direct mechanism of a copy gain of these genes. Finally, it is possible that subject 8's craniosynostosis is secondary to her microcephaly, and duplication of one or more genes distal to *NSD1* causes microcephaly, as opposed to causing craniosynostosis.

Molecular cytogenetic testing in individuals with neurodevelopmental disease and congenital anomalies has led to the discovery of many recurrent microdeletion and microduplication syndromes. The microdeletion syndromes are frequently the first to be characterized, often because the phenotypes are more severe, and more variability is seen with reciprocal microduplications. For some of these characterized reciprocal duplications, like those of distal 1q21.1 and proximal 16p11.2, an apparent opposite effect on growth emerges [Brunetti-Pierri et al., 2008; Shinawi et al., 2010]. We report a cohort of individuals with microduplications reciprocal to the ~2.0-Mb 5q35.2q35.3 deletions that cause the Sotos overgrowth syndrome. The duplications cause growth retar-

dation; the most notable is microcephaly, but height and childhood weight also range from below average to severely restricted. Therefore, duplications of the Sotos syndrome region are another example of a reciprocal duplication demonstrating an opposite effect on growth as compared to the deletion phenotype. With some deletions/duplications, single genes have been implicated in these effects on growth [Golzio et al., 2012], whereas with others the effects may rely on the involvement of a larger region and perhaps inclusion of multiple genes [Lacaria et al., 2012]. Given the growth retardation and small duplication in subject 7 and subject 8's above average stature and only partial duplication of *NSD1*, it is likely that whole-gene duplication of *NSD1* alone is sufficient to cause the growth phenotype, though this remains to be definitively proven. We also show that duplication carriers frequently have DD/LD and occasionally other congenital anomalies, providing a more complete phenotypic picture for these reciprocal Sotos-region duplications.

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