RESEARCH ARTICLE

Human Mutation

OFFICIAL JOURNAL HGVS HUMAN GENOME VARIATION SOCIETY www.hgvs.org

Microdeletions of *ELP4* Are Associated with Language Impairment, Autism Spectrum Disorder, and Mental Retardation

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Communicated by William S. Oetting

Received 6 March 2015; accepted revised manuscript 15 May 2015.
Published online 23 May 2015 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22816

ABSTRACT: Copy-number variations (CNVs) are important in the aetiology of neurodevelopmental disorders and show broad phenotypic manifestations. We compared the presence of small CNVs disrupting the *ELP4-PAX6* locus in 4,092 UK individuals with a range of neurodevelopmental conditions, clinically referred for array comparative genomic hybridization, with WTCCC controls (n = 4,783). The phenotypic analysis was then extended using the DECIPHER database. We followed up association using an autism patient cohort (n = 3,143) compared with six additional control groups (n = 6,469). In the clinical discovery series, we identified eight cases with *ELP4* deletions, and one with a partial duplication of *ELP4* and *PAX6*. These cases were referred for neurological phenotypes including language impairment, developmental delay, autism, and epilepsy. Six further cases with a primary diagnosis of autism spectrum disorder (ASD) and similar secondary phenotypes were identified with *ELP4* deletions, as well as another six (out of nine) with neurodevelopmental phenotypes from DECIPHER. CNVs at *ELP4* were only present in 1/11,252 controls. We found a significant excess of CNVs in discovery cases compared with controls, $P = 7.5 \times 10^{-3}$, as well as for autism, $P = 2.7 \times 10^{-3}$. Our results suggest that *ELP4* deletions are highly likely to be pathogenic, predisposing to a range of neurodevelopmental phenotypes from ASD to language impairment and epilepsy.

Hum Mutat 36:842–850, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: copy number variation; CNV; developmental; neurology; epilepsy and seizures

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsors: Ali Paris Fund for Landau-Kleffner Syndrome Research and Education; Waterloo Foundation; National Institute for Health Research (NIHR); Mental Health Biomedical Research Centre at South London; Maudsley NHS Foundation Trust; King's College London; Charles Sykes Epilepsy Research Trust; European Research Commission via the Seventh Framework Programme "Development and Epilepsy - Strategies for Innovative Research to improve diagnosis, prevention and treatment in children with difficult to treat Epilepsy" (grant agreement no: 602531).

Introduction

Copy-number variation (CNV) plays an important role in the aetiology of neurodevelopmental and psychiatric disorders. Both

recurrent de novo and rare segregating CNVs have begun to explain the overlap of diverse phenotypes in individual cases and families [Cooper et al., 2011; Malhotra and Sebat, 2012]. CNV is a strong risk factor in both focal and generalized epilepsies, and they are also found in 8% of patients with epileptic encephalopathies [Mefford et al., 2010, 2011]. Recent findings in the rare epileptic encephalopathies illustrate the connection among epilepsy, language impairment, and autism spectrum disorder (ASD) through overrepresentation of novel CNVs containing cell adhesion genes (e.g., cadherins and contactins) [Lesca et al., 2012]. However, there are also differences between disorders, for example, specific language impairment cases, while having an increased burden of CNVs, do not in general show enrichment for novel or de novo events [Simpson et al., 2015], whereas rare CNV is an important source of risk in ASD [Pinto et al., 2014].

The examples above indicate that a given genomic alteration can sustain broad susceptibility to several phenotypes depending on the genetic background of the subject. So-called "hotspot" CNVs also manifest this phenotypic variability. The recurrent 15q13.3 microduplication increases the risk for intellectual disability, idiopathic generalized epilepsy, ASD, and schizophrenia [Helbig et al., 2009; Poot et al., 2011]; and deletions at 16p13.11 contribute to a diverse spectrum of epilepsy disorders [Heinzen et al., 2010]. The 16p11.2 hotspot is also pleiotropic; deletions are common in ASD and developmental delay, [Marshall et al., 2008] and duplications have been associated with seizures and speech delay [Shinawi et al., 2010]. Other notable examples of pleiotropy are CNVs of the *CNTNAP2* gene, which are implicated in ASD, Gilles de la Tourette syndrome, schizophrenia and epilepsy, and *AUTS2* with ASD and mental retardation. Interestingly, *AUTS2* and *CNTNAP2* may interact with each other on a molecular level [Poot et al., 2011], indicating emerging convergent pathways for neurodevelopment.

A report of a deletion of the *ELP4* gene (MIM #606985) at 11p13, and adjacent 3′ *PAX6* (MIM #607108) enhancer elements has been described in a case with aniridia, autism, and mental retardation. This case differs from the "classical" *PAX6* gene deletions causing aniridia alone, as only the 3′ enhancer elements are deleted in this case and *ELP4* is included [Davis et al., 2008]. A more recent paper also describes a family with deletion of *PAX6* and *ELP4* with aniridia, ptosis, and mental retardation [Hu et al., 2015], and furthermore, a region responsible for the autistic behavior and severe developmental delay of WAGR syndrome has been narrowed down to 1.6 Mb including *ELP4* and *PAX6* on 11p13 [Yamamoto et al., 2014]. *ELP4* has previously been associated with the electroencephalographic (EEG) signature of the common childhood epilepsy rolandic epilepsy (RE) [Strug et al., 2009], and both EEG abnormalities as well as epilepsy are frequent in ASD and language impairments [Nasr et al., 2001; Parmeggiani et al., 2010]. These examples illustrate again that genomic alterations can show broad phenotypic manifestations during neurodevelopment, as well as incomplete penetrance.

In the present study, we report the presence of a number of deletions of *ELP4* and the regulatory elements of *PAX6* in a UK database of individuals with a childhood onset developmental condition referred for clinical genetic testing (Brain and Body Genetics Research Exchange, BB-GRE). We test the hypothesis that the burden of *ELP4* CNVs is increased in those with neurodevelopmental conditions compared with controls. This phenotypic analysis is then extended using the DECIPHER database of chromosomal imbalances in over 10,000 cases of developmental disorders. Using a CNV-led approach, we then further expand the phenotype associated with *ELP4* microdeletions to cases with ASD and varying comorbidities, and carry out a second case-control analysis. These data support our hypothesis that disruption of *ELP4* and the regulatory regions of *PAX6* contained within its introns can lead to a range of neurodevelopmental conditions with complex interactions between the genotype and phenotype.

Methods

Study Design

We used a three-stage design: first, testing the hypothesis of CNV enrichment at *ELP4* in a clinical discovery sample of developmental disorders (Brain and Body Genetics Research Exchange (BB-GRE, https://bbgre.iop.kcl.ac.uk) and control dataset (WTCCC), both from the UK; second, extending the phenotypic analysis to a larger dataset of developmental disorders (DECIPHER); and finally, replicating the association to neurodevelopmental disorders in ASD cases (Autism Genome Project [AGP] and two Canadian ASD cohorts) compared with a large multicenter control sample set.

Samples

Clinical Dataset—BB-GRE

Children (4,092) referred to Guy's and St Thomas NHS Foundation Trust, southeastern UK from pediatricians and regional hospitals, https://bbgre.iop.kcl.ac.uk. Individuals referred for array comparative genomic hybridization (array-CGH) testing for a range of developmental problems including developmental delay, ASD, speech or language delay, or congenital defects. Individuals had clinical diagnoses made prior to genetic testing, which was part of standard clinical care. Genomic data and referral phenotype information were anonymized and recorded in a clinical database (63% males; August 2014).

Global Clinical Dataset—DECIPHER

We performed a search in the DECIPHER database [Firth et al., 2009] in order to identify additional cases with small CNVs that included and/or disrupted *ELP4*. DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources, http://decipher.sanger.ac.uk), is an interactive Web-based database of over 10,000 cases that enables clinical scientists to maintain records of phenotype and chromosome rearrangement, to aid patient diagnosis by linking to other bioinformatics resources and interactive tools, and to share this information with the clinical research community.

Canadian ASD Samples

The cohort contained 349 probands previously published [Lionel et al., 2011] and 350 additional patients diagnosed with ASD from Canada described below, totaling 699. Individuals were recruited from four different Canadian sites: The Hospital for Sick Children, Toronto; McMaster University, Hamilton; Memorial University, St. John's, Newfoundland, and University of Alberta, Edmonton. All had a clinical diagnosis of ASD, using the Autism Diagnostic Interview-Revised (ADI-R) and/or Autism Diagnostic Observation Schedule (ADOS).

AGP Samples

European ASD cases (2,147) were genotyped as part of a study by the AGP Consortium for rare CNVs affecting autism and are formally described in Pinto et al. (2014). All cases had a clinical diagnosis of autism rated using the ADI-R and/or the ADOS.

Control Populations

A total sample of 11,252 controls from six different datasets were included this study. Group 1 was compared with the BB-GRE cases, and groups 2–6 with the AGP cases: (1) WTCCC, Wellcome Trust Case Control Consortium controls—4,783 population controls from the UK [WTCCC Consortium et al., 2010]; (2) Ottawa Heart Institute (OHI) controls—a cohort of 1,234 control individuals collected as part of a large case control GWA study [Stewart et al., 2009]; (3) German POPGEN controls—a sample of 1,123 individuals of northern German origin (Schleswig-Holstein) [Krawczak et al., 2006]; (4) Ontario Population Genomics Platform (OPGP) controls—a Canadian sample of 416 control individuals of European ancestry (http://www.tcag.ca/facilities/cyto_population_control_DNA.html); (5) HapMap3 controls—a sample of 1,056 individuals from populations from around the world from the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/); (6) controls from the AGP project—consisting of 2,640 of European ancestry assembled from three studies in which subjects had no obvious psychiatric history: "Study of Addiction Genetics and Environment," "Ontario Colorectal Cancer case-control study," and "Health, Aging, and Body Composition."

Genotyping and CNV Analysis

Array-CGH Analysis of BB-GRE Samples

Array-CGH testing was carried out at the Guys and St Thomas' Services cytogenetics CPA accredited laboratory. We have previously described the protocols, analysis, and interpretation using an Agilent oligonucleotide array 60K platform (AMAID 028469 and 017457) and a patient versus patient hybridization strategy and 3-probe minimum aberration call in Ahn et al. (2010, 2013). The average probe density over *ELP4* is 8.5 Kb, giving a limit of around 25 Kb for detection. CNVs in this population are available by application to BB-GRE, https://bbgre.iop.kcl.ac.uk/.

Canadian ASD and Control Groups 1–5

Canadian ASD cases, and control populations 1–5, were genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 with standard protocols. Arrays meeting Affymetrix quality control guidelines of contrast QC > 0.4 were further analyzed. Raw data analysis was carried out using a multiple-algorithm approach to maximize sensitivity and specificity of CNV calling, as described previously [Lionel et al., 2011; Silversides et al., 2012]. Briefly, arrays were analyzed for CNVs with Birdsuite [Korn et al., 2008], iPattern [Pinto et al., 2011], and Affymetrix Genotyping Console and merged into a single dataset. A CNV call was considered high confidence if it was detected by at least two of the calling algorithms and spanned at least 10 Kb and more than five consecutive array probes. Average probe density over *ELP4* was 2.0 Kb, giving a limit of around 10 Kb for detection.

AGP Samples and Control Group 6

A total of 2,147 ASD cases and 2,640 controls were genotyped with the Illumina Infinium 1M SNP microarray. CNV calling was performed using a multialgorithm approach incorporating PennCNV, iPattern, and QuantiSNP [Pinto et al., 2010]. Subsequent analyses focused on those CNVs spanning five or more array probes and detected by at least two algorithms. The analysis is formally described in Pinto et al. (2014). The average probe density over *ELP4* was 2.1 Kb, giving a limit of around 10 Kb for detection.

Association Analysis

A two-tailed Fishers exact test was used to compare frequencies of *ELP4* CNVs in the 4,092 cases in BB-GRE with the 4,783 controls from the WTCCC. Subsequently, another two-tailed Fishers exact test was used to compare the frequency in 2,845 unrelated ASD cases compared with 6,469 controls from control sets 2–6 combined.

Limitations

A limitation of this study is that the CNVs were not identified on the same platform or by the same analysis method between the sample sets. Therefore, there is a chance of false CNV enrichment related to probe density, data quality, and analysis methods. However, all platforms are high-density, with probe coverage shown in Figure 1. This ensures *ELP4* and the surrounding region is well-covered, and indeed the control data are generated on higher density platforms than the BBG-RE cases, resulting in a higher CNV detection power for controls. We have also ensured that all reported CNVs can be called using all three methodologies. For example, the platform used for the BB-GRE cases is the least dense and has a 25-Kb limit for detection. Therefore, no CNV is included from the other case or control groups that is smaller than this size. The Canadian ASD cases and control groups 1–5 also use the same platform and analysis methods as each other. Cases and controls (control group 6) from the AGP study also used the same array and analysis methods as each other. To reduce the chance of error, all of the CNV calling methods from each data set employ published, rigorous quality control measures, as detailed above, ensuring that CNVs called are highly unlikely to be false positives. All of the ASD CNVs have also been validated by orthogonal methods such as qPCR. By also using data sets with different platforms, we have shown that our results are consistent even between the different methods used.

Results

CNV of *ELP4* in the BB-GRE Database

Out of 4,092 individuals referred for neurodevelopmental disorders, we identified nine patients with small (<1 Mb) CNVs disrupting *ELP4* that could also have been detected by the other array methods. Eight CNVs were deletions (Fig. 1; Table 1), varying in size from 26 to 101Kb. The ninth CNV was a 232 Kb duplication of the first seven exons of *ELP4* and the *PAX6* gene. This patient also carried a "hotspot" deletion of 1.2 Mb at 16p13.11, which is also implicated in several neuropsychiatric disorders [Heinzen et al., 2010]. One deletion (patient 117374) is intronic, but does however disrupt regulatory enhancers of *PAX6* and so is included in our analysis. Four of the deletions were maternally inherited and two were paternally inherited, one arose *de novo* and one had unknown inheritance. The inheritance pattern of the duplication was also unknown. Clinical

Table 1. Microdeletions and One Microduplication of *ELP4* on Chr11 Identified in Nine Patients from the BB-GRE Clinical Genetic Database (http://bbgre-dev.iop.kcl.ac.uk), in Nine Individuals from the DECIPHER Database, and in Three Patients from the AGP and Three from a Canadian Study of Autism

ID	Sex	Age at test	Phenotype	hg19Start	hg19Stop	Size (bp)	Inheritance	CNV	Other CNV
BB-GRE patients									
108970	M	5 years	Severe cognitive delay (IQ 20–34) speech and language disorder, reading and spelling development disorder, ASD, epilepsy >24 months at age of onset	31495260	31546276	51,017	Paternal	x1	
119460	M	2 years	Social communication difficulties, speech and language delay	31561220	31625448	64,229	Maternal	x1	Deletion chr5:97302377-97380022. Contains no genes.
129016	F	3 years	Developmental delay (progressing), microcephaly, poor balance	31573422	31674789	101,368	Unknown	x1	
116589	M	3 years	PDD: social interaction difficulties, language disorder, behavior problems	31584329	31642325	57,997	Maternal	x1	
117003	M	8 months	Developmental delay, hypotonia, ventriculomegaly	31601768	31632347	30,580	Maternal	x1	
112031	F	12 years	Developmental delay, hypotonia	31616889	31849574	232,686	Unknown	x3	Deletion chr16: 15048750-16305736 16p13.11 hotspot.
112601	F	1 year	Developmental delay, speech and language disorder, microcephaly (<5th centile), mild cognitive delay, motor skills development disorder	31691270	31722740	31,471	Paternal	x1	Duplication chr5: 93197999-93424468. Disrupts *FAM172A*.
117374	M	20 years	Autism, learning difficulties	31705076	31747631	42,556	Maternal	x1	
130693	M	5 years	Moderate developmental delay mainly affecting language, emerging autistic traits	31760904	31786914	26,010	*De novo*	x1	Deletion chr6: 26440746-26463502. Disrupts *BTN3A3* and *BTN2A1*.
ID	Sex	Age	Phenotype	hg19Start	hg19Stop	Size (bp)	Inheritance	CNV	Other CNV
DECIPHER patients									
257614	M	7 years	Focal epilepsy with cortical dysplasia, mild developmental delay, ADHD, neurinomas, retinal condensation in front of macula in left eye, squint, pitosis, fine motor dyspraxia.	30991456	31564708	573,252	Maternal	x1	
249728	F	24 years	Rieger anomaly.	31118027	31710576	592,549	De novo	x1	
265704	M	<1 year	Aniridia. No further information.	31172410	31775457	603,047	De novo	x1	
292869	F	15 years	Severe intellectual disability, muscle hypotrophy with severe hypotonia and absent gross motor and fine adaptive motor development; no language; severe dysphagia requiring tube feeding; craniofacial abnormalities.	31597322	31802120	204,798	De novo	x1	
287341	M	2 years	Partial aniridia. Currently no signs of neurological impairment.	31605859	31783590	177,731	Maternal	x1	
258970	M	4 years	Developmental delay, behavioral disturbances, regression of language at 18 months to absent at age 4 years, pervasive developmental disorder.	31605859	31775457	169,598	Unknown	x1	

(*Continued*)

Table 1. Continued

ID	Sex	Age	Phenotype	hg19Start	hg19Stop	Size (bp)	Inheritance	CNV	Other CNV
261471	M	4 years	Behavioral and speech disorders, mild mental retardation.	31625389	31775457	150,068	Parent	x1	Deletion chr10:35360169-35605506 disrupts *CUL2*, *CREM*, *CCNY*.
270752	F	9 years	Aniridia, congenital cataract. Mild developmental delay due to processing speed deficiencies largely due to visual impairment.	31735689	31825698	90,009	Paternal	x1	
289275	M	24 years	Aniridia, global developmental delay, autistic behavior.	31742075	31870603	128,528	Unknown	x1	Duplication chrX:46389227-46396390 disrupts intron of *ZNF674*.

ID	Sex	Phenotype	hg19Start	hg19Stop	Size (bp)	Inheritance	CNV	Other CNV
ASD patients								
3617_3	M	Strict autism; no seizures, verbal, language delay; delayed first words (at 24 months), delayed first phrases (at 36 months); verbal IQ >70.	31460506	31655108	194,602	Paternal	x1	
8596_201	M	Strict autism, high functioning; no seizures, verbal, language delay; delayed first words (at 25 months), typical first phrases (at 25 months); verbal IQ >70.	31488890	31607986	119,096	Maternal	x1	Duplication, chr9:115994263-116495631 disrupts *COL27A1*.
NA0285	M	Autism, language delay; delayed first words (at 32 months), no seizures	31518924	31649475	130,551	Maternal	x1	
20130_6005001	M	Strict autism; no seizures, verbal, language delay; delayed first words (at 36 months), delayed first phrases (at 48 months); verbal IQ >70, coordination problems	31576768	31653568	76,800	Maternal	x1	
MM1259-003[a]	F	Autism, language delay; delayed first words (at 21 months), mild developmental delay, motor delay, no seizures	31652219	31764393	112,174	Unknown	x1	Deletion chrX:154772341-154775951 disrupts *TMHLE*.
MM1259-004[a]	F	Autism, language delay; delayed first words (at 18 months), delayed first phrases (at 36 months), expressive language problems, mild developmental delay, motor delay, no seizures	31652219	31764393	112,174	Unknown	x1	Deletion chrX:154772341-154775951 disrupts *TMHLE*.

[a]Sibling sister pair.
AgeAtTest indicates age at array-CGH testing, Age indicates age at last clinical assessment.

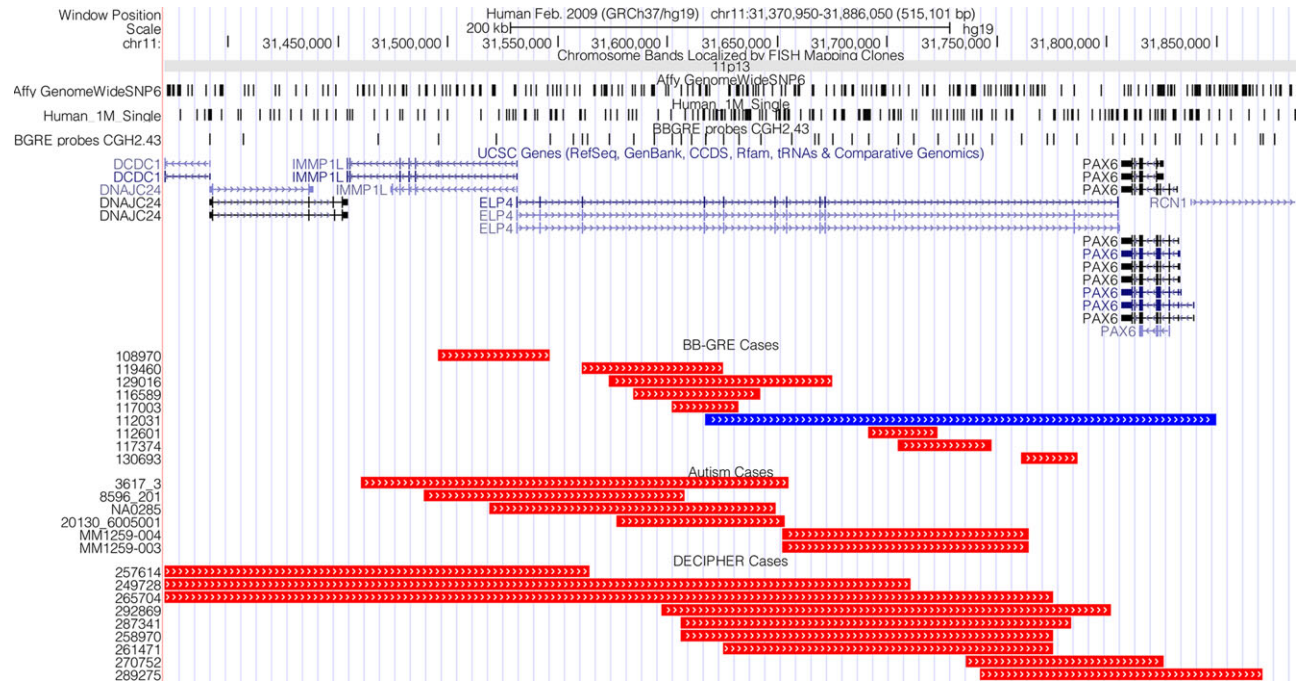

Figure 1. Deletions (red) and a duplication (blue) identified over the *ELP4-PAX6* locus on 11p13 in nine patients from the BB-GRE clinical genetic database with neurodevelopmental phenotypes, six patients with autism from the AGP and Canadian ASD resource, and nine patients with neurodevelopmental phenotypes from the DECIPHER database. Hg19 (http://genome.ucsc.edu/). Tracks showing positions of probes genotyped from the Illumina 1M Single Array, the Affymetrix GenomeWide Human SNP6 Array, and the Custom Agilent oligonucleotide array used for BBGRE patients are above the UCSC gene tracks. Alternatively spliced gene transcripts are shown.

information was not available for the parents as it is not collected for BB-GRE and referring clinicians cannot be contacted. Three of the deletion patients carried a second CNV (Table 1), none of which are predicted to affect the phenotype; patient 119460 had a deletion of unknown inheritance of 77.6 Kb at 5q21 with no genes present in the region, and 112601 had a maternally inherited 226 Kb duplication at 5q15 disrupting *FAM172A*, a potential tumor suppressor. Patient 130693 carried a maternally inherited duplication of 23 Kb at 6p22.2, disrupting the MHC-associated genes *BTN3A3* and *BTN2A1*.

All cases were diagnosed with a neurodevelopmental phenotype: five had speech and language delay or disorder, with one also diagnosed with epilepsy; two had social communication difficulties; and two had a diagnosis of autism, with one further case showing emerging autistic traits. Six of the patients also had a range of cognitive delays (Table 1). Unfortunately, we do not know the age at last neurological assessment for BB-GRE cases; only age at array-CGH testing is recorded. Therefore, some cases may have been too young for some phenotypes to manifest and be reported, for example, 117003.

Only one CNV involving *ELP4* was found in the WTCCC control set: a 221Kb microdeletion (Supp. Table S1). On comparison of the UK BB-GRE samples with the WTCCC controls, the difference in CNV frequency disrupting *ELP4* was significant: P value = 7.5×10^{-3}.

Microdeletions of *ELP4* in the DECIPHER Database

We identified nine individuals with a small (<1 Mb) CNV encompassing *ELP4* in the DECIPHER database (https://decipher.sanger.ac.uk) [Firth et al., 2009]. All were deletions (Table 1), with at least one breakpoint within the gene. Detailed phenotypic information was available for eight of the nine patients; six individuals were diagnosed with developmental delay or intellectual disability. Several cases had speech delay: two had behavioral disorders, one was diagnosed with a pervasive developmental disorder (PDD), most likely ASD, and one further case had ASD. Another case was also diagnosed with ADHD and epilepsy (257614). Three cases were too young at the age of last clinical visit (263619, 265704, and 287341) for a full assessment of neurodevelopmental phenotypes such as ASD.

Two cases, 289275 and 270752, had deletions that disrupted *PAX6* exons that caused aniridia, an abnormality of the iris. Cases 265704, 263741, and 249728 also have aniridia most likely due to *PAX6* enhancer disruption within the large *ELP4* intron 9. A map of *PAX6* regulatory elements can be found in Kleinjan et al. (2008). Case 257614 also has a congenital eye malformation but deletion breakpoints much further from *PAX6*, within *ELP4* intron 3 where it is not thought there are any *PAX6* enhancers, the last being located within *ELP4* intron 4. The remaining three cases, while having breakpoints very similar to those with aniridia, do not share that phenotype, indicating a complex genotype–phenotype relationship.

Two cases carried a second CNV: 289275 had an intronic duplication of *ZNF674* at Xp11.3, and 261471 had a 245Kb deletion at 10p11.21, disrupting *CUL2*, *CREM*, and *CCNY*, genes not involved in neuronal development.

Microdeletions of *ELP4* in Autism Cases

Given that several individuals from BB-GRE and DECIPHER have ASD, PDD, or social communication difficulties, we decided to investigate the prevalence of *ELP4* CNVs in two autism cohorts. Out of 2,446 cases from the AGP [Pinto et al., 2014], three had

microdeletions of *ELP4* (Table 1). All three fulfilled the criteria for a strict definition of autism, and were verbal (verbal IQ >70), but experienced language delay of first words and phrases. None of the cases had a history of seizures or epilepsy. Case 8596_201 also carried a 500Kb maternally inherited duplication disrupting the collagen gene *COL27A1* that is highly unlikely to contribute to the neurological phenotype.

Three out of 699 individuals from the Canadian autism study also carried *ELP4* deletions (Table 1). An affected sister pair both had a 112Kb deletion of half of the gene, and a male case carried a 130Kb deletion of the 3′ (but proximal due to reverse gene orientation) part of *ELP4* and neighboring *IMMP1L*. Again, all three had speech and language delay and the sister-pair had mild developmental delay. Interestingly, the sisters also both carried a deletion of one copy of exon 2 of *TMLHE*, an enzyme involved in carnitine biosynthesis, on Xq28.

A case-control analysis of the frequency of *ELP4* CNVs from unrelated individuals in these 2,845 ASD cases compared with 6,469 control individuals from groups 2–6, where no *ELP4* CNVs were found, yielded a highly significant P value of 2.7×10^{-3}.

Discussion

In this study we have found a strong and consistent pleiotropic association between CNVs disrupting *ELP4* and neurodevelopmental conditions over several experimental platforms. We have described CNVs that can be captured and called from all three high-density platforms/methods. We have also addressed the potential problem of enrichment bias of CNVs in cases, as all control data are generated on higher density platforms than the BB-GRE cases, or the same as ASD/DECIPHER cases, resulting in a higher CNV detection power for controls. CNVs disrupting *ELP4* appear to be rare in the general population given that we found only one CNV in the six control groups studied (total $n = 11{,}252$), and that there are no regions of segmental duplication around the gene (UCSC Segmental Duplication track, [Bailey et al., 2002]). *ELP4* now joins the growing list of genes such as *CNTNAP2*, *SHANK3*, and *NRXN1*, where heterozygous copy number variations are repeatedly associated with a wide range of neuropsychiatric disorders [Gregor et al., 2011; Poot et al., 2011; Lesca et al., 2012].

We have extended the phenotype associated with disruptions of *ELP4* from the EEG signature of RE and speech sound disorder [Strug et al., 2009; Pal et al., 2010] to ASD, social communication difficulties and speech/language disorder, developmental delay, and epilepsy. This corroborates and expands upon the findings of Davis et al. (2008) and Hu et al. (2015) who found inherited deletions of *ELP4* and *PAX6* enhancer elements and exons in patients with autism, aniridia, and mental retardation. The *ELP4* locus may influence the development of language function, as a frequent trait across almost half of the 24 patients described here are speech and language difficulties. There appears to be a genetic crossroads between childhood epilepsy, autism, and speech and language disorders. Several genes and pathways provide a common link such as the cell adhesion genes cadherins and catenins, glutamate receptors *GRIN2A* and *2B*, brain-expressed nuclear proteins such as *AUTS2,* and the transcription factor *FOXP2* [Poot et al., 2011; Graham and Fisher, 2012; Lesca et al., 2012, 2013].

ELP4 is one of six subunits (*ELP1-6*) of the Elongator complex, which plays a role in transcriptional elongation [Wittschieben et al., 1999], tRNA modification, and polarized exocytosis [Huang et al., 2005]. This complex also regulates the migration of multiple cell types, for example, ELP1 colocalizes with filamin A in membrane ruffles, and when depleted creates a disorganized actin cytoskeleton, contributing to motility defects [Johansen et al., 2008]. Impairment of Elongator may be involved in several different neurological disorders [Nguyen et al., 2009], for example, variants within *ELP3* are associated with cases of sporadic ALS, a progressive motor-neuron disease [Simpson et al., 2009]. Furthermore, mutations of *ELP1* cause familial dysautonomia [Slaugenhaupt et al., 2001], a neurodevelopmental and neurodegenerative disorder with EEG abnormalities and seizures, characterized by defects in neuronal development and survival. Elongator also underlies the migration and branching of cortical projection neurons during development and memory consolidation [Creppe et al., 2009]. Thus, there are several mechanisms through which disruption of *ELP4* could result in altered neuronal development and migration, as well as the balance of neuronal excitatory and inhibitory circuits. These changes may disrupt Elongator function in a temporal and regional manner, depending on cellular context and the different array of Elongator targets available.

It is of note that the large intronic regions between exons 9 and 12 of *ELP4* are ultraconserved. They contain long-range *cis*-regulatory enhancers for downstream *PAX6*, which are tissue- or developmental-stage specific in their expression [McBride et al., 2011]. PAX6 is a transcription factor crucial for the correct development of the eyes, spinal cord, several brain regions, and other organs. Deletions of *PAX6* with *WT1* cause Wilms tumor, aniridia, genital anomalies, and intellectual disability (WAGR syndrome). Loss-of-function mutations in *PAX6* also cause aniridia. A rare case of duplication of *PAX6* and the last two exons/introns of *ELP4* has been reported with frontotemporal neonatal seizures, developmental delay, microcephaly, and minor ocular findings [Aradhya et al., 2011]. Recently, *PAX6* has also been proposed as the foremost transcription factor governing glutamatergic neuronal differentiation [Kim et al., 2014], linking it with the major idiopathic focal epilepsy gene glutamate receptor *GRIN2A*. Therefore, disruption of *PAX6* and/or its regulatory elements within *ELP4* and its link via the glutamatergic neurotransmission system described above, as well as the case reports described in the introduction, also make it a prime candidate for involvement in the neurodevelopmental disorders described in some cases here.

The genetic model of disease described here is clearly not monogenic: in 14/24 patients, the *ELP4* CNVs were inherited (13 unrelated events due to the ASD sister pair), four occurred *de novo* and six were of unknown inheritance. The phenotypic status of most parents is unknown and therefore a precise estimation of penetrance will require further segregation studies in a prospective cohort, especially since in other published reports the phenotype is also inherited. However, presuming that many of the parents are unaffected, these inherited CNVs are unlikely to cause a phenotype by reduced expression from haploinsufficiency alone. It is most likely that an interacting model of disease is in action and screening of the second allele of *ELP4* and its regulatory regions can rule out the unmasking of recessive mutations. We note that sequencing of *ELP4* exons has failed to find mutations within RE patients [Reinthaler et al., 2014] and postulate that disruption of the regulatory elements of *ELP4* and/or of *PAX6* within its introns could be causal in the developmental disorders described here.

A two-hit hypothesis can explain CNVs that are nonsyndromic, that is, those that are associated with variable phenotypes and not always inherited, such as the deletions described here [Girirajan and Eichler, 2010]. One hit may reach a threshold to induce some subclinical features and create a sensitized background, onto which the second hit (mutation or second CNV) occurs producing a more severe phenotype. If we assume that these disorders share common

neurodevelopmental pathways, the final disease outcome will then differ depending on the combination of genes affected. Interestingly, a sister pair with ASD in our study who shared the same *ELP4* microdeletion, also shared a microdeletion of exon 2 of the carnitine biosynthesis enzyme gene *TMLHE*, on Xq28. Deletions of *TMLHE* are important in nondysmorphic autism in male–male multiplex families, although with low penetrance [Celestino-Soper et al., 2012], however the significance in females is unclear. It is possible that deletion of the only copy of *TMLHE* is enough of a risk factor for some males to develop ASD; but for females (who normally have two copies of *TMLHE*), further "hits" are necessary, such as the loss of *ELP4* in these sisters. Several other patients also carry a second CNV, as described earlier, but it is unlikely that these specific CNVs contribute to the neurological phenotype. Exome sequencing of the patients without a second causal CNV may uncover coding mutations that would contribute to the developmental burden of *ELP4* loss.

The predominance in our datasets of deletions verses duplications is unlikely to be a platform bias as both array-CGH and SNP arrays were used. Deletion enrichment could be a consequence of undiagnosed duplications, but as this study was not driven by a particular diagnosis this is unlikely. When CNVs are generated by nonallelic homologous recombination between low-copy repeats, a deletion and reciprocal duplication are generated [Malhotra and Sebat, 2012]. A possibility is that the duplications could be selected against due to negative genetic selection, that is, a lower viability or fecundity of carriers. However, since the breakpoints for *ELP4* CNVs differ between cases and there are no low-copy repeats that could explain the generation of CNVs, this mechanism is also unlikely. Instead, there is more in common with deletions seen at *NRXN1*, which may occur by a mechanism involving inverted repeats of variable sizes, or from a significantly higher AT nucleotide content at the breakpoints, generating a rearrangement hotspot of genome instability. These nonrecurrent breakpoints could be generated by a nonhomologous end joining mechanism of double-strand breaks or by replication errors and may be influenced by the genomic architecture of a region in particular people [Enggaard Hoeffding et al., 2014].

Examination of the data from the CNV morbidity map of developmental delay [Cooper et al., 2011] shows four microdeletions (<1 Mb) with breakpoints within *ELP4* (n = 15,767), and five microduplications (Supp. Table S1). All duplication cases had neurological deficits, and two deletion cases and one duplication case had ASD. However, seven microdeletions of *ELP4* were also found in the 8,329 control individuals, one of which is the WTCCC sample reported here. This increase in frequency of smaller CNVs among controls compared with all of the other control datasets used in our study indicates that they may be due to an artifact from the less dense Illumina arrays used by Cooper et al. (2011), compared with the more rigorous platform and methods used to analyze their cases. Indeed, the authors commented that their detection power is substantially higher for cases, the reverse of our study, and will manifest itself as false positive enrichment for CNVs in controls. However, more information (not publicly available) is needed about the specific arrays used for each control with an *ELP4* deletion, their LRR and BAF images, and probe coverage over *ELP4* to draw further conclusions about potential false positives and array bias in their investigation.

Future work will focus on the functional consequences of the *ELP4* deletions by investigation of expression levels of the gene in these cases. Work with cellular and animal models with *ELP4* deletions will help to cement the role of *ELP4* in neurodevelopment through identification of altered interaction networks and developmental pathways such as neuronal migration, branching, and survival.

Acknowledgments

We wish to thank Thomy de Ravel at Leuven University Hospitals who provided phenotype information on DECIPHER patients 289275 and 249728. We also wish to thank Bertrand Isidor at CHU Nantes for helpful discussion on the manuscript. This project and the BBGRE database (http://bbgre-dev.iop.kcl.ac.uk) were funded through a strategic partnership of the South London and Maudsley Trust NIHR specialist Biomedical Research Centre and the Guys and St Thomas Trust NIHR comprehensive Biomedical Research Centre. This study also makes use of data generated by the DECIPHER Consortium. A full list of centers who contributed to the generation of the DECIPHER data is available from http://decipher.sanger.ac.uk and via email from decipher@sanger.ac.uk. Funding for the DECIPHER project was provided by the Wellcome Trust. Those who carried out the initial data collection for DECIPHER bear no responsibility for its further analysis.

David Collier is an employee of, and Laura Addis a contractor for, Eli Lilly and Company.

Compliance with Ethical Standards

The BB-GRE project was approved by the Cambridgeshire Central Research Ethics Committee. In the UK, DECIPHER has been approved by the Eastern MREC 04/MRE05/50 and the project has also been notified to the Information Commissioner in accordance with the Data Protection Act. The DECIPHER Ethical Framework is detailed here: https://decipher.sanger.ac.uk/assets/pdfs/decipher_ethical_framework.pdf

Ethical statements for the AGP and control populations and information on informed consent can be found on their published references. Informed consent was obtained from all individual participants included in this study.

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