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# Implication of *LRRC4C* and *DPP6* in neurodevelopmental disorders

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## Abstract

We performed whole-genome sequencing on an individual from a family with variable psychiatric phenotypes that had a sensory processing disorder, apraxia, and autism. The proband harbored a maternally inherited balanced translocation (46,XY,t(11;14)(p12;p12)mat) that disrupted *LRRC4C*, a member of the highly specialized netrin G family of axon guidance molecules. The proband also inherited a paternally derived chromosomal inversion that disrupted *DPP6*, a potassium channel interacting protein. Copy Number (CN) analysis in 14,077 cases with neurodevelopmental disorders and 8,960 control subjects revealed that 60% of cases with exonic deletions in *LRRC4C* had a second clinically recognizable syndrome associated with variable clinical phenotypes, including 16p11.2, 1q44, and 2q33.1 CN syndromes, suggesting *LRRC4C* deletion variants may be modifiers of neurodevelopmental disorders. *In vitro*, functional assessments modeling patient deletions in *LRRC4C* suggest a negative regulatory role of these exons found in the untranslated region of *LRRC4C*, which has a single, terminal coding exon. These data suggest that the proband's autism may be due to the inheritance of disruptions in both *DPP6* and *LRRC4C*, and may highlight the importance of the netrin G family and potassium channel interacting molecules in neurodevelopmental disorders.

### Keywords

Autism; sensory processing; *LRRC4C*; Netrin G; *DPP6*

## INTRODUCTION

While much progress has been made on the genetics of neurodevelopmental disorders (NDDs), over 50% of cases assessed clinically on any genetic platform are considered idiopathic [Krumm et al., 2014; Sanders et al., 2015]. While more NDD cases would likely be associated with genetic variation if all cases were assessed using the full extent of state-of-the-art technology, different strategies need to be employed to further unravel the genetics of NDDs. We have previously shown that balanced chromosomal rearrangement (BCR) sequencing is a powerful strategy to discover new genes for NDDs [Talkowski et al., 2011; Talkowski et al., 2012c], and here we report on a child who inherited two BCRs, one from each parent, each of which disrupted only a single gene: leucine rich repeat containing 4C (*LRRC4C* [MIM 608817]) and dipeptidyl-peptidase 6 (*DPP6* [MIM 126141]). Analysis of rare copy gains and losses in these genes in thousands of NDD cases suggest that that exon-disrupting CNVs in *DPP6* may be a contributor to NDDs and deletions in *LRRC4C* may be a modifier of genetic lesions associated with variable NDD phenotypes.

## METHODS

### Family recruitment and assessment

We obtained blood from all carriers of a BCR identified by karyotyping in a multigenerational family (46,XY,t(11;14)(p12;p12)mat), from which the proband, and the proband's mother, and grandmother were available for clinical and genetic testing. Participants or their guardians gave informed consent, using consent forms approved by the Douglas Institute Ethics Board. The proband was assessed with both the ADOS and ADI as part of a full clinical work-up done at the Montreal Children's Hospital. The mother of the proband confirmed that he met criteria for autism on both measures. The mother and the grandmother of the proband were assessed with the SCID-I for Axis-I mental disorders and SCID-II for personality disorders according to DSM-IV criteria. Current level of depression was assessed by the Beck Depression Inventory and the Hamilton Depression Rating Scale (HDRS-24). Cognitive functioning for these two individuals was investigated with the Stroop Color Test and the Hayling Sentence Completion test for cognitive inhibition, the Trail Making Test (TMT) A and B for flexibility/shifting, a categorical and semantic verbal fluency test, the National Adult Reading Test (NART) for a raw estimate of verbal IQ, the working memory subscale of the WAIS-III and the Iowa Gambling Task (IGT) for decision-making. Individual cognitive performance was then compared to performance from 81 healthy female controls from the GREFEX control group and 72 healthy female controls from our own database.

### Next generation sequencing of BCRs

Translocation mapping experiments were performed using customized large-insert, or "jumping library", whole genome sequencing [Brand et al., 2014] [Brand et al., 2015] [Talkowski et al., 2012b]. Reads were aligned with BWA and analyzed using Samtools. After data filtering, BAM files were processed using BamStat, a program developed to tabulate mapping statistics and output lists of anomalous read pairs (defined as having ends that map to two different chromosomes, having an abnormal insert size, or unexpected

strand orientations). Anomalous pairs were clustered by their mapped location with readPairCluster, a C++ program which performs a single-linkage clustering of paired-end reads if corresponding ends map within a specified distance (e.g., less than 10 kb) of each other.

## Human gene expression analysis

Post-mortem prefrontal cortex brain tissue from Brodmann Area 46 (BA46) was obtained from the Douglas Brain Bank as described elsewhere [Klempan et al., 2009]. Tissue came from three control individuals, and RNA was extracted using the Qiagen RNAeasy kit (Qiagen, Hilden, Germany). Commercially available RNA from seven additional tissues was obtained from Clontech Laboratories (Mountain View, CA), as follows: Frontal Lobe (Cat#636563), Spinal cord (Cat#636554), Hippocampus (Cat# 636593), Liver (Cat# 636531), Lung (Cat# 636524), Kidney (Cat# 636529) and Fetal brain (Cat# 636526). Reverse transcription was performed using the M-MLV reverse transcriptase enzyme and poly-dT primers to obtain complementary DNA (cDNA). Real-time PCR reactions were performed on an Applied Biosystems (Foster City, CA) 7900 HT system, using 2X iTaq Universal SybrGreen Supermix (BioRad, Saint Laurent, Canada). We used exon boundary-spanning primers, as follows: isoforms 1 and 2 of *LRRC4C* (leucine rich repeat containing 4C) were quantified together (NM_020929.2 and NM_001258419.1; F: TAAGTGGGTTCCAGTTTTGC / R: CCAACAGGTATTGATCTTCCTGAG). For *DPP6* (dipeptidyl-peptidase 6), we quantified the expression of isoform 3 (NM_001039350; primers F: AACGTGATGGAGCTGGTG / R: CCGCTGGTGTCAGAAGTATG).

## Magnetic Resonance Imaging (MRI)

Two members of the family, II-6 and III-2, underwent a structural magnetic resonance imaging (sMRI) session. Scanning sessions were conducted at the Douglas Institute Brain Imaging Centre, on a 3T Siemens Magnetom MRI scanner. Structural scans consisted of a high resolution, whole brain T1 acquisition. T1 weighted data were acquired using a flow-compensated 3D RF-spoiled GRE sequence with TR/TE/flip angle = 18 ms/10 ms/30°, Nex 1, and a 256×256×180 matrix with 1 $mm^3$ isotropic voxels. To avoid reading bias, scans from six females, including those from II-6 and III-2, were read by an experienced radiologist (NM) blinded to the genetic status of the subjects but aware of their age. A systematic assessment was run, which included: movement artifacts, atrophy (pons, vermian, cortical, corpus callosum), white matter hyperintensities (infratentorial, cerebellar, pons, dentate nuclei, cerebellar peduncles, mesencephalon, periventricular, deep, juxtacortical, basal ganglia, semi ovale centrum, corticospinal tract, corpus callosum), Scheltens and Koedam scores, enlargement (ventricular, olfactive sulcus), infarct (territorial, lacunar, junctional), perivascular spaces dilatation, fluid cavities, and basal ganglia signal abnormalities.

## Cloning and luciferase assays

We cloned wildtype (long fragment, 776bp) and CNV deletion model (short fragment, 137 bp) constructs of the 5′UTR of *LRRC4C* into a luciferase vector (Invivogen), using the TOPO cloning kit, following manufacturer's instructions (Invitrogren, Carlsbad, CA). cDNA was amplified using the following primers.

LRRC4C-F8-BsrG1-sense: actgag TGTACA agtgagaaagaaggga

LRRC4C-R1-Nco1-sense: ctagct CCATGG ctccactgggggtctcta

LRRC4C-F8-Nco1-antisense: actgag CCATGG agtgagaaagaaggga

LRRC4C-R1-BsrG1-antisense: ctagct TGTACA ctccactgggggtctcta

LRRC4C-F1-BsrG1-sense: agtttt TGTACA tggcttacttttggcgg

LRRC4C-F1-Nco1-antisense: agtttt CCATGG tggcttacttttggcgg

All fragments were cloned in the TOPO IV vector and Sanger sequenced to confirm orientation and to determine that fragments were mutation-free. To transfer inserts from the TOPO IV vector to the luciferase-containing vector, both vectors were digested with *Bsr*G1 and *Nco*1 enzymes. Inserts were gel purified and ligated at 4°C overnight, where the ligation product was transformed into GT115 competent cells. Positive transformants were identified by Sanger sequencing. To measure luciferase activity, 250 ng of construct was transfected in HEK 293 cells using the Jetprime reagent with an equivalent amount of pGL3 control vector used for signal normalization. Luciferase activities were quantified using the dual luciferase reporter (Promega, Madison, WI) kit 24 h after transfection in the cell medium and cellular extracts.

## Clinical CNV cohort

From Signature Genomic Laboratories (SG), we analyzed a total of 14,077 non-prenatal NDD samples submitted for clinical genetic testing using oligonucleotide-based whole-genome array comparative genomic hybridization (aCGH), either a 105K-feature platform (SignatureChip OS version 1 or 4; custom-designed by SG, manufactured by Agilent Technologies, Santa Clara, CA) or a 135K-feature platform (SignatureChip OS version 2 or 3; custom-designed by SG, manufactured by Roche NimbleGen, Laval, Canada). This cohort has been fully defined and characterized in our previous work [Talkowski et al., 2012c]. The ethnic distribution in the samples from SG was estimated from a sampling cross-section where 75% were white individuals, 7% African American individuals, and 18% individuals of other race/ethnicity. The sex distribution was 59% male and 41% female.

## Control cohorts

Controls used were a combination of several datasets, for a total of 8,960 individuals. We used control datasets from SickKids Toronto (OHI n=1,234, POPGEN n=1,123[Krawczak et al., 2006], and the Ontario Population Genomics Platform (OPGP), n=416 (http://www.tcag.ca/facilities/cyto_population_control_DNA.html), all of whom (n=2,773) had CNVs called from Affymetrix 6.0 SNP arrays, and all of which were carefully screened for false positives. The POPGEN cohort is a sample of control individuals from Northern Germany, while the OHI cohort is a set of control individuals from the Ottawa Heart Institute. The OPGP cohort are control individuals from Ontario consented for use as controls in genomic studies. From a Swedish control cohort [Bergen et al., 2012; Szatkiewicz et al., 2014], all subjects were born in Sweden and identified via the Swedish Hospital Discharge Register containing all individuals hospitalized in Sweden since 1973. There were 5,917 psychiatrically screened controls that were hospitalized only for non-

psychiatric reasons. DNA was extracted from blood from the Swedish control cohort using standard methods at the Karolinska Institutet. All genotyping was conducted at the Broad Institute of Harvard and MIT, and genotypes and CNVs were called using the Birdsuite algorithm using the Affymetrix (Santa Clara, CA) 6.0 platform, as previously described. CNVs present in > 0.5% of controls were removed from analyses, and only CNVs >50Kb were included. All (n=8,960) controls are Caucasian.

## RESULTS

### Two independent BCR in a subject with autism, a sensory processing disorder, and apraxia

Individual II-6, who was recruited at the Douglas Hospital for an unrelated study, reported that her daughter, III-2, had a 46,XX,t(11;14)(p12;p12) translocation (Fig. 1), identified after four miscarriages, and a son with autism and other developmental problems (described below). To understand better the transmission and precise breakpoints of this reported chromosomal rearrangement, we collected DNA from II-6, III-2, and IV-1. After performing an aCGH (OMNI 2.5 array) on III-2 and IV-1, which revealed no other genetic anomalies, we performed jumping library sequencing [Talkowski et al., 2011] in all three subjects. We defined the translocation breakpoint, identical in all three subjects, at (GRCh37/hg19) Chr11:40614960 and ChrUn_gl000220:140245 (Fig. 1A; Supplemental Table I). gl000220.1 is a 161.8 kb unplaced genomic contig, which has still not been placed into the newest primary reference genome assembly (hg38); however, our results show gl000220.1 localizes at least to 14p12. Acrocentric short arms contain highly repetitive sequences, with most shared with other acrocentric short arms and which contain rDNA genes [Bandyopadhyay et al., 2001a; Bandyopadhyay et al., 2001b]. Additional characterization, outside the scope of this work, would be necessary to establish whether this sequence is unique to 14p or shared among more acrocentric short arms. The Chr11 breakpoint results in the direct disruption of *LRRC4C*, which encodes the netrin G1 ligand important in cortical and thalamic axon guidance [Lin et al., 2003]. The disruption occurs in intron three of this seven-exon gene. In the male with autism, IV-1, we also detected a paternally inherited Chr7 inversion [inv(7)(q34q36.2)]. We localized the breakpoints (hg19) to Chr7:140416412 and Chr7:153725012 (Fig. 1B, Supplemental Table SII). The Chr7:140416412 breakpoint localizes to an intergenic region, 9,965 bp from the 3′UTR of *NDUFB2* and 17,952 bp from the 3′UTR of *BRAF*. The Chr7:153725012 breakpoint localizes to a highly polymorphic region of intron 1 of the longest and lowest expressed isoform of *DPP6* (Dipeptidyl peptidase-like protein 6), a component of Kv4 channel complexes that may be important in the neuronal A-type potassium current [Maffie et al., 2013]. Our previous work suggested revised terminology for these findings [Ordulu et al., 2014], so the next-generation cytogenetic nomenclature for this case is:

> 46,XY,inv(7)(q34q36.2)pat,t(11;14)(p12;p12)mat.seq[GRCh37/hg19] inv(7)(pter->q34(140,416,412)::GGATACTGTATCTGGATCCT::q36.2q34(153,725,012-140,416,421)::ATGTACATAGT::q36.2(153,725,220)- >qter),t(11;Un_gl000220)(Un_gl000220(+)(140,236)::TGTCTTTTGTGATA::11p12(40,614,960)- >11qter;11pter->11p12(40,614,957)::TATATAG::Un_gl000220(+)(140,245))

### Clinical and neurological information for members with chromosomal rearrangements

Individual IV-1 (born in: 2009) is the only son of III-2 (Fig. 1C). Pregnancy and delivery were without issue, though the family noticed lack of eye contact at an early age and delayed milestones including complete lack of speech until age 3 and walking after age 2. The proband was evaluated at a specialty clinic at the Montreal Children's Hospital and diagnosed with autism using the ADOS and ADI. He also experiences some intellectual disability in that by age 5 he could only read some words, say the alphabet, and count as high as 30. He is able to socialize with family members on his own terms but does not like to be touched and rarely makes eye contact when speaking. He is energetic, has no concept of danger, struggles with transitions from one activity to the next, and has some trouble balancing and jumping. Physiotherapy and speech and language training after age 3 led to significant improvements in speech, implying a lack of regression.

Besides meeting criteria for autism, the mother reports other particular features specific to the proband. Specifically, that he never chews food or moves his tongue despite the ability to do so. Because of this, he was assessed for an oral sensory processing disorder which revealed extreme hypersensitivity to most textured foods in his mouth, unrelated to taste or desire for food. He also has a severe case of apraxia, two examples of which are his lack of response to painful stimuli (despite being able to sense pain), and not going to the bathroom despite acknowledging the need to do so. He has a fixation with trains that interferes with daily activities.

II-6 (born in 1950) is a 64-year-old woman and has five siblings. Her mother, I-1, suffered from major depression and committed suicide at age 54. No DNA is available for individual I-1, and the family was unsure of any history of miscarriages she may have had. II-6 reported a history of panic disorder associated with depressive symptoms for which she received successful treatment with psychological therapy and later recurrence was successfully treated with an antidepressant throughout a 9-month period. She had two miscarriages. She has completed high school and has a 2-year college diploma. The cognitive battery showed that II-6 is within the 33% lowest performance for TMT-B, categorical verbal fluency and IGT, and the 10% lowest performance for TMT-A and Stroop Interference score and errors.

Individual III-2 (born in 1980) suffered from anorexia nervosa at age 8 during a 1-year period and again at age 14, when she was followed in a specialty clinic. III-2 presented with two major depressive episodes at 16 and 20 years of age and was treated with several antidepressants. She has had four miscarriages and has a current diagnosis of ADHD. III-2 was within normal range for all psychiatric assessment and her highest level of education completed is a Bachelor's degree. Neuropsychological profiling showed that III-2 is within the 33% lowest performance for IGT, category verbal fluency, Stroop interference score and error, and TMT-A. We conclude that the translocation does not associate with strong effect cognitive or structural deficits.

There was limited information available on III-1, the father of IV-1 who carries the inv(7)(q34q36.2), whom we did not consent to a clinical evaluation. Spousal report suggests he is a healthy individual, who is shy and works in computing.

Because the translocation disrupts a gene encoding an axon guidance-related molecule [DeNardo et al., 2012; Kim et al., 2006], which could affect brain structure, we tested II-6 and III-2 using structural MRI and cognitive performance evaluations. II-6's MRI scan revealed moderate cortical and vermian hypoplasia, a moderate ventricular enlargement, and a moderate olfactive sulcus enlargement compared to six control individuals (Supplemental Fig. S1). There were no anomalies in III-2's MRI scan.

## Translocation and inversion breakpoints are located in genomic regions that are transcribed in human brain

To assess whether the chromosomal rearrangements, that is, inv(7) and t(11;14), were in transcriptional products, we undertook RNA analysis in healthy tissues. There are only two protein-coding transcript variants of *LRRC4C*. Two other transcripts produced from the 3' end of this locus are out of frame and non-coding. Both of the gene's protein-coding isoforms, NM_020929.2 and NM_001258419.1 [Rajasekharan and Kennedy 2009], are predicted to be disrupted by the translocation (Fig. 2A). Primers targeting both isoforms show expression in adult human brain regions as well as in fetal brain, while only mild expression was detected in spinal cord with extremely low or absent expression in the kidney, liver, or lung (Fig. 2A).

There are three predicted isoforms of *DPP6*, all of which have distinct transcription start sites. The inversion identified in family member IV-1 disrupts the promoter and exon 1 of isoform 3 (NM_001039350). The 5'UTR for *DPP6* isoforms 1 and 2, which were the first to be identified [Wada et al., 1992], start ~171 kb and 423 kb downstream from exon 1 of isoform 3, respectively, and ~25 kb from the inversion breakpoint. Still, we observed expression of *DPP6* isoform 3 in adult human brain regions as well as in fetal brain, while only mild expression was detected in spinal cord and low or absent expression in the kidney, liver, or lung (Fig. 2B).

## Examination of CNVs in *LRRC4C*

To determine if *LRRC4C* variation are associated with NDDs, we took advantage of cases referred to Signature Genomics for clinical microarrays (N=14,077 NDD cases, no prenatal samples). To assess significance of *LRRC4C*-specific disruptions, we calculated a two-tailed Chi-square value using seven NDD cases (Fig. 3A and Table I) from a pool of 14,077 cases, and compared these with the 8,690 control cases where there was one control with an exon disrupting CNV ($X^2 = 2.23$, p=0.14). We conclude that CNVs in *LRRC4C* have a non-significant association with NDDs.

## Examination of CNVs in *DPP6*

There is high CNV variation in isoform 3 exon 1 of *DPP6* (hg19; Chr7: 153485627-153682815, occurring in 26 control subjects) in the database of genomic variation, and this CNV is also within the site of the inversion breakpoint, >25kb distant from the transcription start sites and promoter of isoforms 1 and 2. This CNV was excluded from all analyses in *DPP6*. We identified six rare CNVs in *DPP6* in NDD cases in isoforms 1 and 2 and no controls (Fig. 3B and Table I). Two-tailed $X^2$-square test revealed a marginally non-

significant value ($X^2 = 3.7$, p=0.05). Similar to *LRRC4C*, we suggest that variation in *DPP6* has a suggestive but non-significant association with NDDs.

*DPP6* mutations leading to increased mRNA have been previously associated with familial ventricular fibrillation [Alders et al., 2009]. There was no cardiac phenotype reported in any of the NDD cases with CNVs over this locus; however, to further assess the potential association between *DPP6* variation and heart anomalies, we analyzed *DPP6* CNVs derived from 7,006 prenatal Signature samples (Supplemental Table SIII). Nine cases had CNV duplications, six of whom had a heart anomaly noted on the ultrasound. Two of three cases with CNV deletions affecting DPP6 had heart anomaly. There are two important caveats for this observation: (i) heart anomalies are unlikely to be rare from a population of prenatal cases sent for genomic analysis: (ii) These are structural heart deficits observed by ultrasound and so are very different the ventricular fibrillation phenotype identified by Anders et al.

### *In vitro* functional analysis of *LRRC4C* 5'UTR length

The identified translocation in *LRRC4C* generates a predicted loss of the 5' portion of *LRRC4C*, affecting the first three exons; however, only the terminal exon of *LRRC4C* is coding, meaning that this gene has a long, spliced 5'UTR. While this suggests a haploinsufficiency model for the proband since the promoter is also translocated, we wanted to explore this unusually long 5'UTR since several NDD cases from the Signature cohort had intragenic deletions in this region. To assess the affect of the length of the *LRRC4C* 5'UTR on gene expression, we cloned both a long 5'UTR and a short 5'UTR into a vector with a minimal promoter and the *Luciferase* gene (Fig. 4A–D). We found that a shorter length of 5'UTR led to a strong increase in activity of *Luc* compared to a longer form, suggesting that the 5'UTR of *LRRC4C* might be an important regulatory mechanism to dampen expression.

### *LRRC4C* as a potential genetic modifier of NDDs

In the Signature cohort, we found that 3/5 subjects with deletions only in *LRRC4C* also have a second genomic abnormality (Table I), while a single subject with a deletion in *DPP6* had a CNV associated with a genomic syndrome CNV (1/6). The pathogenic CNVs associated with *LRRC4C* mutations are all known to be associated with syndromes of variable expressivity, including 1q44 [Thierry et al., 2012] (deletion), 2q33.1 [Docker et al., 2014] (duplication), and 16p11.2 [Hanson et al., 2010] (deletion). No *LRRC4C* duplication cases had co-incident secondary pathogenic CNVs, implying that if a genetic modifier effect exists it may be specific to deletions. We identified another subject from the non-NDD Signature cohort, who was too young (1-month old) to diagnose with an NDD (Table I; subject 56338), and who had a secondary anomaly consistent with variable expressivity (2q11.2 deletion [Riley et al., 2015]). Finally, we also accessed all cases from DECIPHER that have CNVs affecting *LRRC4C* (Supplemental Table SIV). Four of eight cases with intragenic deletions in *LRRC4C* had secondary lesions, three of which were on chromosome 16, with one on chromosome 8. No lesions were in genomic regions unambiguously associated with disease.

### Assessment of other Netrin G family members in neurodevelopmental disorders

*LRRC4C* is also known as the netrin G ligand 1 (*NGL-1*), which is one member of a highly specialized family of neuronal guidance molecules. The netrin G family includes *LRRC4* (*NGL-2*) and *LRRC4B* (*NGL-3*) [Woo et al., 2009b]. Receptors for netrin G ligands include *NTNG1* (which binds *LRRC4C*), *NTNG2* (which binds *LRRC4*), and *PTPRF* (aka *LAR*) [Takahashi and Craig 2013], which binds *LRRC4B* [Nishimura-Akiyoshi et al., 2007]. Netrin G ligands and receptors are expressed in non-overlapping patterns in mouse brain, specifically with differences between the thalamus and cortical regions, which are suggested to guide thalmocortical and corticothalamic projections [Nakashiba et al., 2002; Yin et al., 2002]. We confirmed this relationship in human brain using microarray gene expression data from the six available adult brains from the Allen Brain Atlas and extracted all microarray probes for netrin G ligands and receptors (Supplemental Fig. S2)

Netrin G members regulate excitatory synapse formation [Kim et al., 2006; Kwon et al., 2010; Linhoff et al., 2009; Matsukawa et al., 2014; Takahashi and Craig 2013; Woo et al., 2009a], and *NTNG1* has been previously implicated in neurodevelopment and sensory processing in different investigations [Aoki-Suzuki et al., 2005; Nectoux et al., 2007; O'Roak et al., 2012]. Similarly, absent startle responses have been identified in mice with deletions in *NTNG2* or *LRRC4*, despite normal hearing [Zhang et al., 2008], supporting a potential role for netrin G ligands and receptors in sensory processing. These data together with the particular sensory phenotype of the probands in this study, led us to reason that the netrin G family of ligands and receptors may be important for sensory processing disorders in human. In the case and control datasets used here, we examined clinically reported CNV data for *LRRC4, LRRC4B, NTNG1, NTNG2*, and *PTPRF* (Supplemental Table SV). These data suggest that *LRRC4B* (Two NDD duplications and one NDD deletion; zero controls) and *NTNG2* (three NDD duplications and zero controls) may be important in neurodevelopmental disorders and warrant further investigation in larger cohorts. Larger datasets should provide a more complete assessment of the potential role of CNVs affecting the Netrin G family and NDDs.

## DISCUSSION

We identified a boy with autism, apraxia, and a sensory processing disorder who had a maternally inherited translocation (46,XY,t(11;14)(p12;p12)mat) and a paternally inherited inversion of chromosome 7. Similar to our previous studies [Talkowski et al., 2012a], this study highlights the strength of BCR sequencing to discover new genes important in NDD, complemented by follow-up in many thousands of affected subjects.

The relationship between the phenotype and the translocation genotype within the family was ambiguous, though we could identify clinically relevant psychiatric features in all generations. There are some possible explanations as to why the affected son with autism, apraxia, and a sensory processing disorder had such a profound but dissimilar phenotype compared to his parents or grandparents (i) The combination of inv(7) and t(11;14) produced a novel phenotype not previously observed in the family (Fig. 5). (ii) The translocation breakpoint in *LRRC4C* shows a more deleterious phenotype in males, consistent with the female protective effect in some inherited ASD loci [Jacquemont et al., 2014]. Finally, (iii)

While there were no clinically relevant CNVs in this case as assessed by clinical aCGH, the subject may harbor an unidentified mutation not detected in our assays.

We found suggestive but non-significant evidence for the involvement of *LRRC4C* and *DPP6* mutations in NDDs. We used a cohort of 14,077 non-prenatal samples with an NDD indication and found non-significant *P*-values for independent association of each gene; one caveat for even a suggestive association here is that the control cohort is called with lower resolution arrays, so it is possible that the frequency of these CNVs in controls is higher than we observed.

We investigated the functionality of intragenic deletion CNVs in *LRRC4C*. Our investigations into this suggested that the 5'UTR of *LRRC4C* functions as a negative regulatory element. We do not suggest these mutations are pathological, but rather that they may be a predisposing factor to disease, dependent upon genomic background for actual disease expression. Notably, the translocation case was missing not only three exons (so had a truncated 5'UTR), but also had no promoter, meaning that the mechanism of action in the translocation case may be different than in *LRRC4C* CNV deletion cases. The lack of conclusive evidence for a direct effect of *LRRC4C* disruption on any clinical phenotype is consistent with it not being a strong-effect contributor to NDDs, yet the presence of this chromosomal rearrangement in a child with autism suggests that a contribution of the *LRRC4C* disruption may act to modify the penetrance or expression of NDD lesions. This modifier effect is supported by the finding that 3/5 cases with *LRRC4C* deletions had secondary clinically significant genetic lesions associated with reduced penetrance. Together these data argue that exonic deletions of *LRRC4C* may modify other genetic lesions.

The data reported here represent information from a family that carried a translocation in three generations, where the proband also inherited an inversion from his father. We sequenced the breakpoints from these karyotypic anomalies and found that they disrupted two different genes, *LRRC4C* and *DPP6*. We suggest that the co-incident inheritance of both BCRs may explain the autism and sensory deficits in the probands.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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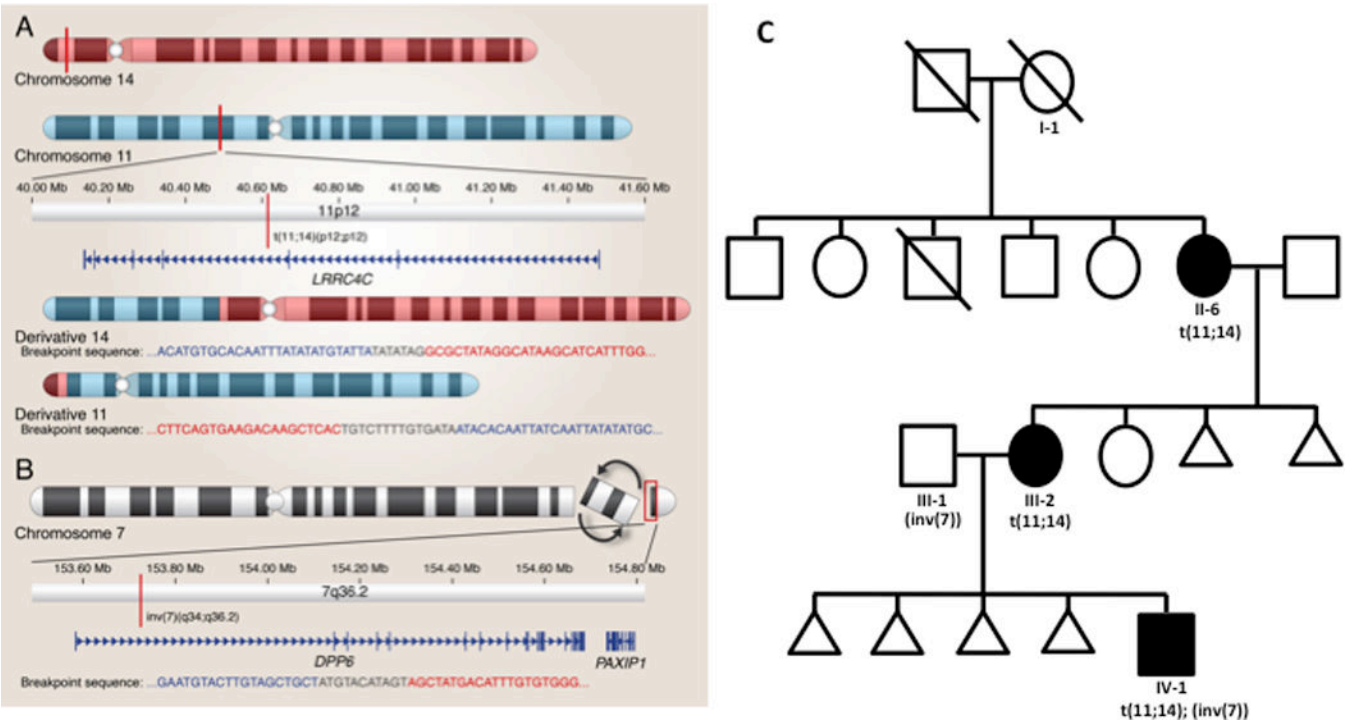


**FIG. 1.**

A subject with a sensory processing disorder, apraxia, and autism carries both a maternally inherited translocation affecting *LRRC4C* and a paternally inherited inversion on chromosome 7 affecting *DPP6*.

**(A)** Balanced translocation of chromosomes 11 and 14 that disrupts a highly repetitive region with no known genes on Chr14 and on Chr11 at Chr11:40614960, within the gene *LRRC4C* that encodes a netrin G1 ligand important in cortical and thalamic axon guidance. This disruption occurs in intron 3 of this seven exon gene. **(B)** Inversion with breakpoints mapped to Chr7:140416412, which falls in an intergenic region, and Chr7:153725012, which is in intron 1 of isoform 3 of *DPP6* (dipeptidyl peptidase-like protein 6), a component of Kv4 channel complexes and thought to be important in the neuronal A-type potassium current and dendritic stability during neurodevelopment. **(C)** The family pedigree: I-1 died by suicide at age 54 and had multiple episodes of major depressive disorder (MDD) but is of unknown carrier status. II-6 had two serious episodes of panic disorder at age 27 and 40 years, both of which were successfully treated. III-2 had anorexia at 8 and 14 years and two episodes of MDD at 16 and 20 years. IV-1 was diagnosed with autism spectrum disorder, an oral hypersensory disorder, and apraxia, at age 3.5 years. No other members of this pedigree were assessed for translocation status or clinical phenotype.

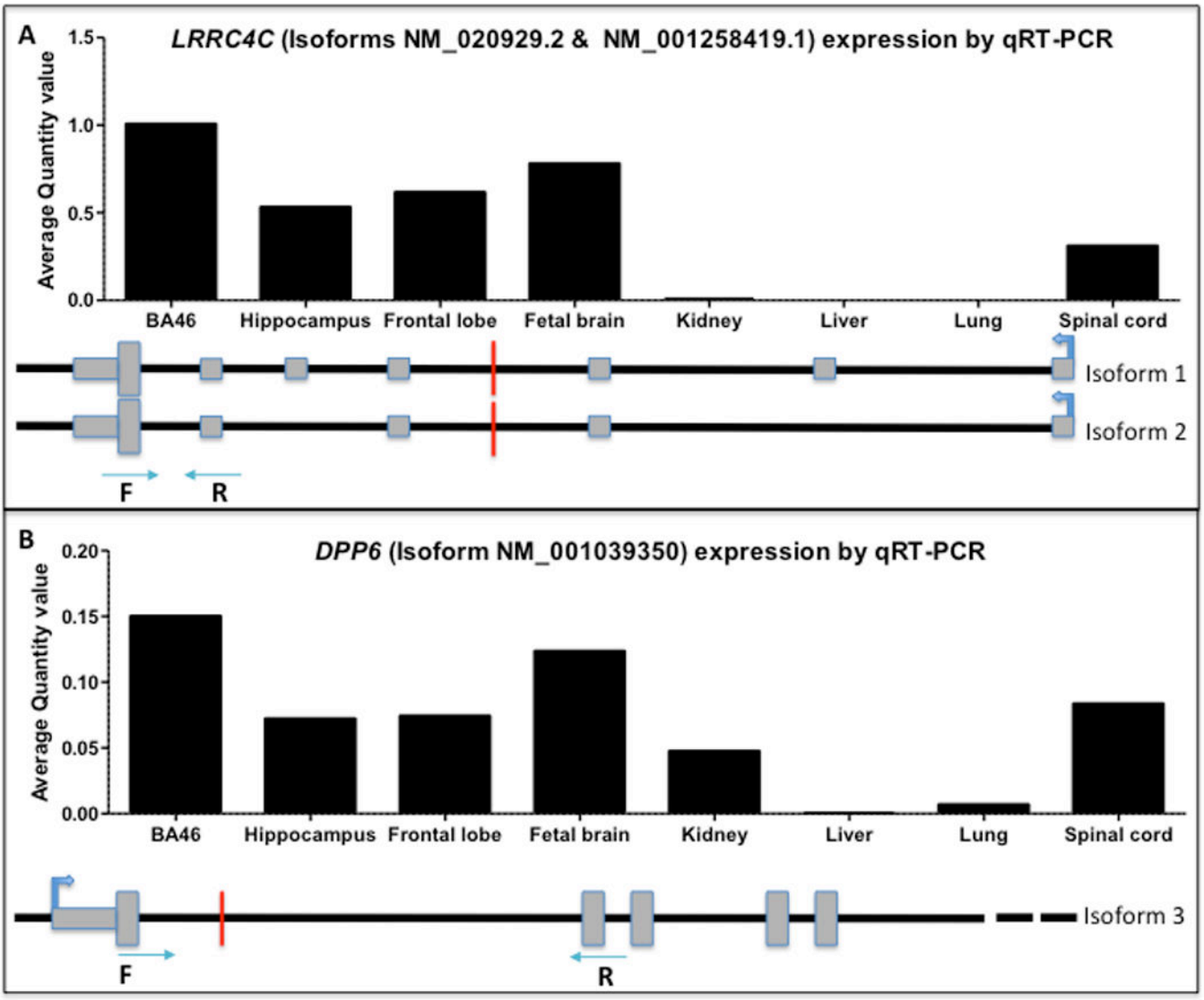


**FIG. 2.**

Translocation breakpoints are in genomic regions encoding for mRNAs in control human brain.

**(A)** mRNA expression of *LRRC4C* was quantified for both isoforms disrupted by the balanced translocation, NM_020929.2 and NM_001258419.1, in adult human brain regions (frontal cortex, BA46, and hippocampus) and fetal brain, as well as spinal cord, kidney, liver, and lung tissues. **(B)** mRNA expression of *DPP6* was quantified for isoform 3 (NM_001039350) in adult human brain regions (frontal cortex, BA46, and hippocampus) and fetal brain, as well as spinal cord, kidney, liver, and lung tissues, as this isoform was the only one affected by the Chr7 inversion.

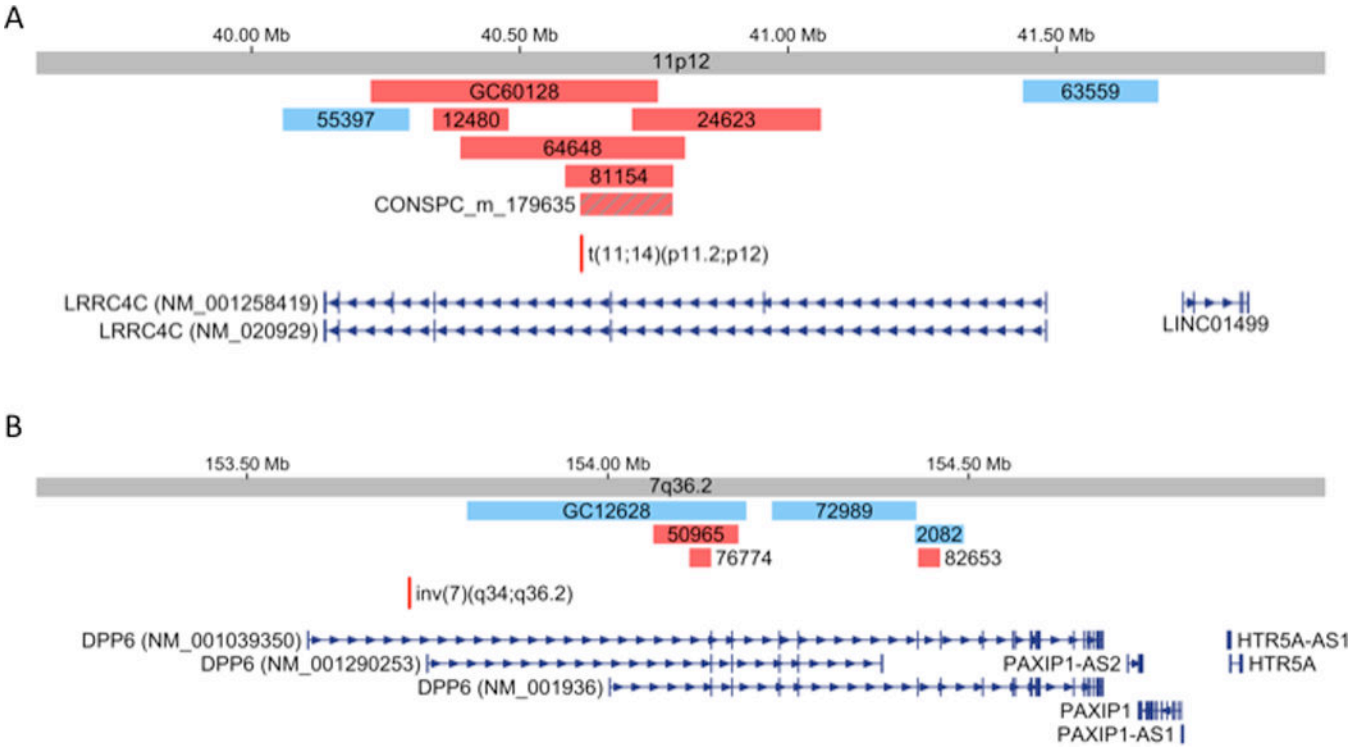


**FIG. 3.**

Copy number analysis of *LRRC4C* and *DPP6* in subjects referred for genetic diagnostic screening.

A total of 14,077 non-prenatal NDD subjects referred to Signature Genomics for clinical genetic testing were screened for intragenic exon-disrupting copy number variants (CNVs). Deletion cases (Red bars), duplication cases (Blue bars).

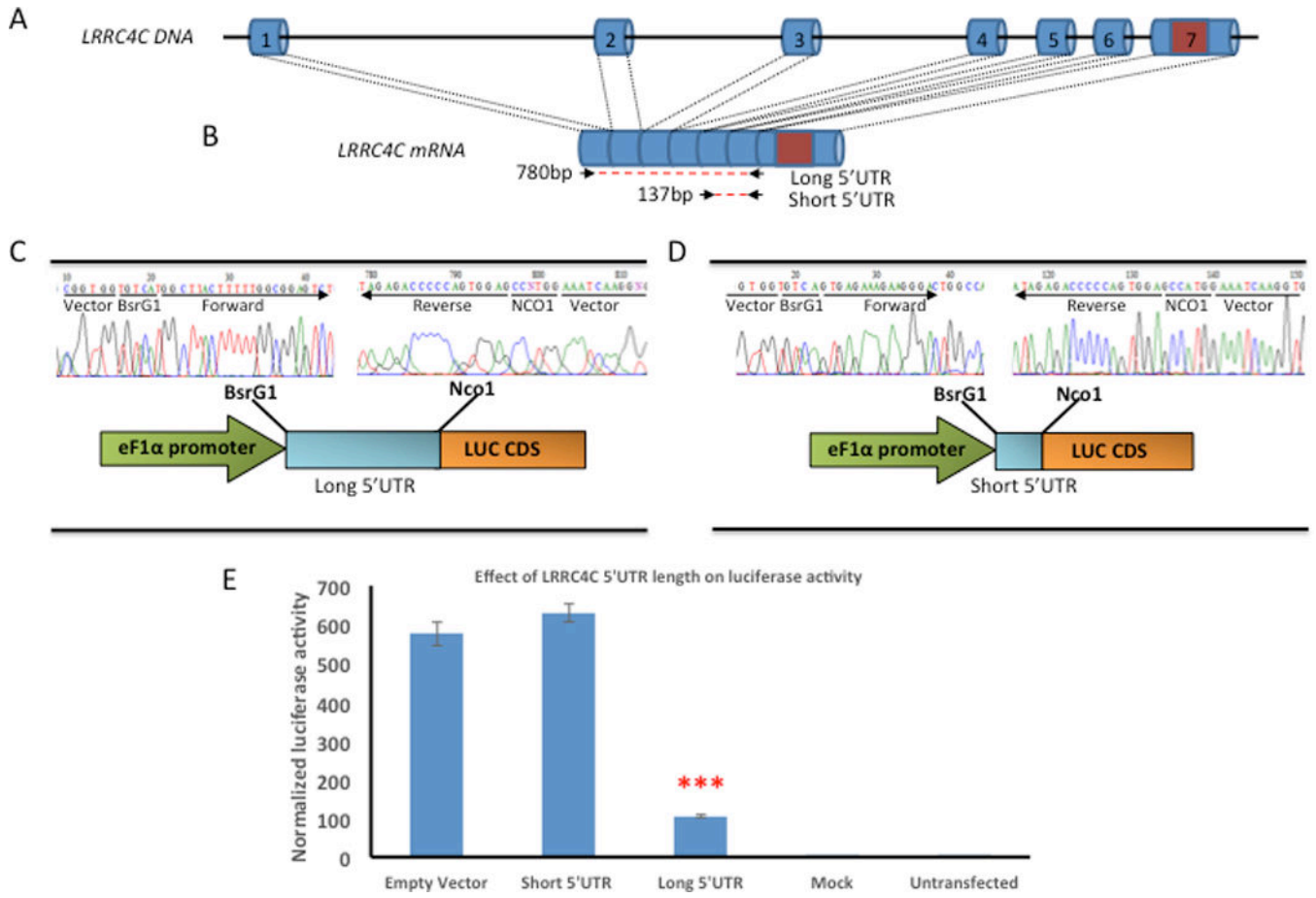


**FIG. 4.**

Functional impact of *LRRC4C* 5'UTR length on expression.

**(A)** Genomic locus of *LRRC4C*, with exons numbered. Non-coding exons are colored blue, while the orange exon represents the single protein coding exon. **(B)** mRNA of *LRRC4C* with approximate positions of long and short cloning targets shown with red-dotted line. Numbers represent the cloning product length, in basepairs. **(C)** Sanger sequencing of vector-amplicon junction demonstrating successful cloning of *LRRC4C* long fragment. Cartoon fragment shows vector with promoter (green), restriction enzyme sites, cloned amplicon (blue), and luciferase gene (green). **(D)** Sanger sequencing of vector-amplicon junction demonstrating successful cloning of *LRRC4C* short fragment. Cartoon fragment shows vector with promoter (green), restriction enzyme sites, cloned amplicon (blue), and luciferase gene (green). **(E)** Luciferase assay: No signal in untransfected or mock cells, no significant difference between the short 5'UTR and promoter-only constructs (p=0.237), but a very significant decrease in *Luc* activity with the long *LRRC4C* 5'UTR compared to the short *LRRC4C* 5′UTR ($p=1.2\times10^{-9}$).

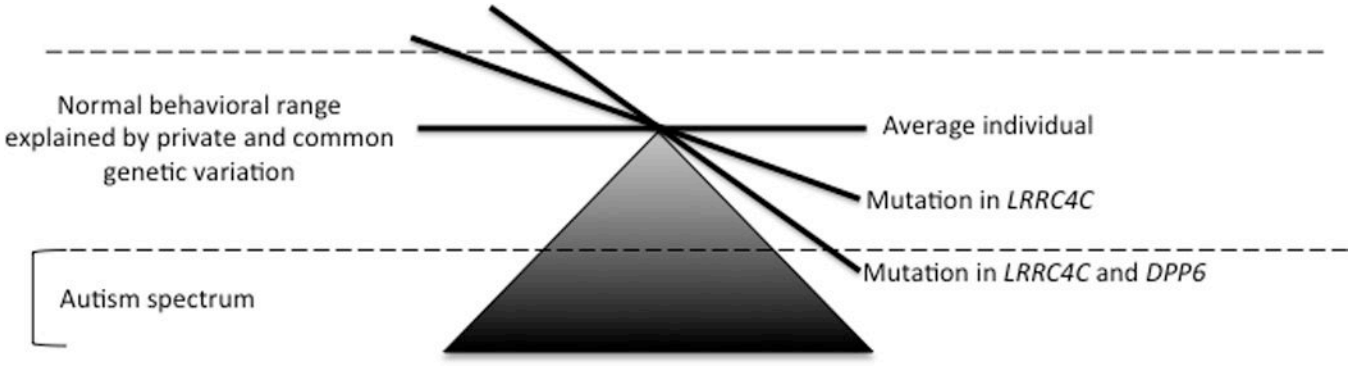


**FIG. 5.**

Potential model for the influence of genetic variation in *DPP6* and *LRRC4C* on behavior and disease.

*Am J Med Genet A*. Author manuscript; available in PMC 2018 March 02.

**Table I**

Intragenic CNVs in *LRRC4C* and *DPP6* from 14,077 neurodevelopmental disorder cases referred for clinical microarrays

| GCAD ID | Sex | Age | Indication | Inheritance | Platform | CNV type | Max coordinates (hg19) | Array ISCN (hg18 unless otherwise specified) |
|---|---|---|---|---|---|---|---|---|
| ***LRRC4C*** | | | | | | | | |
| 60128 | Male | 2y | Mixed development disorder, Multiple congenital anomalies, Hearing loss | Unknown | Signature v2.0 12-plex | Loss | chr11:40223248-40756156 | none |
| 12480 | Female | 10y | Developmental Delay, Seizure Disorder | Unknown | Signature v1.1 2-plex | Loss | chr11:40339674-40477655 | arr cgh 1q44(243,006,404->244,452,092)x1 |
| 24623 | Male | 5y | Developmental Delay | Maternal | Signature v1.1 Rev. B 2-plex | Loss | chr11:40710283-41059922 | arr 2q33.1(203,099,433-203,183,738)x3 dn |
| 64648 | Female | 7y | ADHD, Congenital heart defect | Unknown | Signature v3.0 12-plex | Loss | chr11:40390398-40806705 | arr 16p11.2(29,564,890-30,100,123)x1 |
| 81154 | Male | 6y | Dysmorphic features, Speech delay | Unknown | Signature v4.0 4-plex – CGH | Loss | chr11:40585689-40784701 | none |
| 55397 | Male | 9y | Autism spectrum disorder | Unknown | Signature v2.0 12-plex | Gain | chr11:40059697-40292517 | none |
| 63559 | Male | 7y | Developmental delay, ADHD, Other psychological or physical stress, not elsewhere classified | Unknown | Signature v3.0 12-plex | Gain | chr11:41439036-41688684 | none |
| 56338 | Male | 1M | Clover leaf shaped head, Hypospadias, Dilated renal pelvis, Intrauterine growth restriction | Unknown | Signature v3.0 12-plex | Loss | chr11:40756156-41107103 | arr 2q11.2(96,104,157-97,384,378)x1 mat |
| ***DPP6*** | | | | | | | | |
| 50965 | Female | 11m | Delayed milestones | Unknown | Signature v2.0 12-plex | Loss | chr7: 154063870-154179914 | none; |
| 76774 | Male | NA | Autism spectrum disorder | Unknown | Signature v4.0 4-plex | Loss | chr7: 154113977-154142083 | none |
| 82653 | Female | 5y | Developmental delay | Unknown | Signature v4.0 4-plex – CGH | Loss | chr7: 154430846-154459512 | arr[hg19] 7q36.1(150,844,263-151,091,892)x1 |
| 2082 | Male | NA | Autism | Unknown | Signature v2.0 12-plex | Gain | chr7: 153805929-154190828 | none; |
| 72989 | Male | 0m | Fetal loss/stillbirth/POC, Premature rupture of membranes, Central nervous system malformation in fetus | Unknown | Signature v3.1 12-plex | Gain | chr7: 153522549-153653554 | none |
| GC12628 | Male | 15y | Autism | Maternal | Signature v4.0 1-plex | Gain | | none |