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Reversed clinical phenotype due to a microduplication of Sotos syndrome region detected by array CGH: Microcephaly, developmental delay and delayed bone age

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5q35 duplication presents with psychiatric and undergrowth phenotypes mediated by *NSD1* overexpression and mTOR signaling downregulation

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Declarations: Not applicable

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Availability of data and material: Copy number variants reported will be made available to public databases.

Animal Research (Ethics) : Not needed (flys are not covered in animal welfare)

Plant Reproducibility: N/A

Clinical Trials Registration: N/A

Image Manipulation: The authors attest that results are presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation (including image based manipulation

Web Resources
HUGO Gene Nomenclature Committee (HGNC): http://www.genenames.org/
Online Mendelian Inheritance in Man (OMIM): http://www.omim.org/
UCSC Genome Browser: http://genome.ucsc.edu/
WHO growth charts: http://www.who.int/childgrowth/standards/chts_wfa_girls_z/en/index.html
NIH Clinical Trials: https://clinicaltrials.gov/ct2/home
FlyBase: http://flybase.org/
Genotype-Tissue Expression (GTEx) project: https://gtexportal.org/home/

Supplementary Data
Detailed Patient information. Recommendations for initial evaluation of patients diagnosed with this microdeletion syndrome.

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Abstract

Purpose: Nuclear receptor binding SET domain protein 1, *NSD1*, encodes a histone methyltransferase H3K36. *NSD1* is responsible for the phenotype of the reciprocal 5q35.2q35.3 microdeletion-microduplication syndromes. We expand the phenotype and demonstrate the functional role of *NSD1* in microduplication 5q35 syndrome.

Methods: Through an international collaboration, we report nine new patients, contributing to the emerging phenotype, highlighting psychiatric phenotypes in older affected individuals. Focusing specifically on the undergrowth phenotype, we have modeled the effects of *Mes-4/NSD* overexpression in *Drosophila melanogaster.*

Results: The individuals (including a family) from diverse backgrounds with duplications ranging in size from 0.6–4.5 Mb, have a consistent undergrowth phenotype. *Mes-4* overexpression in the developing wing causes undergrowth, increased H3K36 methylation, and increased apoptosis. We demonstrate that altering the levels of insulin receptor (IR) rescues the apoptosis and the wing undergrowth phenotype, suggesting changes in mTOR pathway signaling. Leucine supplementation rescued *Mes-4/NSD* induced cell death, demonstrating decreased mTOR signaling caused by NSD1.

Conclusion: Given that we show mTOR inhibition as a likely mechanism and amelioration of the phenotype by leucine supplementation in a fly model, we suggest further studies should evaluate the therapeutic potential of leucine or branched chain amino acids as an adjunct possible treatment to ameliorate human growth and psychiatric phenotypes and propose inclusion of 5q35-microduplication as part of the differential diagnosis for children and adults with delayed bone age, short stature, microcephaly, developmental delay, and psychiatric phenotypes.

Keywords

NSD1; Delayed Growth; Microduplication syndrome; Psychiatric; mTOR

INTRODUCTION

Copy number variants (CNVs) in 5q35.2q35.3 have been suggested as an addition to the list of reciprocal loci microdeletion/duplication syndromes with “reverse” phenotypes, with the critical gene being Nuclear receptor binding SET domain protein 1-*NSD1*(MIM 606681) (Rosenfeld et al. 2013). This gene is a part of a family of three histone methyltransferases (HMT) encoded by *NSD1* (5q35.2q35.3), *NSD2* (4p16.3, MIM 602952), and *NSD3* (8p11.23, MIM 607083), which share 70–75% sequence homology and identical functional domains. It is the catalytic SET (Suppressor of variegation, Enhancer of zeste, and Trithorax) domain within each protein that determines the HMT specificity (Douglas et al. 2005). NSD family proteins are recognized as critical participants in chromatin integrity. Many studies link acquired mutations of the NSD family to a variety of cancers, including acute myeloid leukemia, multiple myeloma, lung, breast and prostate cancers (Vougiouklakis et al. 2015). Heterozygous microdeletions and single nucleotide variants (SNVs) of *NSD2* are associated with Wolf-Hirschhorn syndrome (Lozier et al. 2018).

Haploinsufficiency of HMTs and demethylases are implicated in multiple developmental disorders (Faundes et al. 2018). *NSD1* specifically modifies histone proteins through the transfer of methyl groups to lysine residues at position 36 of histone 3 (H3K36) and other residues, thus regulating chromatin integrity and gene expression (Li et al. 2009). *NSD1* is expressed in all tissues with the highest expression in brain, endocrine tissues, and reproductive organs, and plays fundamental roles in cell growth, keratin biology, and bone morphogenesis through its regulation of gene expression (Lucio-Eterovic et al. 2010). Haploinsufficiency of *NSD1* either by loss-of-function (LoF) SNVs or microdeletions leads to autosomal dominant Sotos syndrome 1 (SOTOS1) (MIM 117550). SOTOS1 has a well-defined presentation with overgrowth phenotypes including macrocephaly, advanced bone age, developmental delay (DD), and characteristic facial features (Tatton-Brown et al. 2005). Microduplication of 5q35.2q35.3 (microdup-5q35) patients exhibit undergrowth phenotypes including microcephaly, short stature, and delayed bone age, in addition to DD (Chen et al. 2006; Dikow et al. 2013; Franco et al. 2010; Jamsheer et al. 2013; Novara et al. 2014; Rosenfeld et al. 2013; Zhang et al. 2011). The recurrent ~1.9 Mb CNVs of 5q35 result from unequal crossing over through non-homologous recombination of same orientation low-copy repeats flanking *NSD1* (Kurotaki et al. 2005). The ‘reverse phenotype’ is further exemplified by the opposite DNA methylation signature in five individuals with NSD1 microduplication versus patients with NSD1 LoF (Aref-Eshghi et al. 2020; Peeters et al. 2020).

We report 9 new patients, along with the previously published patients, for a combined total of 39 patients with microdup-5q35. We expand the emerging phenotype, the long-term prognosis, penetrance, and expressivity of this microduplication syndrome. We identify the predisposition to psychiatric disorders, such us major depressive disorder (MDD), Attention-Deficit with/without Hyperactivity Disorder (ADHD/ADD), and possibly psychosis as an under-recognized feature of this syndrome and suggest that microdup-5q35 syndrome should be considered in the differential diagnosis for adults who present with developmental delay and psychiatric comorbidities with microcephaly, delayed bone age and short stature.

We then investigated potential molecular mechanisms underlying this disorder to gain insight on potential therapeutic approaches. Since *NSD1* LoF compared to overexpression results in opposite growth syndromes in humans, and in opposite DNA methylation signature (Aref-Eshghi et al. 2020; Peeters et al. 2020), we hypothesized that the PI3K/mTOR growth pathway might be involved in *NSD1*-associated effects on signaling. This pathway is a central regulator of body and organ growth. Its main component is the serine/threonine kinase mammalian target of rapamycin (mTOR), which exhibits an essential role in cell growth and development through protein synthesis and autophagy. Branched-chain amino acids (BCAA such as leucine, isoleucine, valine), and nutrients can also directly activate mTOR to connect to the endomembrane system in lysosomes, thus encountering the recombination-activating gene (RAG)- Ras homolog enriched in brain (RHEB) complex (Sancak et al. 2010). The mTOR pathway also interacts with epigenetic regulators (i.e. H3K36) required for modifying chromatin structure and function to control gene expression. Because altered nutrient states exert both individual and transgenerational phenotypic changes, mTOR signaling to chromatin effectors may have a significant role in mediating the effects of diet and nutrients on the epigenome (Laribee 2018).

Furthermore, decreased leucine levels have been found in unmedicated and non-duplicated patients with MDD and bipolar disorder (BD)(Fellendorf et al. 2018), leading the authors to suggest reduced mTOR activation as a factor contributing to the symptomology. We demonstrate that providing a leucine-rich diet throughout all larval stages rescues *Mes-4/NSD1* overexpression- induced cell death. Thus, we propose that leucine supplementation may trigger mTOR activation, thus rescuing cell death phenotype and possibly ameliorating not only the growth, but also the brain phenotype of this microduplication syndrome.

MATERIALS and METHODS

Patient Identification, Phenotyping, and Variant Identification

Through an international collaboration, we identified nine additional patients of diverse ethnic backgrounds with microdup-5q35 between 618 Kb to 4.5 Mb in size, encompassing a minimum of 21 and a maximum of 61 RefSeq genes (Figure S1). Clinical whole genome chromosomal microarray analysis was performed on blood from patients 1, 2, 3, 4, 5, and 6 using different platforms (Table S1). Patients 4, 7, and 8 were tested by metaphase and interphase FISH (Table S1).

Fly Stocks and Immunohistochemistry

Fly experiments were conducted as previously described (Ferguson and Martinez-Agosto 2014). Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose and 7% dry yeast. Canton-S was used as the wild type (wt) strain for all performed experiments. The strains apterous-gal 4(II)/Cyo, GFP, UAS-Mes-4/NSD (BL 22044, BL 15384), and UAS-IRwt were obtained from the Bloomington Drosophila Stock Center. We generated a UAS-Mes-4/NSD; UAS-IRwt stock using standard methods.

Brightfield images of third instar larvae and adult flies were obtained using a Leica light microscope. Larvae images were obtained at a magnification of 2.0X, adult flies at 1.6X

and adult wings at 3.2X. Third instar larvae tissues were dissected then fixed at room temperature in 3.7% formaldehyde. Tissues were washed in 0.4% Triton X-100 in primary antibody overnight at 4°C, washed, stained in secondary antibody for 2 hours, mounted using Vectashield (Vector Laboratories) and imaged using a Zeiss LSM5. Antibodies used include: Cleaved Caspase-3 (Cell Signaling; 1:50), Phospho Histone H3 (Cell signaling; 1:50), Gigas (DHSB, 1:20), and H3K36me2 (EMDMillipore, 1:100).

RESULTS

Clinical presentation

This study adds nine new patients (four females, five males), from four families of diverse backgrounds (Asian, Hispanic, Caucasian), to the emerging microdup-5q35 syndrome. Our patients have the typical features described for this syndrome such as intellectual delay and undergrowth phenotypes including microcephaly, short stature, delayed bone age, and minor skeletal anomalies, and failure to thrive (Tables 1, S1). Importantly, this cohort demonstrates an enrichment of neuro-psychological disorders from the general population. Behavioral phenotypes in the younger individuals (ages 5–7 years old) include attention deficit with/without hyperactivity, poor sociability, and oppositional behavior. Psychiatric phenotypes in older individuals (onset in their 20s) include severe depression, personality changes and behavioral disturbances (i.e. psychotic features) requiring hospitalization and affecting the ability to perform their job. The mother of four half-siblings with this syndrome is unhoused and has substance abuse issues, which resulted in all 11 of her offspring (from 10 different partners) being placed in foster care. Although this mother was not tested, given the pedigree and phenotype, the clinical team assumes she harbors the duplication present in 4 of her 11 offspring. Detailed description of each patient neuropsychological data is found in the supplemental information.

NSD overexpression downregulates mTOR signaling and leads to growth restriction through caspase-mediated cell death.

To elucidate the role *NSD1* microduplication might play in humans, we utilized *Drosophila melanogaster* as a model organism to analyze the effects of overexpression of *Mes-4/NSD,* the fly homolog of *NSD1.* The fly is an excellent tool for multiple reasons: flies typically lack gene redundancy and only have one gene copy of protein families (unlike vertebrates) and the fly wing size is a clear read-out of over/undergrowth phenotypes. We were able to specifically target and overexpress *Mes-4/NSD* in the wing without affecting other tissues by using the GAL4-UAS system with a wing-specific promoter in the *apterous* gene allowing for easy comparison of the wing growth to wild-type. Since final adult fly size is determined during larval stages, we focused on the growth of imaginal discs at the third instar larval stage. *Mes-4/NSD* overexpression in the developing wing and scutellum (plate covering the upper rear thorax) leads to an increase in H3K36 di-methylation marks as expected for its known function (Figure 2b” compared to 2a”), confirming that Mes-4 overexpression is associated with increased methyltransferase activity. This is associated with apoptosis detected through an increase in cleaved caspase staining compared to normal control imaginal wing discs (Figure 2b’ compared to 2a’). *Mes-4/NSD* overexpression results in growth restriction of the wing in the adult fly (Figures 2b and S3, compared to

2a). Since cell proliferation (using Phospho-Histone 3 (PH3) as a marker) is not affected by *Mes-4/NSD* overexpression (Figure 2b"", compared to 2b""), the observed increase in apoptosis demonstrates that the small wing size is due to increased cell death (Figure 2b'). These findings suggest that Mes-4/NSD overexpression induces apoptosis associated with a smaller wing size.

Similar undergrowth phenotypes have been observed in previous studies in which the mTOR pathway has been analyzed using the fly model (Katewa and Kapahi 2011; Potter et al. 2003). Since the PI3K/mTOR signaling pathway regulates cell and tissue growth, and the Insulin Receptor (IR) stimulates this pathway, we hypothesized that overexpression of *IR* may rescue the undergrowth phenotype. In contrast to the *Mes-4*/NSD overexpression associated decreased wing size (Figure 2b) and increase in cleaved caspase-3 staining (Figure 2b' compared to 2a'), overexpression of *IR* results in increased wing size (Figure 3c) and reduction of apoptosis (Figure 3c'). Furthermore, co-overexpression of both *Mes-4/NSD* and *IR* results in increased wing size (Figure 3d). The overexpression of both *Mes-4/NSD* and *IR* also decreased the apoptosis phenotype seen with overexpression of *Mes-4/NSD* alone (Figure 2d'). These findings suggest that under conditions of high *Mes-4*/NSD levels, additional IR expression modifies the decreased growth and increased cell death observed when *Mes-4/NSD* is overexpressed (Figure 4b).

NSD overexpression alters mTOR signaling and can be rescued through leucine supplementation

IR signaling activates the PI3K signaling cascade, which ultimately leads to activation of mTOR, a potent regulator of tissue growth through modulation of cell proliferation, cell size, and proliferation. mTOR is activated by direct binding of RHEB (Long et al. 2005), and TSC counteracts activation through its role as a GTPase activating protein converting active GTP-bound RHEB into inactive GDP-bound RHEB (Tee et al. 2003). Insulin stimulation leads to the PI3K signaling pathway activating AKT and thus promoting mTOR activity through TSC inhibition. In the developing Drosophila wing imaginal disc, mTOR inhibition induces apoptosis. We therefore hypothesized that a potential mechanism by which *Mes-4/NSD* overexpression could induce apoptosis and affect wing size, could be due to its effects on mTOR signaling. To further investigate mTOR inhibition, we determined levels of TSC, a known negative regulator of mTOR. Gigas (TSC2 fly homolog) expression is increased with *Mes-4/NSD* overexpression (Figure 2b"' compared to 2a"') suggesting that increased TSC levels may contribute to decreasing mTOR signaling (Figure 4b).

Given the increased Gigas/TSC2 levels, we hypothesized that bypassing this blockade through direct activation of mTOR would restore tissue growth. Leucine has been shown to be a powerful activator of mTOR function independent of IR-mediated PI3K signaling (Anthony et al. 2000). Leucine supplementation to the fly diet throughout larval stages was tested for its ability to rescue the observed apoptosis phenotype. While leucine supplementation has no effect on wild-type imaginal discs (Figure 3b compared to 3a), it reduced *Mes-4/NSD* overexpression-induced cell death as visualized by decreased activated caspase-3 staining (Figure 3b' compared to 3a', 4c). Feeding developing larvae with an

mTOR inhibitor (rapamycin) activates cell death in the developing imaginal disc (data not shown). Rapamycin minimally enhances the cell death phenotype in the presence of *Mes-4/NSD* overexpression (data not shown) suggesting that the observed apoptosis is due to an NSD-mediated decrease in mTOR activity. This finding coupled with the effects of leucine supplementation on reversing the observed apoptosis further suggest the role of mTOR signaling deficiency in mediating the *NSD* overexpression phenotype.

DISCUSSION

In this study, we expand the clinical phenotype of *NSD1* microdup-5q35 syndrome and propose a mechanism for the undergrowth phenotype observed in patients using an *Mes-4/NSD* overexpression experimental paradigm in *Drosophila melanogaster*. We expand the phenotypic spectrum of microdup-5q35 syndrome by reporting an additional nine individuals from four families (Figure 1). In all patients, the microduplication includes the entire *NSD1* gene (Figure S1), and one patient (# 4) demonstrates that duplication of *NSD1* alone is sufficient to recapitulate the undergrowth and psychiatric phenotype. The additional gene content (Figure S2) in the non-overlapping duplicated regions cannot explain their shared phenotype. Our participants, along with 30 patients previously reported, confirm that this microduplication has a recurring defined phenotype: short stature, DD/ID, microcephaly, low birth weight, and digital anomalies usually associated with delayed bone age (when assessed) (Tables 1 and S1, Figure S1 and S2). Other less common features include abnormal facies, teeth abnormalities, ear, kidney and heart abnormalities, hypotonia, constipation, and tremors. The family of four affected half-siblings and mother represent the complete penetrance but variable expressivity observed for the same microduplication, which has previously been reported in three other families (Dikow et al. 2013; Rosenfeld et al. 2013).

While we and others (Jeong et al. 2018) have previously proposed that *NSD1* overexpression may underlie the mechanism responsible for the undergrowth phenotype, here we demonstrate that overexpression of *NSD* is sufficient to mediate the growth phenotypes through its effects on the PI3K/AKT/mTOR pathway. Since the PI3K/mTOR pathway is a master regulator of cell proliferation, protein synthesis and cell survival, we hypothesized that *NSD1* overexpression affects this pathway. Our results are consistent with *Mes-4/NSD1* overexpression altering mTOR signaling, increasing apoptosis without affecting cell proliferation (Figure 2). Notably, patient 2 did not respond to growth hormone (GH) therapy, and others have ruled out GH deficiency as an endocrine cause of short stature in this syndrome (Jamsheer et al. 2013; Novara et al. 2014), further suggesting that a decrease in mTOR signaling may contribute to the undergrowth observed in these microdup-5q35 patients. mTOR signaling can be activated independently of PI3K with the addition of BCAA, such as leucine. We demonstrate that L-leucine supplementation rescued the caspase-induced cell death caused by *Mes-4/NSD* overexpression in the fly (Figure 3), thus further implicating the involvement of the cascade activating the PI3K/mTOR pathway. Multiple studies have demonstrated that dietary leucine supplementation increases tissue protein synthesis in weanling piglets (Suryawan et al. 2012) and increases hepatic mitochondrial DNA levels preventing mitochondrial dysfunction in neonatal pigs with intrauterine growth restriction (Su et al. 2017). This process involves cellular uptake

of L-leucine through the membrane-bound receptor SLC7A5/SLC3A2 triggering activation of mTOR in a Rag GTPase-dependent manner (Jewell et al. 2015), which leads to upregulation of translation. Leucine supplementation in other models of syndromic mTOR downregulation also has promising results. *TBCK*-related ID syndrome (MIM 616900) results from functional loss of *TBCK,* which leads to downregulation of mTOR signaling. Lymphocytes from affected individuals show a reduction in mTOR activation. Leucine addition to patients' lymphocytes cultures rescued mTOR activity (Bhoj et al. 2016). Roberts syndrome (RBS, MIM 268300) is due to SNVs in *ESCO2* eliminating the acetyltransferase activity that is necessary to hold sister chromatids together (Vega et al. 2010). *ESCO2* morphant zebrafish, like RBS patients, display an undergrowth phenotype. Both mutants and RBS patient's cells had a global increase in apoptotic cells mediated by caspases, and leucine supplementation suppressed levels of apoptosis, linking it to the mTOR pathway (Xu et al. 2013). Notably, zebrafish transgenic Cornelia de Lange syndrome (CdLS, MIM 122470, another undergrowth syndrome) mutants (*rad21*, *nipbla/b* and *smc3*) when treated with leucine, display partially rescued mTOR function, thus improving protein biosynthesis and cell division while inhibiting cell death (Xu et al. 2015). Thus, we conclude that while the genetic of human growth is rather complex and hardly reducible to the sole alteration of the PI3K /mTOR pathway, the decreased mTOR function is an emerging common mechanism for the growth restriction phenotypes observed in microdup-5q35 and similar undergrowth syndromes. Because of its relative low risk of harm, oral L-leucine is currently being tested in clinical trials for Diamond Blackfan anemia (NCT01362595, Phase 2), Type 1 diabetes mellitus (Desikan et al. 2010), body weight (NCT00683826)(Yao et al. 2016), muscle loss/sarcopenia (Band et al. 2018), and, notably, MDD (NCT03079297, Phase 2). Interestingly, in our cohort, four patients (#2, 3, 4, and 9), have debilitating depression (MDD), BD, and/or behavioral issues (Table S1). Others have noted in microdup-5q35 patients frequent and extreme mood swings (from extremely active and motivated to dysphoric and withdrawn), requiring psychiatric care and/or hospitalization, aggressive or emotional reactions, and restlessness (Dikow et al. 2013; Franco et al. 2010), or recurrent MDD (Table 1)(Novara et al. 2014; Rosenfeld et al. 2013). Of the 12 individuals older than 15 years reported to date, all but one has a psychiatric disorder, highlighting that this phenotype is also part of the clinical presentation of this syndrome, and not a prodromal manifestation of a late onset specific neurodegenerative disorder. Most individuals with microdup-5q35 reported to date are under the age of six, thus we strongly recommend close psychiatric monitoring, as these patients seem to be at high risk for developing depression, ADHD/ADD, and possibly psychosis later in life. However, reports of additional patients with similar mental illness would be helpful to further confirm and strengthen this association (Table 1).

In this study, we demonstrated *Mes-4/NSD* overexpression in *Drosophila* resulted in an increase of caspase activation through the alteration of mTOR signaling by TSC upregulation and decreased mTOR activity. Of note, altered expression of pro-apoptotic factors in postmortem brain tissue from BD patients and decreased AKT1 and mTOR mRNA expression levels in unmedicated BD patients during depressive episodes have been previously demonstrated (Machado-Vieira et al. 2015). These findings have made activation of the mTOR pathway in the medial prefrontal cortex of the brain a new therapeutic target

for depression (Réus et al. 2017). Thus, in the microdup-5q35 syndrome the erratic mood swings, diagnoses of ADHD in childhood and severe depression as an adult, may be directly impacted by the possible upregulation of caspases (increased apoptosis) in the brain similar to the findings observed in BD. However, given that our experiments did not evaluate fly brain tissue, additional studies are needed to confirm this hypothesis. Furthermore, recently it has been shown that mTOR activation is required for the rapid-antidepressant effects by ketamine, an NMDA receptor antagonist (Gerhard and Duman 2018), and a leucine amino acid sensor can directly and selectively activate the mTOR pathway to produce effects similar to those of ketamine for the treatment of MDD (Wolfson et al. 2016). Further studies should evaluate if long-term supplementation with this or a combination of many other BCAA at an early age may help serve as an adjunct therapy to lessen or delay the onset of psychiatric symptoms. While leucine supplementation has been promoted for a variety of reasons, potential risks of oral L-leucine supplementation when ingested with glucose, have been noted, such as synergistically stimulating insulin secretion and lowering blood glucose (Kalogeropoulou et al. 2008). Clinical trials, however, have noted no significant adverse effects between those who received leucine versus placebo. (Martinez-Arnau et al. 2020; Yoshimura et al. 2019). A number of preclinical and clinical reports, also supports the use of dietary supplementation with balanced amino acid formulas containing BCAAs to prevent cognitive function decline, disability and prolong healthy life expectancy of elderly subjects, and to enhance the cognitive recovery of patients with severe traumatic brain injury(Aquilani et al. 2005; D'Antona et al. 2010; Valerio et al. 2011).

In summary, we report on nine new patients with microdup-5q35 syndrome, who present with a core characteristic phenotype: short stature, intellectual disability/developmental delay (ID/DD), microcephaly, low birth weight, and digital anomalies. We provide evidence to suggest that psychiatric disorders including MDD with or without psychotic features, and ADHD/ADD should be considered part of the core phenotype. The microdup-5q35 syndrome should be included in the differential diagnosis for children with delayed bone age, short stature, microcephaly, ID/DD and psychiatric comorbidities. We demonstrate in Drosophila the effect and mechanism of *Mes-4/NSD* dosage on growth; *Mes-4/NSD* overexpression results in growth restriction through increased cell death signaling; and a leucine-rich diet rescues *Mes-4/NSD* induced cell death. Using this model, we demonstrate that *Mes-4/NSD* overexpression is the responsible gene within this regional duplication, solidifying its association with opposing phenotypes in human syndromes. There is human data supporting that an enriched mixture of BCAA, is more efficient in activating mTOR signaling in vivo compared to L-leucine alone (Valerio et al. 2011). Thus, we propose that supplementation with leucine or a mixture of BCAAs postnatally for these patients at the time of diagnosis could ameliorate growth and improve psychiatric symptoms. However, further studies are needed to examine the therapeutic potential of mTOR activation via L-leucine or BCAA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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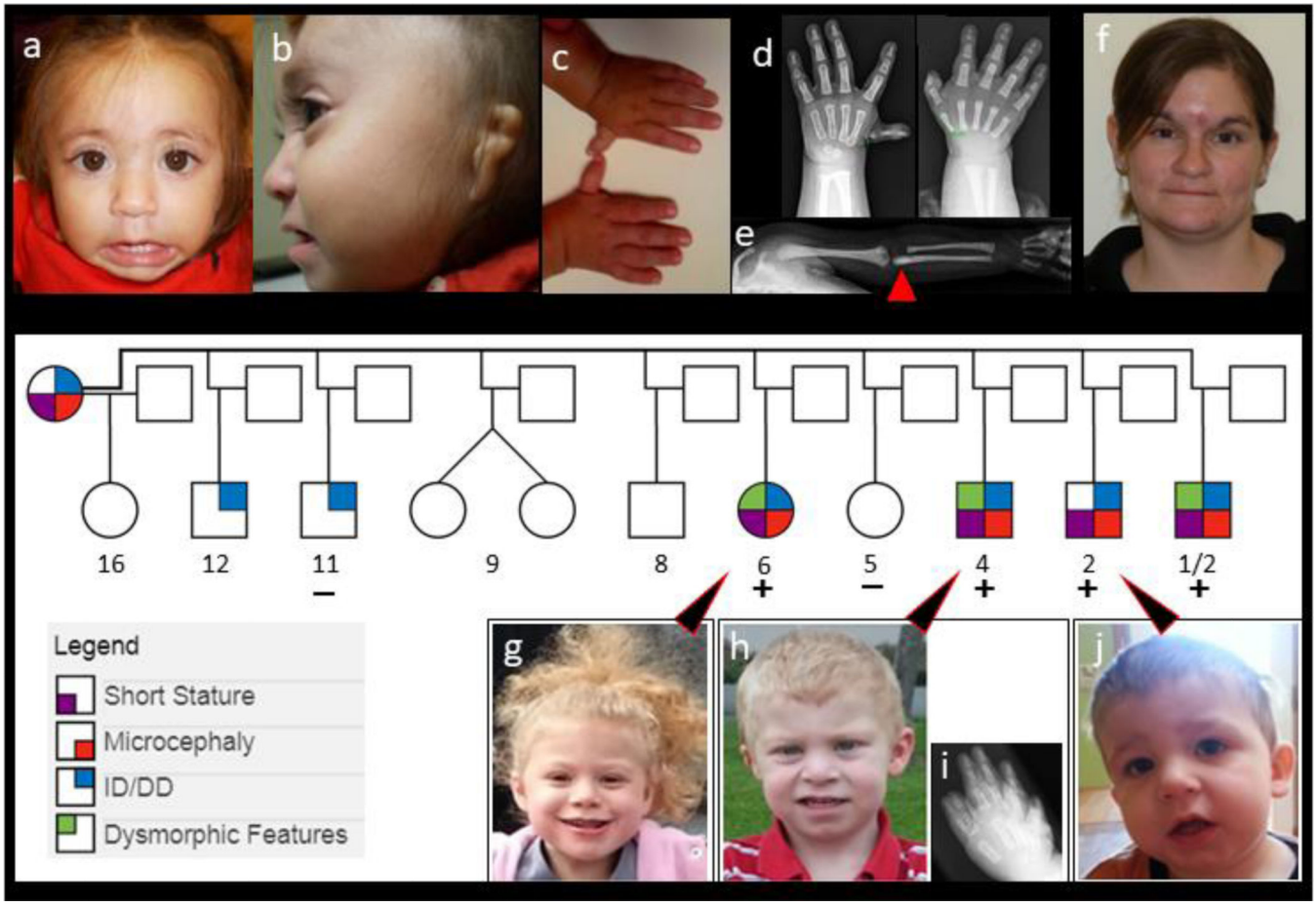

Fig. 1.

Clinical Features. Patient 1, front view, facial soft tissue asymmetry, flat nasal bridge (a) and side view, microtia grade 3 lobular type (b). Hand abnormalities including bilateral clinodactyly, tapered fingers (c): Hand radiographs demonstrating a subluxed left thumb extending at a 90-degree angle to the remainder of the hand, with marked shortening of the first metacarpal bone (d), proximal shortening of radii (arrow) (e). Patient 4, front view (f) deep set eyes and a prominent nose with a bulbous tip, thin upper and lower vermillion borders, and prognathism. Pedigree (Patients 5–9) with genotypes and images of each patient (g-j) and radiographic finding of duplicated right thumb (arrow) (Patient 6) (i)

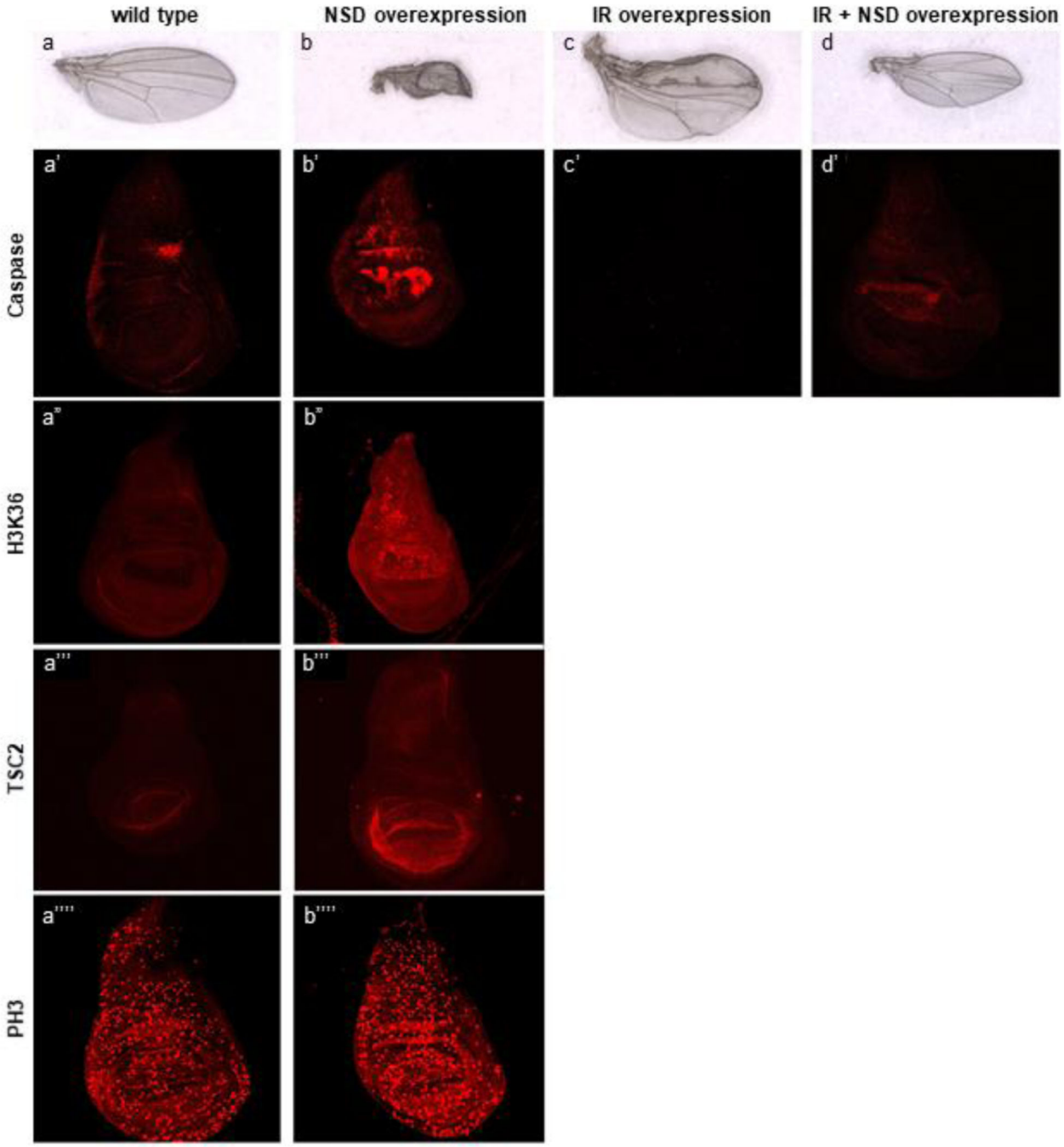

Fig. 2.

Tissue-specific Mes-4/NSD overexpression model in *Drosophila*. Comparative wild type *Drosophila melanogaster* wings in (a-a””). Overexpression of *Mes-4/NSD* results in smaller wing size (b), an increase of activated caspase staining (b’), and an increase of H3K36 methylation (b”) and Gigas/TSC2 levels (b”‘). Overexpression of *Insulin Receptor* (IR) results in larger wing size (c), and in reduced number apoptosis (c’). Co-overexpressed IR and *Mes-4/NSD* results in rescued wing size (d), and in rescued Mes-4/NSD mediated

cell death (d'). Phospho-Histone 3 (PH3) staining is comparable between fly wings overexpressing *Mes-4/NSD* (b"") and wild-type (a"")

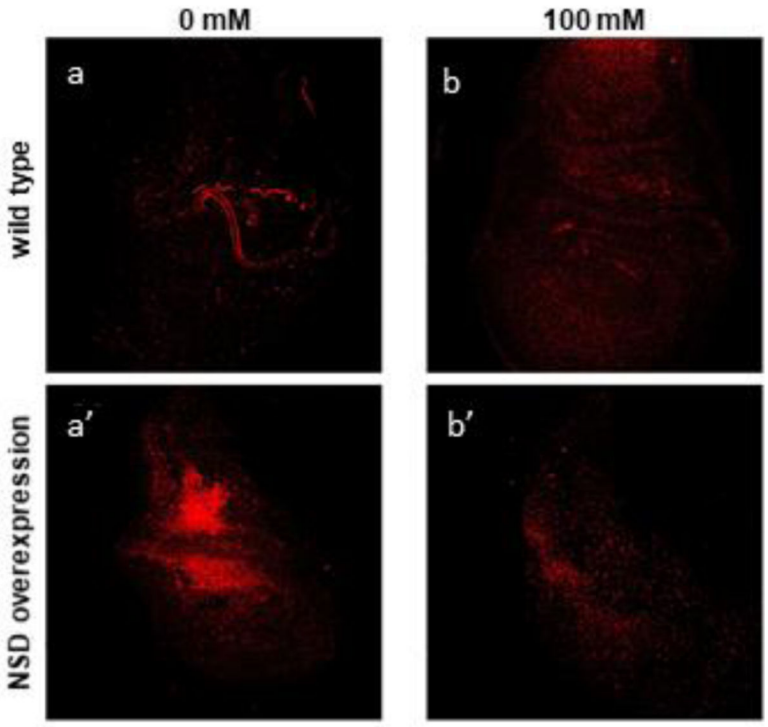

Fig. 3.

Tissue-specific *Mes-4/NSD* overexpression induced apoptosis is rescued with leucine supplementation. Overexpression of *Mes-4/NSD* results in an increase of activated caspase staining (a') compared to wild type (a). Leucine supplementation rescues caspase-mediated cell death in wing imaginal discs overexpressing *Mes-4/NSD* (b')

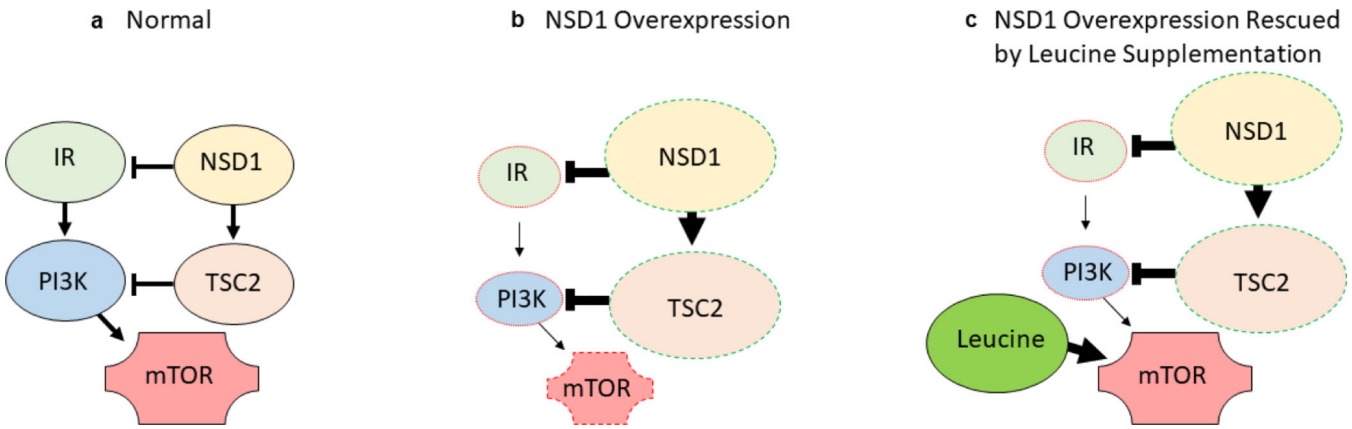

Fig. 4.

Model of NSD role in mTOR pathway signaling. Overexpression of *NSD* results in increased TSC levels that can be ameliorated by IR overexpression, and changes in mTOR signaling (b). mTOR activity is rescued through addition of exogenous leucine (c). Representation of expression: larger, green circles and thick lines, increased; smaller, red circles and thin lines, decreased

Table 1

Common phenotypes associated with 5q35.2q35.3/*NSD1* microduplication syndrome

Feature	Present (n=39*)	% Patients
Short Stature (<10th percentile)	35	90
Developmental/Intellectual Delay	34	87
Microcephaly (<2nd-3rd percentile)	29	74
Low Weight (<10th percentile)	25	64
Digital Anomalies	21	54
Behavioral Problems: • Mood Swings (6) • Drug/Alcohol abuse (2) • Aggression (5) • Depression (3) • ADD/ADHD (4) • Psychosis (3)	18 (n=42) **	43 **
Delayed Bone Age	7 (n=12) ***	58 ***

* Tbis study, Chen, Dikow, Franco, Jamsheer, KirchofE Novara, Reis, Rosenfeld, Sachwitz, Zhang, Zilina

** Estimate includes 3 mothers, 11 of 12 (92%) >15 y.o. have psychiatric phenotypes

*** Only 12 patients had an evaluation of bone age