

De Novo Mutations in *SON* Disrupt RNA Splicing of Genes Essential for Brain Development and Metabolism, Causing an Intellectual-Disability Syndrome

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The overall understanding of the molecular etiologies of intellectual disability (ID) and developmental delay (DD) is increasing as next-generation sequencing technologies identify genetic variants in individuals with such disorders. However, detailed analyses conclusively confirming these variants, as well as the underlying molecular mechanisms explaining the diseases, are often lacking. Here, we report on an ID syndrome caused by de novo heterozygous loss-of-function (LoF) mutations in *SON*. The syndrome is characterized by ID and/or DD, malformations of the cerebral cortex, epilepsy, vision problems, musculoskeletal abnormalities, and congenital malformations. Knockdown of *son* in zebrafish resulted in severe malformation of the spine, brain, and eyes. Importantly, analyses of RNA from affected individuals revealed that genes critical for neuronal migration and cortex organization (*TUBG1*, *FLNA*, *PNKP*, *WDR62*, *PSMD3*, and *HDAC6*) and metabolism (*PCK2*, *PFKL*, *IDH2*, *ACY1*, and *ADA*) are significantly downregulated because of the accumulation of mis-spliced transcripts resulting from erroneous *SON*-mediated RNA splicing. Our data highlight *SON* as a master regulator governing neurodevelopment and demonstrate the importance of *SON*-mediated RNA splicing in human development.

Recent advances in whole-exome and whole-genome sequencing have accelerated the identification of the genetic etiologies of intellectual disability (ID) and developmental delay (DD), facilitating appropriate care and therapy for affected individuals and their families. So far, mutations in more than 1,500 genes have been implicated in ID and DD disorders,^{1–9} and de novo single-nucleotide variants and copy-number variations (CNVs) have been identified as a major cause of severe ID and/or DD.^{5,7} Recently, two independent studies reported on a single individual with ID and/or DD and a de novo mutation in *SON* (*SON* DNA binding protein [MIM: 182465]), which encodes a protein required for proper RNA splicing. However, the level of evidence required for securely implicating mutations in this gene as disease causing was lacking.^{5,10,11} Including these two individuals, we recruited a total of 20 unrelated individuals with mild to severe ID and/or DD

(Figure 1A and Table S1) and report on the delineation of an ID syndrome caused by de novo LoF mutations in *SON*. This study was approved by the local institutes under the realm of diagnostic testing.

We compared in detail the phenotypic characteristics of all 20 individuals with *SON* LoF mutations. Clinical examination showed that all affected individuals had mild to moderate facial dysmorphisms, including facial asymmetry, midface retraction, low-set ears, downslanting palpebral fissures, deep-set eyes, horizontal eyebrows, a broad and/or depressed nasal bridge, and a short philtrum (Figures 1B and Figure S1). Interestingly, brain MRI, available for 19 affected individuals, revealed that 17 of them had significant abnormalities, including abnormal gyration patterns (polymicrogyria, simplified gyria, and periventricular nodular heterotopia), ventriculomegaly, Arnold-Chiari malformations, arachnoid cysts, hypoplasia of the

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corpus callosum, hypoplasia of the cerebellar hemispheres, and loss of periventricular white matter (Figures 1C–1E). 11 of 20 individuals developed seizures and/or epilepsy with an age of onset ranging from 1 to 6 years. 17 of 20 individuals presented with musculoskeletal abnormalities, comprising hemivertebrae, scoliosis or kyphosis, contractures, hypotonia, and hypermobility of the joints. Vision problems, including cerebral visual impairment, hypermetropia, optic atrophy, and strabismus, were present in 15 of 20 individuals. In addition, the vast majority of individuals showed congenital malformations consisting of urogenital malformations (6/20), heart defects (5/20), gut malformations (3/20), and a high and/or cleft palate (2/20). Short stature was present in ten individuals, and craniosynostosis involving both the metopic ($n = 1$) and the sagittal sutures ($n = 2$) was noted in 3 of 20 individuals. Metabolic screening was performed in 9 of 20 individuals, confirming mitochondrial dysfunction in individuals 2 and 11 and an O-glycosylation defect in individual 20 (a clinical summary is provided in Table 1, and details are listed in Table S2). Apart from individuals 13 (II-1 in family 13; Figure 1A), 15 (II-3 in family 15), and 20 (II-1 in family 20), none of the individuals had additional coding-sequence mutations that explained (part of) the phenotype (Table S2). Individual 13 was clinically diagnosed with dyskeratosis congenita, for which a maternally inherited pathogenic *TERT* (MIM: 187270) mutation was identified (Table S2). Individual 13 was, however, more severely affected than could be explained by a *TERT* mutation alone. Simi-

larly, none of the other coding variants identified in individual 15 or the additional genes deleted by the 384 kb deletion CNV in individual 20 were likely to explain the phenotype of these individuals (Table S2).

SON (GenBank: NM_138927.2) is composed of 12 exons (Figure 2A) and encodes a protein (GenBank: NP_620305.2) containing an arginine/serine (RS)-rich domain and two RNA-binding motifs (a G-patch and a double-stranded RNA binding motif) (Figure 2A).^{12–14} 17 of 20 mutations are frameshift mutations, including a recurrent 4-bp deletion (c.5753_5756del) in four independent individuals (Table S1 and Figures 2A and 2B). The remaining mutations include a nonsense mutation, an in-frame deletion of eight amino acids, and a complete gene deletion (Table S1). Importantly, parental DNA was available for testing in 19 of 20 individuals and indicated that all mutations had occurred de novo (Figure S2 and Table S1). Interestingly, de novo truncating mutations in SON have not been observed in over 2,000 control individuals,^{4,15–18} and SON, with a Residual Variation Intolerance Score of -1.88 , belongs to the 2% most intolerant human protein-coding genes.¹⁹ Furthermore, interrogation of large databases (such as the Exome Aggregation Consortium [ExAC] Browser) has shown that, after sequence context and mutability are considered, SON is significantly depleted of LoF variants according to multiple LoF metrics ($pLI = 1.00$, and the false-discovery rate of the LoF depletion score is $p = 1.68 \times 10^{-6}$).^{20,21} Although these population genetic signatures of intolerance cannot

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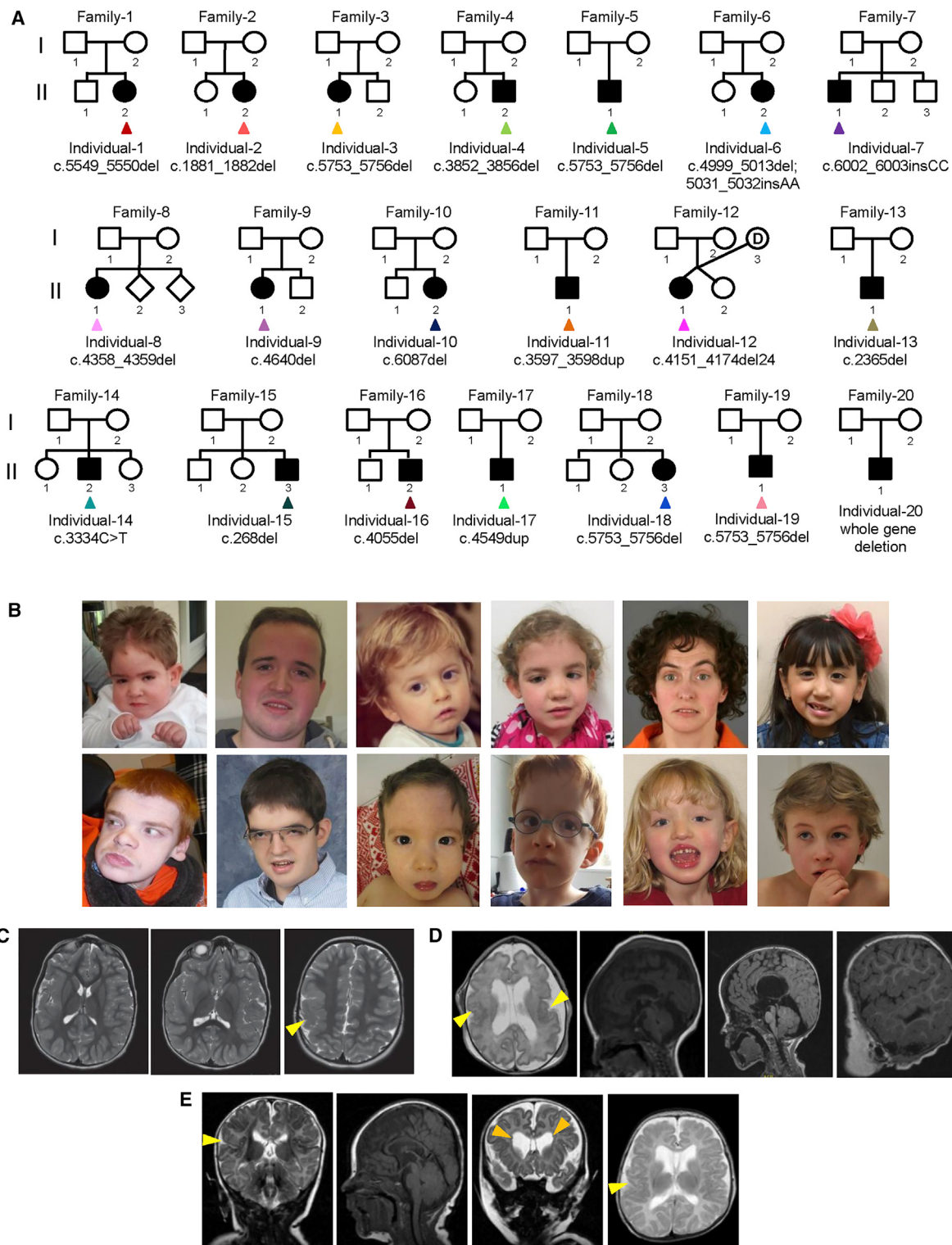


Figure 1. Pedigree Structures, Photos, and Brain MRI of Individuals with SON Mutations

(A) Family pedigrees of individuals carrying mutations in *SON*.

(B) Top row from left to right: photos of individuals 2 (at age 5 years), 4 (age 19 years), 5 (age 2 years), 6 (age 6 years), 8 (age 34 years), and 10 (age 6 years). Bottom row from left to right: photos of individuals 11 (age 21 years), 13 (age 14 years), 15 (age 15 months), 16 (age 5 years), 18 (age 6 years), and 19 (age 10 years). Shared facial dysmorphisms include facial asymmetry, midface retraction, low-set ears, downslanting palpebral fissures, deep-set eyes, horizontal eyebrows, a broad and/or depressed nasal bridge, and a short philtrum.

(C) Axial T2-weighted fast spin-echo MRI of the brain of individual 1 at age 3 years. Three panels show ascending images (left to right) revealing that the individual's insular cortex on the right is thickened and featureless. Less impressive areas of similar change were noted in the posterior aspect of the left insular cortex, which revealed bilateral perisylvian and parietal polymicrogyria (yellow arrowhead).

(legend continued on next page)

Table 1. Clinical Features of Individuals with SON Haploinsufficiency

	Percentage	Number of Affected Individuals
Intellectual disability	100%	20/20
Brain malformation	89%	17/19
Ventricular enlargement	74%	14/19
Corpus callosum abnormality	53%	10/19
Cortex malformation	37%	7/19
White-matter abnormalities	21%	4/19
Cerebellar abnormalities	21%	4/19
Other	11%	2/19
Neurological features	85%	17/20
Seizures	55%	11/20
Hypotonia	75%	15/20
Musculoskeletal abnormalities	85%	17/20
Hypermobility	40%	8/20
Scoliosis or kyphosis	20%	4/20
Hemivertebrae	10%	2/20
Contractures	10%	2/20
Other	85%	17/20
Eye and/or vision abnormality	75%	15/20
Strabismus	55%	11/20
Suspicion of CVI	20%	4/20
Hypermetropia	30%	6/20
Heart defect	25%	5/20
Gastrointestinal malformation	15%	3/20
Urogenital malformation	30%	6/20
Horseshoe kidney	10%	2/20
Other	20%	4/20
Facial dysmorphism	100%	20/20
Short stature	50%	10/20
Craniosynostosis	15%	3/20

The following abbreviation is used: CVI, cortical visual impairment.

be considered sufficient evidence of causality on their own, they support the hypothesis that *SON* LoF mutations are under strong purifying selection in the human population

and that their occurrence most likely contributes to severe clinical phenotypes.

Transcripts bearing a premature stop codon are likely to be degraded by nonsense-mediated mRNA decay. To confirm that LoF mutations result in reduced dosage of *SON*, we used three different PCR primer sets (Table S3) to perform qRT-PCR to determine the amounts of the *SON* transcript in peripheral-blood mononuclear cells (PBMCs) isolated from trio 1 (I-1, I-2, and II-2 in family 1), trio 3 (I-1, I-2, and II-1 in family 3), individual 5 (II-1 in family 5; Figure 1A), and an unrelated healthy donor (Figure 2C). All three primer sets showed that compared to mRNA from the parental samples and the unrelated healthy donor, *SON* mRNA in the affected individuals was significantly downregulated (Figure 2C). Subsequent western blotting using PBMC lysates from trio 1 and two different *SON* antibodies consistently showed the reduction of *SON* in individual 1 (Figures 2D and 2E), indicating that de novo *SON* LoF mutations result in *SON* haploinsufficiency.

To examine the effect of *SON* haploinsufficiency on embryonic development, we utilized *Danio rerio* (zebrafish), which has a well-conserved homolog of human *SON* (NCBI Gene: LOC565999; Figures S3 and S4). We assessed the developmental effects of *SON* haploinsufficiency in vivo with morpholino (MO)-mediated knockdown of *son* in zebrafish embryos. Interestingly, embryos injected with a *son* MO had a host of developmental defects that ranged from bent tails (63.6%) to eye malformations and microcephaly (17.1%) and shortened or gnarled tails, deformed body axes, and massive body curvatures (2.1%) 24 hr post-injection (hpi) (Figure 3A and Figure S5). Embryos that survived 72 hpi progressed to more severe phenotypes including extreme spinal malformations (22.2%), head and eye malformations with brain edema (37.2%), and profound developmental abnormalities (10.1%; Figure 3B), mimicking features observed in the affected individuals.

SON is a nuclear speckle protein able to bind to both DNA and RNA, and its cellular functions include regulation of RNA splicing and gene transcription, as well as proper cell-cycle and embryonic stem cell maintenance.^{13,22–25} To identify molecular mechanisms underlying the clinical features of individuals with *SON* haploinsufficiency, we examined global expression patterns upon *SON* knockdown in cellular systems. Hereto, we re-analyzed microarray-based RNA-expression profiling and RNA-sequencing datasets generated upon *SON* knockdown in HeLa cells^{13,22} and human embryonic

(D) Sagittal T1-weighted and axial T2-weighted MRI of the brain of individual 2. The two images on the left (age 1 day; gestational age 34 + 6 weeks) reveal enlarged lateral ventricles, cavum septum pellucidum, a hypoplastic cerebellar hemisphere, a broad cistern magna, a small fourth ventricle, and a thin corpus callosum. The cortex shows a simplified gyration pattern, and the perisylvian and frontotemporal areas are suspect for polymicrogyria (yellow arrowheads). The two panels on the right (age 2 years) show the fissure Sylvie with an abnormal cortical border, an Arnold Chiari malformation, and hydrocephalus.

(E) Frontal T2-weighted, sagittal T1-weighted, axial T2-weighted, and sagittal T1-weighted MRI of the brain of individual 7 (II-1 in family 7) at the age of 2 months. The cortex shows deep sulci and perisylvian areas suspect for polymicrogyria (yellow arrowheads), as well as discrete heterotopic nodules (orange arrowheads). A thin corpus callosum, a small fourth ventricle, enlarged frontal horns of the lateral ventricles, and cavum septum pellucidum are present.

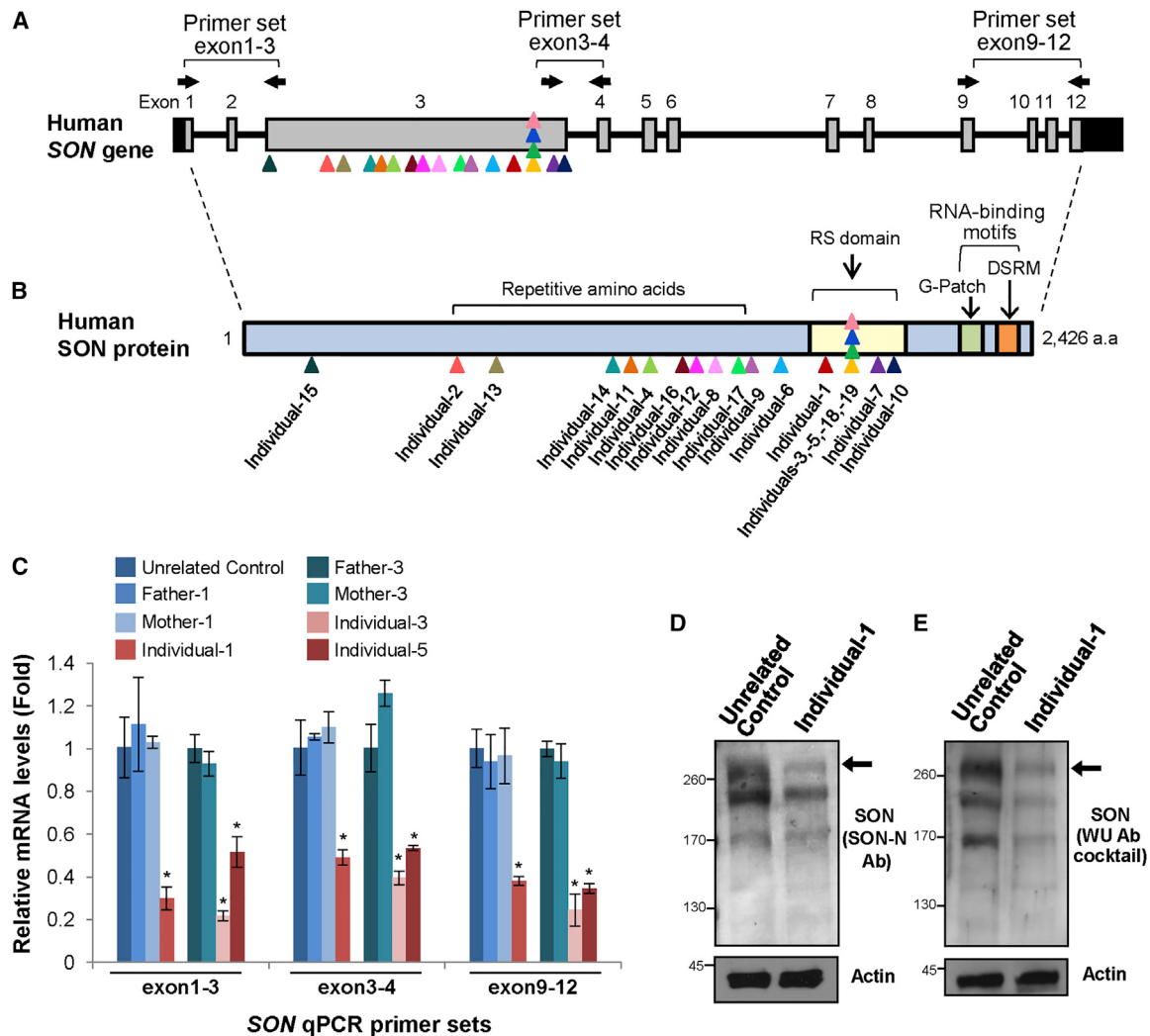


Figure 2. SON Mutations and Their Functional Effect at the RNA and Protein Levels

(A and B) Schematic representation of SON (A) and SON (B) shows the position of the mutations identified in the 20 affected individuals with color-coded arrowheads. The locations of the PCR primer sets are indicated by black arrows.

(C) Real-time qPCR with three different primer pairs showed that SON mRNA from the affected individuals was overall downregulated in comparison to mRNA from the parents and unrelated normal individual. Error bars represent mean $\pm$ SD. * $p < 0.001$.

(D and E) Western blotting demonstrated reduced expression of SON. SON-N antibody (1:1,000) was generated against amino acids 74–88 of the human SON (amino acid sequence DTELRYKPDLEKGSR). The cocktail of WU SON antibodies was a mixture of three different SON antibodies (WU09 [1:100], WU14 [1:2,000], and WU21 [1:200]). The epitopes of WU SON antibodies were as follows: MDSQMLATSS for WU09, CEESKTKSH for WU14, and SMMSAYERS for WU21. SON-N antibody (D) and the cocktail of SON WU antibodies (E) showed similar results. The bands indicated by the black arrow represent full-length SON. Other bands, which could represent potential isoforms, were also detected. Besides the bands present in samples from both normal and affected individuals, no other specific bands were detected in the affected individuals.

stem cells.²⁵ Surprisingly, from these previous datasets, we noticed that a group of genes playing pivotal roles in neuronal cell migration, embryonic survival, metabolism, and mitochondrial function, including *TUBG1* (MIM: 191135), *FLNA* (MIM: 300017), *PNKP* (MIM: 605610), *WDR62* (MIM: 613583), *PSMD3*, *HDAC6* (MIM: 300272), *PCK2* (MIM: 614095), *PFKL* (MIM: 171860), *IDH2* (MIM: 147650), *ACY1* (MIM: 104620), and *ADA* (MIM: 608958), showed significantly decreased expression upon SON knockdown (Tables S4 and S5).^{13,22,25} To investigate whether genes involved in regulating brain development and in metabolism are also downregulated in individuals

with SON LoF mutations, we measured the levels of RNA expression of these genes in PBMCs from trio 1, trio 3, and individual 5, as well as from an unrelated healthy donor (primers are listed in Table S3). Using qPCR analysis, we confirmed that all 11 genes were indeed significantly downregulated in individuals with SON haploinsufficiency (Figures 4A and 4B).

SON functions as a splicing co-factor that promotes correct and efficient RNA splicing of weak splice sites and alternative splice sites by facilitating spliceosome recruitment to the elongating RNA polymerase II complex.¹³ Prominent features observed upon SON knockdown in HeLa cells

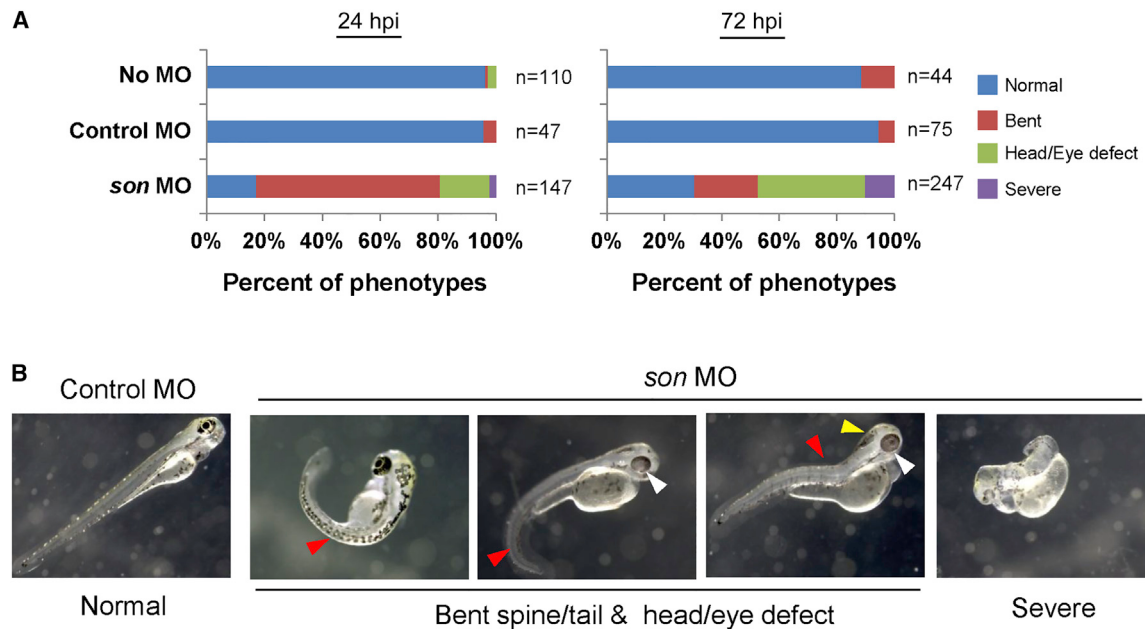


Figure 3. Targeted *son* Knockdown in Developing Zebrafish Causes Impaired Head Development and Spinal Malformations

(A) Zebrafish injected with a splice-blocking *son* morpholino (MO; 5'-TGGTCCTGGATATAACAGACAGATT-3', 6.25 ng) that targeted the junction between intron 9 and exon 10, a control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3', 6.25 ng), or no MO showed a normal phenotype, a bent spine or tail, a head or eye defect, or a severe phenotype at 24 and 72 hpi. The percentages of embryos with each phenotype are shown in the bar graphs, and the number of embryos examined is listed next to each bar.

(B) Representative images of the phenotype observed 72 hr after MO injection (red arrow, bent spine or tail; white arrow, eye defects; and yellow arrow, brain edema).

and human embryonic stem cells have included intron retention and exon skipping, which have been shown at the gene level for *TUBG1*, *HDAC6*, and *ADA*.^{13,22,25} We next sought to determine whether RNA splicing of these 11 genes is also impaired in our individuals with *SON* haploinsufficiency. To this end, we analyzed the pre-mRNA sequences of the remaining eight genes to predict weak splice sites that could be potential targets of *SON*-mediated RNA splicing (Table S6). We performed RT-PCR by using DNase-treated RNA samples isolated from trio 1, trio 3, individual 5, and an unrelated healthy donor and using primers designed to detect intron retention and exon skipping (Tables S6 and S7). We not only confirmed that these sites were indeed mis-spliced in HeLa cells upon *SON* knockdown (Figure S6) but also found that all three affected individuals showed significant intron retention (*TUBG1*, *FLNA*, *PNKP*, *WDR62*, *PSMD3*, *PCK2*, *PFKL*, *IDH2*, and *ACY1*) and exon skipping (*HDAC6* and *ADA*) at the predicted sites of the target pre-mRNAs and that this resulted in the accumulation of mis-spliced products (Figures 4C and 4D). In contrast, mis-spliced RNA products were absent in the parents and unrelated donor (Figures 4C and 4D). Together, these results indicate that *SON*-mediated RNA splicing is severely compromised in individuals with *SON* haploinsufficiency.

Our data have revealed that the complex neurodevelopmental disorder observed in these affected individuals is due to compromised *SON* function, which causes insufficient production of downstream targets as a result of erroneous *SON*-mediated RNA splicing. Moreover, the roles of

several downregulated genes are well-known causes of ID and/or DD in humans (Tables S4 and S5).^{4,6,26–35} For instance, *FLNA* haploinsufficiency is the most common cause of periventricular nodular heterotopia (MIM: 300049),³⁵ a rare brain malformation that we also found among our cohort with *SON* LoF mutations. Similarly, de novo LoF mutations in *TUBG1* are known to result in cortical malformations (MIM: 615412),³² also frequently observed in our cohort of affected individuals. Because we have shown that a substantial number of essential developmental genes are significantly downregulated upon *SON* haploinsufficiency, *SON* thus represents a master regulator of genes essential for human neurodevelopmental processes.

In summary, we have identified de novo LoF mutations in *SON* as a cause of a complex neurodevelopmental disorder associated with ID and/or DD and severe brain malformations. In addition, we have revealed the underlying molecular mechanism by showing that *SON* haploinsufficiency leads to defective RNA splicing of multiple genes critical for brain development, neuronal migration, and metabolism. Our findings thus greatly contribute to our understanding of how defective RNA splicing leads to human neurodevelopmental disorders.

Supplemental Data

Supplemental Data include six figures and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2016.06.029>.

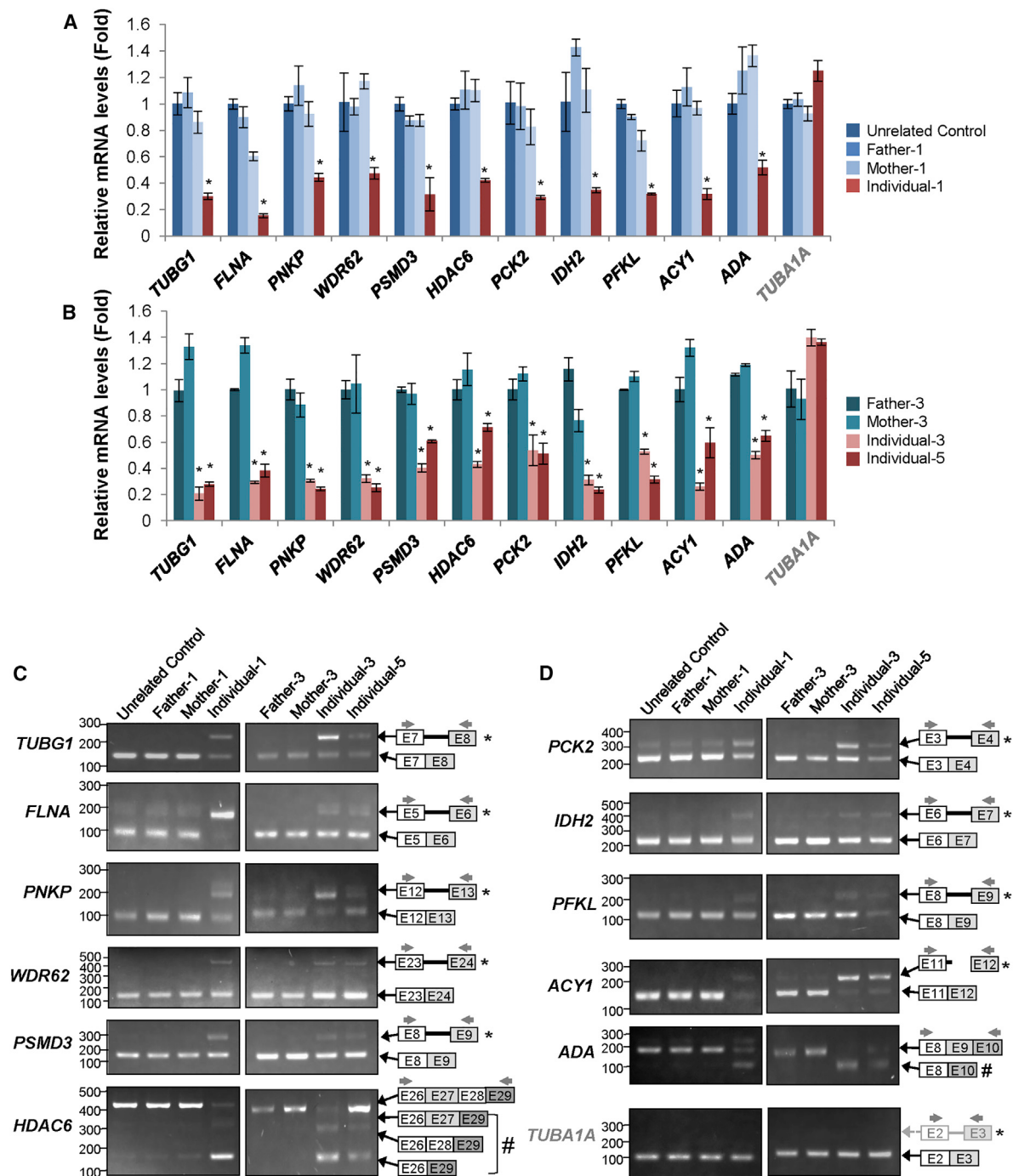


Figure 4. Individuals Carrying Heterozygous SON LoF Mutations Have Defective RNA Splicing of Genes Associated with the Pathophysiology of ID and/or DD and Metabolic Disorders, Resulting in Their Reduced Expression

(A and B) Multiple genes associated with the pathophysiology of ID and/or DD (A) and metabolic disorders (B) in the affected individuals were downregulated in comparison to genes from parents and unrelated healthy individuals. *TUBA1A* mRNA served as a negative control (unaffected transcript). Error bars represent mean $\pm$ SD. * $p < 0.001$.

(C and D) Intron retention and exon skipping of genes involved in ID and/or DD when mutated (C) and genes involved in metabolic disorders when mutated (D) in the individuals with *SON* mutations. The locations of the primers used for PCR are marked by gray arrows above the exons. Analysis of *TUBA1A* pre-mRNA, which served as a negative control, demonstrated that splicing of this transcript is not impaired in the affected individuals. *, intron-retained products; #, exon-skipped products.

Conflicts of Interest

D.N.S., S.T., and D.E. are employees of Ambry Genetics, Inc. M.T.C., K.M., G.D., B.B., A.B., and T.F. are employees of GeneDx, Inc.

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Web Resources

Clinical Genome Database, <http://research.nhgri.nih.gov/CGD/>
 DECIPHER, <https://decipher.sanger.ac.uk/>
 ESEfinder, <http://rulai.cshl.edu/tools/ESE/>
 ExAC Browser, <http://exac.broadinstitute.org/>
 Genic Intolerance, <http://genic-intolerance.org/>
 NCBI Gene, <http://www.ncbi.nlm.nih.gov/gene>
 OMIM, <http://www.omim.org/>
 RefSeq, <http://www.ncbi.nlm.nih.gov/refseq/>

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