Deregulation of EIF4E: a novel mechanism for autism

M Neves-Pereira, B Müller, D Massie, J HG Williams, P CM O'Brien, A Hughes, S-B Shen, D St Clair and Z Miedzybrodzka

J. Med. Genet. published online 25 Jun 2009;
doi:10.1136/jmg.2009.066852

JMG Online First, published on June 25, 2009 as 10.1136/jmg.2009.066852

Deregulation of *EIF4E*: a novel mechanism for autism

M Neves-Pereira[1], B Müller[2], D Massie[3], J H G Williams[1], P C M O'Brien[4], A Hughes[1], S-B Shen[2], D St Clair[1] & Z Miedzybrodzka[1,3].

[1]School of Medicine, and [2]School of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland AB25 2ZD. [3]Department of Medical Genetics, NHS Grampian, Foresterhill, Aberdeen, Scotland AB25 2ZD. [4]Molecular Cytogenetics Group, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK.

Correspondence should be addressed to D. St Clair (d.stclair@abdn.ac.uk) and Z. Miedzybrodzka (zosia@abdn.ac.uk).

Professor David St. Clair
University of Aberdeen
Institute of Medical Sciences
Foresterhill, Aberdeen
Scotland, UK.
Tel: +44 (0)1224 557950
Fax: 01224 557400

Dr Zosia Miedzybrodzka
University of Aberdeen
Department of Genetics
Polwarth Building
Foresterhill, ABERDEEN AB25 2ZD
Telephone : + 44 1224 559399
Fax : + 44 1224 555766
Email Zosia@abdn.ac.uk

Keywords: mTOR, translation, synaptic plasticity, autism, ASD

Word count: 3,689

ABSTRACT

Autism is a common childhood onset neurodevelopmental disorder, characterized by severe and sustained impairment of social interaction and social communication, as well as a markedly restricted repertoire of activities and interests. Its aetiology is multifactorial with a strong genetic basis. EIF4E is the rate-limiting component of eukaryotic translation initiation, and plays a key role in learning and memory through its control of translation within the synapse. EIF4E mediated translation is the final common process modulated by the mTOR, PTEN and Fragile X mental retardation protein (FMRP) pathways which are implicated in autism. Linkage of autism to the *EIF4E* region on chromosome 4q has been found in genome wide linkage studies.

Here we present evidence that directly implicates *EIF4E* in autism. In a boy with classic autism we observed a *de novo* chromosome translocation between 4q and 5q and mapped the breakpoint site to within a proposed alternative transcript of *EIF4E*. We then screened for mutations 120 autism families. We found two unrelated families where in each case both autistic siblings and one of the parents harboured the same single nucleotide insertion at position -25 in the basal element of the *EIF4E* promoter. Electrophoretic mobility shift assays and reporter gene studies show that this mutation enhances binding of a nuclear factor and *EIF4E* promoter activity. These observations implicate *EIF4E*, and more specifically control of *EIF4E* activity, directly in autism. Our findings raise the exciting possibility that pharmacological manipulation of EIF4E may provide therapeutic benefit for those with autism caused by disturbance of the converging pathways controlling EIF4E activity.

INTRODUCTION

Autism (OMIM 209850) is a common childhood onset neurodevelopmental disorder, characterized by severe and sustained impairment of social interaction and social communicative abilities, as well as a markedly restricted repertoire of activities and interests. Although multifactorial in origin, autism has a strong genetic basis. For autistic disorder, monozygotic twin concordance is 60%, and when a broader spectrum of autistic features is considered, concordance rates approach 90%, in contrast to 0% and 10% for dizygotic twins [1]. Autism is clinically heterogeneous with up to 10% of cases associated with well defined neurological disorders such as tuberous sclerosis and fragile X syndrome [1]. Genome wide linkage studies yielded several linkage peaks including 2q, 5p, 7q, 11p 17q, and, of interest here, 4q [2-5]. Rare mutations have been found at several loci including the neuroligin, neurexin and SHANK3 genes [6-8]. Microscopic chromosomal rearrangements are seen in 3-6% of autism [9], submicroscopic copy number variations (CNVs) are found in at least 10% of sporadic, but less than 2% in familial cases, and many individual loci remain to be identified [10;11].

EIF4E activity is the rate-limiting component of eukaryotic translation initiation, which directs ribosomes to the mRNA 5' cap structure for initiation of protein synthesis. In brain, EIF4E activity is fundamental to the regulation of lasting alterations in synaptic strength or plasticity, and of long-term potentiation (LTP): these are important in learning and memory [12;13]. Increased activity in these systems can lead to repetitive, perseverative behaviour patterns [12]. *EIF4E* is highly conserved across species and no germline mutations have been reported to date.

Synaptic translation is the final step in pathways already implicated in autism. Deregulation of PTEN/PI3K and tuberous sclerosis pathways, which converge on mammalian target of

rapamycin (mTOR), an upstream regulator of EIF4E, have been implicated in conditions co-morbid with autism [14]. Likewise, in Fragile X syndrome, absence of FMRP upregulates synaptic translation through failure of recruitment of CYFIP1, a newly recognised EIF4E binding protein [15;16]. Local protein synthesis is required for synaptic plasticity, a mechanism that underpins learning and memory [17-21].

Regulation of EIF4E activity is known to play a key role in learning and memory through its control of translation within the synapse [12;13]. Genome wide linkage studies in autism patients have shown linkage to the region containing the *EIF4E* locus on chromosome 4q [2;4]. Here we present evidence from three independent families that strongly implicate EIF4E in autism.

METHODS

Subjects. The child with the translocation was assessed using an informal clinical interview and the Autism Diagnostic Observation Schedule (ADOS; module 1 of generic version) at 4 yrs 9 months, and the Autism Diagnostic Interview Revised (ADI-R) at 6 yrs 7 months [22;23]. Blood samples were obtained from the translocation carrier and his parents with informed consent. The study protocol was approved by the Grampian Local Research Ethics Committee. A lymphocyte cell line was derived from the translocation carrier by transformation with EBV (European Collection of Cell Cultures, Health Protection Agency Culture Collections, Salisbury UK).

Cases of non-syndromic autism were obtained from the Autism Genetic Research Exchange collection (http://www.agre.org/). Cases were selected on the basis of availability of DNA from both parents and two siblings with a definite diagnosis of autism (120 multiplex families).

One thousand and twenty control DNA samples from healthy individuals with no evidence of neuropsychiatric disorder were screened for the mutations found in autism cases. Putative mutations identified in autism cases were screened for in family members.

Cytogenetic analysis Conventional cytogenetic analysis was performed on GTG banded metaphase nuclei at the 550 band level. Nuclei were obtained from a lithium-heparinised peripheral blood sample following standard cytogenetic culture and harvest protocols [24]. Fluorescent in-situ hybridization (FISH) was performed with commercial unique sequence telomere-specific probes (Vysis), bacterial artificial chromosomes (BACs), and fosmid clones from the regions flanking the cytogenetic breakpoints. The BACs and fosmids were selected using the University of California at Santa Cruz Genome Bioinformatics Browser (http://genome.ucsc.edu/) and obtained from BACPAC Resources (Children's Hospital Oakland Research Institute). Genomic DNA was labelled by direct incorporation of fluorochromes by nick translation (Vysis nick translation kit). BACs were hybridized for 24 hours, followed by 2 min washes in 0.4 x SSC/0.1% IPEGAL CA-630 (Sigma) at 72°C visualized at 100x magnification (Zeiss Neofluar objective) using an epifluorescence microscope (Zeiss Axiscop) and an Applied Imaging analysis system using the MacProbe version 4.3 software.

Chromosome flow-sorting Derivative chromosomes were separated from their homologues by dual laser flow sorting at the Molecular Cytogenetics Group, University of Cambridge. Generation of chromosome-specific paint probes followed previously described methods [25;26].

Detailed analysis of the breakpoint regions DNA from der (4) and der (5) was amplified using the GenomePlex® Single Cell Whole Genome Amplification Kit (WGA4) from Sigma-Aldrich. PCR primer pairs from across the region of interest were used to amplify DNA of derivative

chromosomes. Primer pairs were selected to walk across both breakpoints to characterize the translocation by direct sequencing. Copy-number variation analysis on the Affymetrix human Gene-Chip 10K array was used to exclude other cryptic rearrangements.

Mutation analysis Direct sequencing was used to examine the coding regions and the promoter of the *EIF4E* gene. PCR products were purified by a Y-100 column (Fisher Scientific) and direct sequencing was performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were analysed using an ABI 3100 Genetic Analyzer and results were analyzed using the programs SEQUENCHER 3.1.1 (Gene Codes Corp.). Putative mutations were validated by sequencing DNA from the affected sibling (and both parents) showing a variation, and 56 controls. Variants found in affected sibs but not controls were additionally screened for in 1020 anonymous control samples using denaturing high performance liquid chromatography (dHPLC) on a Transgenomic WAVE apparatus, with a positive control on each run.

Binding reactions and EMSA Binding reactions with 80 fmol ^{32}P 5' end-labelled double-stranded oligonucleotides were performed in 16 mM Hepes-KOH (pH 8), 16 % glycerol, 80 mM KCl, 0.16 mM EDTA, 0.8 mM DTT and 10 mg/ml HeLa cell nuclear extract (Abcam). After 30 min incubation on ice, reactions were analysed by 5 % polyacrylamide gel electrophoresis, and visualised using autoradiography or a Fuji Phosphoimager with AIDA software for quantitation. Double-stranded DNA molecules used were the wild type genomic sequence 5'-TTTCCTCTTACCCCCCCTTCTGGAGCGGTT (C_7-4EBE) and the derivative C_8-4EBE with an additional C added to the C_7 stretch element. Where indicated, 200-fold or 500-fold excess of cold double-stranded competitor DNA was added.

Luciferase assays A 410 base pair *EIF4E* promoter fragment spanning the region from a PstI site up to the major transcription initiation site (Position +1 in Fig. 3a) was amplified using the Roche Expand High Fidelity PCR kit, with oligonucleotides to create a C_7-4EBE and a C_8-4EBE version. PCR fragments were inserted into pGEM-T easy (Promega) and verified by sequencing. The primers contained KpnI and HindIII restriction sites that were used to insert the promoter fragments into the firefly luciferase reporter vector pGL3 basic (Promega). Near confluent HeLa cells grown in 24 well plates were transfected with 0.7 µg pGL3 based reporter, and with 100 ng pRL-Tk (Promega) expressing Renilla luciferase. Cells were lysed 24 h - 48 h after transfection and luciferase activities were determined using a dual luciferase assay (Promega). Firefly luciferase activity was standardised with respect to Renilla luciferase. Transfections were done in triplicates and each transfection was measured three times. Shown are the average and standard deviation calculated from the three parallel transfections.

RESULTS

Routine cytogenetic screening identified a *de novo* balanced 46,XY,t(4;5)(q23;q31.3) translocation in a boy with classic autism (fig 1a). There is no family history suggestive of autistic traits, he had no dysmorphic features, other than a double hair whorl on the crown, and no malformations. On the Autism Diagnostic Observation Schedule- Generic (Module) the child scored 21 points on algorithm items and on the Autism Diagnostic Interview Revised (2000) [22;23] he scored at ceiling level for most algorithm items with a total of 28 points in the domain of reciprocal social interaction, 14 points for non-verbal communication and 8 points for repetitive behaviours. Thus, he demonstrated a typical and severe autistic phenotype and on all

measures, DSMIV criteria for the diagnosis of Autistic disorder were fulfilled. Development was apparently normal up until the age of two. This was followed by a period of severe regression characterized by loss of speech, social interaction and communication skills, and the development of stereotyped and repetitive patterns of behaviour. At the age of 6.5 years his speech consisted of little other than stereotyped vocalizations but did not display co-morbid global developmental delay.

Fluorescent *in situ* hybridization (FISH) analysis with bacterial artificial chromosomes (BACs) and fosmid clones localized the sites of the breakpoints in immortalised lymphocytes. BAC clone RP11-911N10 spans the breakpoint on chromosome 4 (fig 1b, fig 1c), and fosmids mapped the breakpoint on chromosome 5 to a 47.6 kb interval (142,854,992-142,902,586) (data not shown). Fine mapping was performed by PCR using DNA amplified from flow sorted derivative chromosomes [25;26]. The breakpoint was further characterized by Sanger sequencing [27] (fig 1d). Chromosome translocation occurred by breakage and re-ligation of chromosomes 4 and 5 and involved the addition of few (1-4) residues at the breakpoint. Copy-number variation analysis using the GeneChip Human Mapping 10K 2.0 Array (Affymetrix) excluded other cryptic rearrangements (data not shown).

The breakpoint on chromosome four is located in a region linked to autism [2] and maps 56 kb downstream of the *EIF4E* reference sequence. The breakpoint on chromosome five is not in a linked region, the nearest gene being *NR3C1*. Families with heterozygous mutation of *NR3C1* are reported with hypertension, hypokalaemia and female masculinisation, but not autistic features [28]. Our subject by contrast is normotensive with a normal urinary screen for catecholamines. Thus our finding supports previous data implicating the chromosome 4q region in autism.

To further explore a role of EIF4E in autism, we screened for mutations in 120 multiplex families with two autistic siblings obtained from the Autism Genetic Research Exchange collection (AGRE). In two independent families, direct sequencing revealed a heterozygous single base C insertion in the *EIF4E* promoter region in the proband. In both of these families, the variant was also present in the second autistic sibling and the father. The variant was not found in 1020 anonymous control samples. In fig 2, the chromatograms from the analysis of one family are shown, clearly revealing the presence of the insertion in the father and the two affected offspring. Inheritance of the mutation is identical for the second family (data not shown). AGRE SNP data from Affymetrix 10k 2.0 arrays confirmed that the two families are unrelated. This sequence variant is not present in 2040 control chromosomes. It is located in a region previously identified as the EIF4E basal promoter element (4EBE) that binds hnRNPK and contains a stretch of 7 C nucleotides (C_7-4EBE) [29;30] (fig 2b). The C insertion extends it to an 8 C nucleotide run (C_8-4EBE). A number of rare base changes were also present in the autism families (see Table S1).

To determine whether this mutation changes the properties of the *EIF4E* promoter we performed binding studies with short double-stranded DNA fragments derived from 4EBE, using HeLa cell nuclear extract as source of binding factors. Electrophoretic mobility shift assays (EMSA) demonstrate that the C_8-4EBE DNA sequence variant has an increased affinity for an abundant nuclear protein, probably hnRNPK [30]. This is revealed in binding experiments where excess unlabeled C_8-4EBE DNA competes more efficiently with binding of the nuclear factor to ^{32}P-labelled C_7-4EBE DNA then an identical excess of unlabeled C_7-4EBE DNA (fig 3a).

Promoter activity assays were performed using a 0.4 kb *EIF4E* promoter fragment with either a C_7- or a C_8-4EBE upstream of the firefly luciferase reporter gene, resulting in a construct where

luciferase expression is exclusively controlled by one or the other of these elements. Both variants were transfected into HeLa cells and showed a significant increase in expression compared to the promoterless control construct. Importantly, the single C insertion (C_8-4EBE) resulted in a significant two-fold increase in *EIF4E* promoter activity compared to the wild type (C_7-4EBE) promoter (fig 3b). Thus this mutation causes de-regulation of *EIF4E* expression.

All four of the affected children with this promoter variant scored highly within all domains of the ADI-R interview [22] and had marked language delay. One showed regression. The ADI-R is not designed to provide an index of severity but scores indicate that all subjects had a high level of symptoms.

DISCUSSION

Our findings implicate the *EIF4E* gene in the pathogenesis of autism, and provide a strong case for further study of EIF4E and related pathways in autism. Our evidence is twofold. First, the chromosomal translocation in the boy with autism implicates the region containing *EIF4E* in autism. Second, we observed a heterozygous C insertion in the *EIF4E* promoter in two further unrelated families. In both families, this mutation changes a basal promoter element of EIF4E, shown to be the binding site of hnRNPK [30]. Importantly, the mutation leads to a higher affinity for its binding protein, and causes a two-fold increase in promoter activity.

Genomic data indicates that the *EIF4E* gene has at least two different transcription start sites. The transcript ENST00000280892, a member sequences of the human *EIF4E* consensus CDS set CCDS34031, is transcribed from the proximal promoter that contains 4EBE (fig 2B) [29;31],

whereas transcription of NM_001968.3 is initiated about 1.5 kb further upstream. Our data implicate the C insertion in the development of autism and suggest increased activity of the proximal promoter EIF4E promoter as a mechanism.

In both families, the C insertion is shared by a reportedly unaffected parent and two children diagnosed with autism. Variable penetrance and expression is frequently seen in families with autism. Likewise, in tuberous sclerosis, a disorder caused by TSC1 and TSC2 mutations associated with aberrant mTOR signaling, variation in severity of phenotype is common even within families. Furthermore, these parents may have mild behavioral features that are not catalogued with the AGRE collection. Of the five cases of autism we describe with deregulation of EIF4E, all had a high level of symptoms, and two of five exhibited regression. In contrast to findings at other autism loci, none of the affected cases with mutations in this study had either associated mental retardation or epilepsy.

The translocation may have its effect in two ways. The effect of downstream brain specific regulatory elements may be disrupted by the translocation. The translocation also disrupts an alternative *EIF4E* transcript. The GENSCAN algorithm [32] predicts an *EIF4E* transcript NT_016354.401 that encodes a larger protein encompassing the EIF4E reference sequence including the cap-binding domain required for the role of EIF4E in translation initiation. NT_016354.401 has three additional downstream exons, and two of these exons map downstream of the breakpoint (fig 4). Hybridisation to probes 2778989 and 2778996 on the Affymetrix Human Exon 1.0 ST array indicates that the two proximal downstream exons of NT_016354.401, which flank the breakpoint, are expressed at elevated levels in the cerebellum. Our own data revealed widespread expression of the terminal exon in the amygdala, hippocampus and cerebral cortex, regions of the brain implicated in autism (not shown).

Disruption of NT_016354.401 by the translocation would most likely result in the degradation of the mRNA by a mechanism related to nonsense-mediated mRNA decay, and cause downregulation of EIF4E activity.

Clearly, further investigation of function is limited by the unavailability of brain material from the affected individuals. EIF4E levels in transformed lymphocytes derived from the translocation case and the children carrying the C insertion were similar to levels in control transformed lymphocyte cell lines (not shown). Expression of the four genes flanking the breakpoint (see fig 1) was similar in the lymphocyte cell line from the translocation case and in control cells (not shown). We reviewed the available genomic data across the *EIF4E* locus for evidence of alternative isoforms. Our analysis of EST data revealed that there is little alternative splicing, with some transcripts containing a cryptic second exon. Differences between *EIF4E* transcripts are concentrated at the 5' end and 3' ends. Differences at the 5' end reflect at least in part the different promoter usage. In addition, a significant proportion of mRNAs lack the first exon. Differences at the 3' end are mostly due to mRNAs ending prematurely and lacking the terminal exons. Many of these differences may be artificially caused by the methods used for analysis. However it is possible that there might be several *EIF4E* transcripts, some of which may be brain specific. Future investigations will focus on identifying such transcripts and on the effects of deregulating *EIF4E* expression on synapse function in appropriate models.

A number of independent lines of evidence support a role of *EIF4E* in autism. First, it is located in a linkage hot spot implicated through linkage studies [2]. Second, EIF4E activity is regulated by the highly conserved PTEN/PI3K and tuberous sclerosis pathways. These pathways converge on mammalian target of rapamycin (mTOR), an upstream regulator of EIF4E. In tuberous sclerosis complex (TSC), where 25 - 50% have autistic features, mutations in *TSC1* and *TSC2*,

remove inhibition of mTOR and increase EIF4E activity [13;33]. Similarly, individuals with germline mutations in PTEN often have associated autistic features [14]. Mice with knockouts of the intracellular receptor mediator of rapamycin activity, *Fkbp12,* display repetitive and other behavioural features like those found in autism [34]. Deregulation of these signaling pathways can result in abnormalities of brain growth and synaptic plasticity in a manner analogous to Fragile X syndrome, a learning disability disorder with prominent autistic features, where inactivation of FMRP causes upregulation of synaptic translation mediated through CYFIP1, a recently recognised EIF4E binding protein [16]. Deregulation of glutamate signaling is also seen in both Fragile X and TSC. Cap-dependent translation is active during mGluR-LTD, and both MEK-ERK and PI3K-mTOR signaling pathways regulate EIF4E activity [35]. Indeed, on the basis of the association of mutations in FMRP, TSC1/2 and PTEN with autism, Kelleher et al. (2008) [36] hypothesized that "defects in translational repression may represent a possible mechanism leading to autistic phenotypes". Finally, mutation in another sub-unit of the translation initiation complex, *EIF2B*, causes the pediatric neurological disorder, leukoencephalopathy with vanishing white matter [37]. We previously reported decreased white matter and increased grey matter in autism [38]. Thus variation in *EIF4E* or interacting proteins is consistent with the white matter variation observed in autism.

Regulation of synaptic plasticity by EIF4E is highly complex and probably governed by as yet unknown additional EIF4E binding proteins [12]. In oncogenesis, increased EIF4E activity results in specific upregulation of translation of particular mRNAs that are normally inefficiently translated [13]. Subtle deregulation, either up or down, of synaptic EIF4E through modification of translation of specific brain transcripts may significantly impact on delicate processes such as synaptic consolidation. Penetrance and expressivity of such genetic variants will depend upon

genetic background and environmental factors, possibly at specific stages in development. This could account for our finding of reportedly asymptomatic carriers of the insertion mutation among the parents of the affected cases. Larger studies are now required to determine the prevalence and penetrance of EIF4E mutations.

EIF4E then is the endpoint of a number of pathways already implicated in autism. Our work showing direct involvement of EIF4E therefore provides key additional support for Kelleher's protein synthesis hypothesis for autism [36]]. The drug rapamycin suppresses EIF4E expression through its effects on mTOR. Exciting preliminary observational data from trials of rapamycin in TSC, suggest that rapamycin may not only control tumour growth, but may also improve behaviour [39]. Our findings raise the exciting possibility that in cases of autism caused by deregulation of translation, pharmacological manipulation EIF4E expression, either directly or, for example, through manipulation of mTOR signaling, could provide therapeutic benefit.

ACKNOWLEDGEMENTS

We thank Helen Murdoch for putting us in contact with the translocation case; Elaine Durward, Ben Milner, Ronggai Li, Guoqing Liu and Lynne Gray for technical assistance; Caroline Clark and Christine Bell for technical advice and Dee Rasalam for advice on diagnostic criteria for autism; the Medical Genetics and Tau Rx groups for sharing physical resources; and Irwin McLean and Ian Booth for invaluable comments. We gratefully acknowledge the family of our translocation case, participating AGRE families and the Autism Genetic Resource Exchange (AGRE) Consortium. The Autism Genetic Resource Exchange is a program of Cure Autism Now and is supported, in part, by grant MH64547 from the National Institute of Mental Health to

Daniel H. Geschwind (PI). Flow sorting was made possible by a Wellcome Trust grant to Malcolm Ferguson-Smith for the Cambridge Resource Centre for Comparative Genomics. This project was funded by the Scottish Government Chief Scientist Office, NHS Grampian Endowments and an anonymous grant to the University of Aberdeen Development Trust.

Reference List

[1] Bailey AF, Le Couteur AF, Gottesman IF, Bolton PF, Simonoff E FAU - Yuzda, Yuzda E FAU - Rutter, Rutter M. Autism as a strongly genetic disorder: evidence from a British twin study. Psychol Med 1995; **25**:63-77.

[2] Yonan AL, Alarcon M, Cheng R, Magnusson PK, Spence SJ, Palmer AA, Grunn A, Juo SH, Terwilliger JD, Liu J, Cantor RM, Geschwind DH, Gilliam TC. A genomewide screen of 345 families for autism-susceptibility loci. Am J Hum Genet 2003; **73**:886-897.

[3] Trikalinos TA, Karvouni A, Zintzaras E, Ylisaukko-oja T, Peltonen L, Järvelä I, Ioannidis JP. A heterogeneity-based genome search meta-analysis for autism-spectrum disorders. Mol Psychiatry 2006; **11**:29-36.

[4] Schellenberg GD, Dawson G, Sung YJ, Estes A, Munson J, Rosenthal E, Rothstein J, Flodman P, Smith M, Coon H, Leong L, Yu CE, Stodgell C, Rodier PM, Spence MA, Minshew N, McMahon WM, Wijsman EM. Evidence for multiple loci from a genome scan of autism kindreds. Mol Psychiatry 2006; **11**:1049-1060.

[5] Autism Genome Project Consortium. Mapping autism risk loci using genetic linkage and chromosomal rearrangements. Nat Genet 2007; **39**:319-328.

[6] Jamain S, Quach H, Betancur C, Rastam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T, Paris Autism Research International Sibpair Study. Mutations of the X-linked neuroligins NLGN3 and NLGN4 are associated with autism. Nat Genet 2003; **34**:27-29.

[7] Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, Nygren G, Rastam M, Gillberg IC, Anckarsäter RH, Sponheim E, Goubran-Botros H, Delorme R, Chabane N, Mouren-Simeoni MC, de Mas P, Bieth E, Roge B, Heron D, Burglen L, Gillberg C, Leboyer M, Bourgeron T. Mutations of the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet 2006; **39,**25-27.

[8] Kim HG, Kishikawa S, Higgins AW, Seong IS, Donovan DJ, Shen Y, Lally E, Weiss LA, Najm J, Kutsche K, Descartes M, Holt L, Braddock S, Troxell R, Kaplan L, Volkmar F, Klin A, Tsatsanis K, Harris DJ, Noens I, Pauls DL, Daly MJ, MacDonald ME, Morton CC, Quade BJ, Gusella JF. Disruption of neurexin 1 associated with autism spectrum disorder. Am J Hum Genet 2008; **82**:199-207.

[9] Vorstman JA, Staal WG, van Daalen E, van Engeland H, Hochstenbach PFR, Franke L. Identification of novel autism candidate regions through analysis of reported cytogenetic abnormalities associated with autism. Mol Psychiatry 2006; **11**:18-28.

[10] Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, Walsh T, Yamrom B, Yoon S, Krasnitz A, Kendall J, Leotta A, Pai D, Zhang R, Lee YH, Hicks J, Spence SJ, Lee AT, Puura K, Lehtimäki T, Ledbetter D, Gregersen PK, Bregman J, Sutcliffe JS, Jobanputra V, Chung W, Warburton D, King MC, Skuse D, Geschwind DH, Gilliam T, Ye K,

Wigler M. Strong association of de novo copy number mutations with autism. Science 2007; **316**:445-449.

[11] Weiss LA, Shen Y, Korn JM, Arking DEPh, Miller DT, Ph.D., Fossdal R, Saemundsen EBA, Stefansson H, Ferreira MAR, Green T, Platt OS, Ruderfer D, Walsh CA, Altshuler D, Chakravarti A, Tanzi REPh, Stefansson K, Santangelo S, Gusella JF, Sklar P, Wu BL, Daly MJ, for the Autism Consortium. Association between microdeletion and microduplication at 16p11.2 and autism. NEJM 2008; **358**:667-675.

[12] Klann E, Dever TE. Biochemical mechanisms for translation in synaptic plasticity. Nature Reviews Neuroscience 2004; **5**:931-942.

[13] Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature 2005; **433**:477-480.

[14] Butler MG, Dasouki MJ, Zhou XP, Talebizadeh Z, Brown M, Takahashi TN, Miles JH, Wang CH, Stratton R, Pilarski R, Eng C. Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. J Med Genet 2005; **42**:318-321.

[15] Pfeiffer BE, Huber KM. Current Advances in Local Protein Synthesis and Synaptic Plasticity. J Neurosci 2006; **26**:7147-7150.

[16] Napoli I, Mercaldo V, Boyl PP, Eleuteri B, Zalfa F, De Rubeis S, Di Marino D, Mohr E, Massimi M, Falconi M, Witke W, Costa-Mattioli M, Sonenberg N, Achsel T, Bagni C. The Fragile X Syndrome Protein Represses Activity-Dependent Translation through CYFIP1, a New 4E-BP. Cell 2008; **134**:1042-1054.

[17] Job C, Eberwine J. Localization and translation of mRNA in dentrites and axons. Nat Rev Neurosci 2001; **2**:889-898.

[18] Job C, Eberwine J. Identification of sites for exponential translation in living dendrites. Proceedings of the National Academy of Sciences of the United States of America 2001; **98**:13037-13042.

[19] Steward O, Schuman EM. Protein synthesis at synaptic sites on dendrites. Annual Review of Neuroscience 2001; **24**:299-325.

[20] Tang SJ, Reis G, Kang H, Gingras AC, Sonenberg N, Schuman EM. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proceedings of the National Academy of Sciences of the United States of America 2002; **99**:467-472.

[21] Inamura N, Hoshino Si, Uchiumi T, Nawa H, Takei N. Cellular and subcellular distributions of translation initiation, elongation and release factors in rat hippocampus. Molecular Brain Research 2003; **111**:165-174.

[22] Lord C, Rutter M, Lecouteur A. Autism Diagnostic Interview-Revised - A Revised Version of A Diagnostic Interview for Caregivers of Individuals with Possible Pervasive Developmental Disorders. Journal of Autism and Developmental Disorder 1994; **24**:659-685.

[23] Lord C, Risi S, Lambrecht L, Cook JEH, Leventhal BL, DiLavore PC, Pickles A, Rutter M. The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. J Autism Dev Disord 2000; **30**:205-223.

[24] Rooney D, Czepulkowski B. Human Cytogenetics: constitutional analysis. A practical approach. 3 ed. Oxford University: Oxford University Press, 2001.

[25] Telenius H, Pelmear AH, Tunnacliffe A, Carter NP, Behmel A, Ferguson-Smith MA, Nordenskjöld M, Pfragner R, Ponder BA. Cytogenetics analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. Genes Chromosomes Cancer 1992; **4**:257-263.

[26] Yang F, Carter NP, Shi L, Ferguson-Smith MA. A comparative study of karyotypes of muntjacs by chromosome painting. Chromosoma 1995; **103**:642-652.

[27] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977; **74**:5463-5467.

[28] Karl M, Lamberts SW, Detera-Wadleigh SD, Encio IJ, Stratakis CA, Hurley DM, Accili D, Chrousos GP. Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 1993; **76**:683-689.

[29] Johnston KA, Polymenis M, Wang S, Branda J, Schmidt EV. Novel Regulatory Factors Interacting with the Promoter of the Gene Encoding the mRNA Cap Binding Protein (eIF4E) and Their Function in Growth Regulation. Mol Cell Biol 1998; **18**:5621-5633.

[30] Lynch M, Chen L, Ravitz MJ, Mehtani S, Korenblat K, Pazin MJ, Schmidt EV. hnRNP K binds a core polypyrimidine element in the Eukaryotic Translation Initiation Factor 4E (eIF4E) promoter, and its regulation of eIF4E Contributes to neoplastic transformation. Mol Cell Biol 2005; **25**:6436-6453.

[31] Jaramillo M, Pelletier J, Edery I, Nielsen PJ, Sonenberg N. Multiple mRNAs encode the murine translation initiation factor eIF-4E. J Biol Chem 1991; **266**:10446-10451.

[32] Burge C, Karlin S. Prediction of complete gene structures in human genomic DNA. J Mol Biol 1997; **268**:78-94.

[33] Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous Sclerosis Complex Gene Products, Tuberin and Hamartin, Control mTOR Signaling by Acting as a GTPase-Activating Protein Complex toward Rheb. Current Biology 2003; **13**:1259-1268.

[34] Hoeffer CA, Tang W, Wong H, Santillan A, Patterson RJ, Martinez LA, Tejada-Simon MV, Paylor R, Hamilton SL, Klann E. Removal of FKBP12 Enhances mTOR-Raptor Interactions, LTP, Memory, and Perseverative/Repetitive Behavior. Neuron 2008; **60**:832-845.

[35] Banko JL, Hou L, Poulin F, Sonenberg N, Klann E. Regulation of Eukaryotic Initiation Factor 4E by Converging Signaling Pathways during Metabotropic Glutamate Receptor-Dependent Long-Term Depression. J Neurosci 2006; **26**:2167-2173.

[36] Kelleher III RJ, Bear MF. The Autistic Neuron: Troubled Translation? Cell 2008; **135**:401-406.

[37] Leegwater PAJ, Vermeulen G, Könst AAM, Naidu S, Mulders J, Visser A, Kersbergen P, Mobach D, Fonds D, van Berkel CGM, Lemmers RJLF, Frants RR, Oudejans CBM, Schutgens RBH, Pronk JC, van der Knaap MS. Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. Nat Genet 2001; **29**:383-388.

[38] Waitera GD, Williams JHG, Murraya AD, Gilchristc A, Perrettd DI, Whitend A. Structural white matter deficits in high-functioning individuals with autistic spectrum disorder: a voxel-based investigation. Neuroimage 2005; **24**:455-461.

[39] Cahoon L. BIOMEDICINE: A New View on--and Hope for--an Old Disease. Science 2009; **323**:203-205.

FIGURE LEGENDS

Figure 1: A chromosomal translocation in a case of classical autism. (a) GTG banding of chromosomes 4 and 5 and der (4) and der (5). (b) FISH analysis of breakpoint. FISH analysis with bacmids and fosmids was performed as described. RP11-911N10 maps to base pairs 99887465-100079064 on chromosome 4, RP11-359H9 and RP11-659D8 map to base pairs 146356329-14651515 and 142011154-142179956 on chromosome 5, respectively. RP11-911N10 labels both derivative chromosomes and thus spans the breakpoint. RP11-359H9 and RP11-659D8 (indicated by an arrow and a diamond) flank the chromosome 5 breakpoint. (c) Schematic representation of the breakpoint regions of chromosomes 4 and 5, and derivative chromosomes der (4) and der (5). The breakpoints are indicated with arrows. Representation was prepared using the UCSC Genome Bioinformatics Browser. Not all forms of the reference genes are shown, nor are all exons. Chromosome coordinates are based on the human genome build 36.2. (d) Breakpoint sequences. Shown are chromatograms of the breakpoint sequence on der (4) and der (5) obtained by direct sequencing. Indicated are the sequences derived form chromosomes 4 and 5, respectively. In the sequence alignment, chromosome 4 sequences are in red letters and chromosome 5 sequences are in blue capital letters. Residues in black capital letters at the break point are not derived from either chromosome 4 or chromosome 5. Note that an AC dinucleotide at the breakpoint in chromosome 5 has been duplicated and occurs in der (4) and der (5).

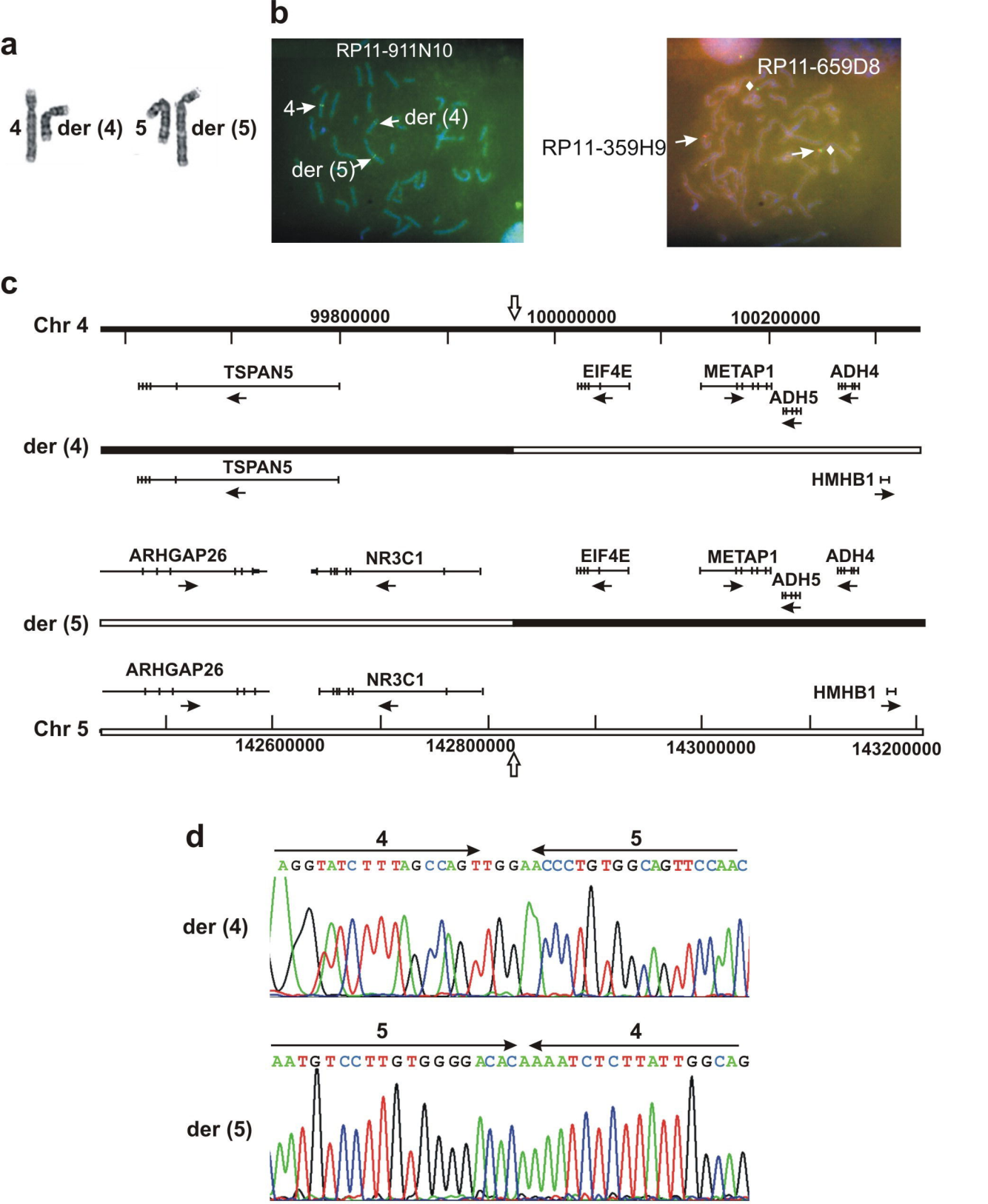
Figure 2: An inherited C insertion in the *EIF4E* promoter element 4EBE in families with children with autism (a) Insertions of a C in the EIF4E-4EBE promoter element were detected by sequencing in two independent families of the AGRE cohort (C_8-4EBE). Pedigree and sequencing traces are shown for one family only as the inheritance pattern and sequence are the

same in both families. (b) *EIF4E* promoter region showing transcription initiation sites (capital letter), the start of the *EIF4E* coding sequence (boxed) and the 4EBE promoter element (4EBE, underlined) with a stretch of 7 nucleotides (C_7-4EBE) [29]. The major transcription initiation site is indicated with +1 [31].

Figure 3. The C insertion into the 4EBE promoter element increases binding of a nuclear factor and *EIF4E* promoter activity (a) Effect of the C insertion on binding of nuclear factors. Binding reactions with wild type C_7-4EBE element or the derivative C_8-4EBE and HeLa nuclear were done and analyzed by EMSA followed by visualisation using autoradiography or a Fuji Phosphoimager as described. Where indicated a 200-fold or 500-fold excess of unlabelled competitor DNA was included in the binding reaction. The histogram summarizes the proportion of ^{32}-P labelled DNA in complexes. The proportion of complexed DNA in reaction without competitor was defined as 100 %. Shown are data from two independent experiments (in black and white, respectively). (b) C insertion increases *EIF4E* promoter activity. Dual luciferase assays were done by transfecting HeLa cells with pGL3 basic (no promoter) or pGL3 with the 0.4 kb *EIF4E* promoter fragments containing either the C_7-4EBE or the C_8-4EBE. The major *EIF4E* transcription initiation site (Position +1 in fig 2b) and two natural myc binding sites are included in both constructs. Plasmid pRL-Tk was co-transfected and Renilla luciferase activity was used as control. Transfections were done as described and shown are relative firefly luciferase units 24 h after transfection, standardized with respect to Renilla luciferase expression. This data is representative of three independent experiments.

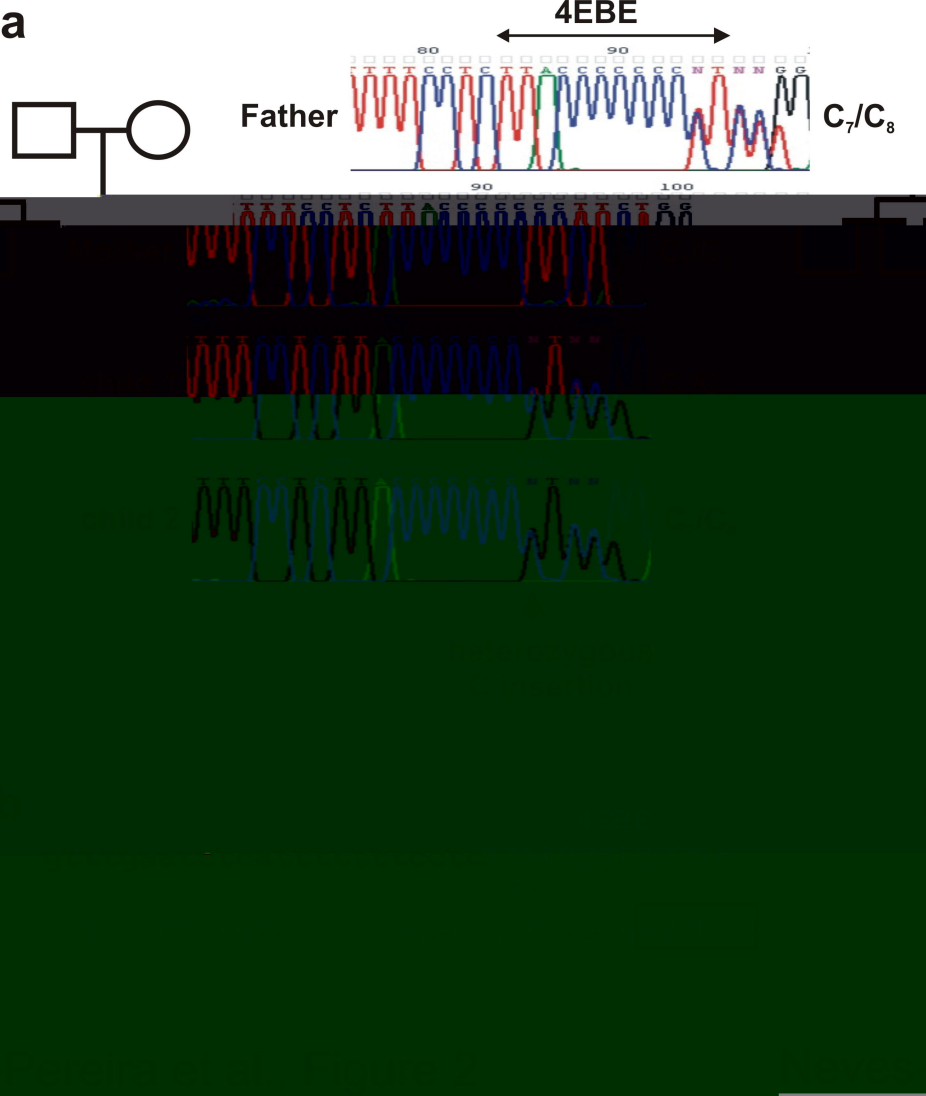
Figure 4: *EIF4E* transcripts (a) Schematic representation of *EIF4E* transcripts. Shown are the intron/exon structures of *EIF4E* transcript ENST00000280892 (top) and of the predicted alternative transcript (NT_016354.401; bottom) (2 additional 5' exons have been omitted for

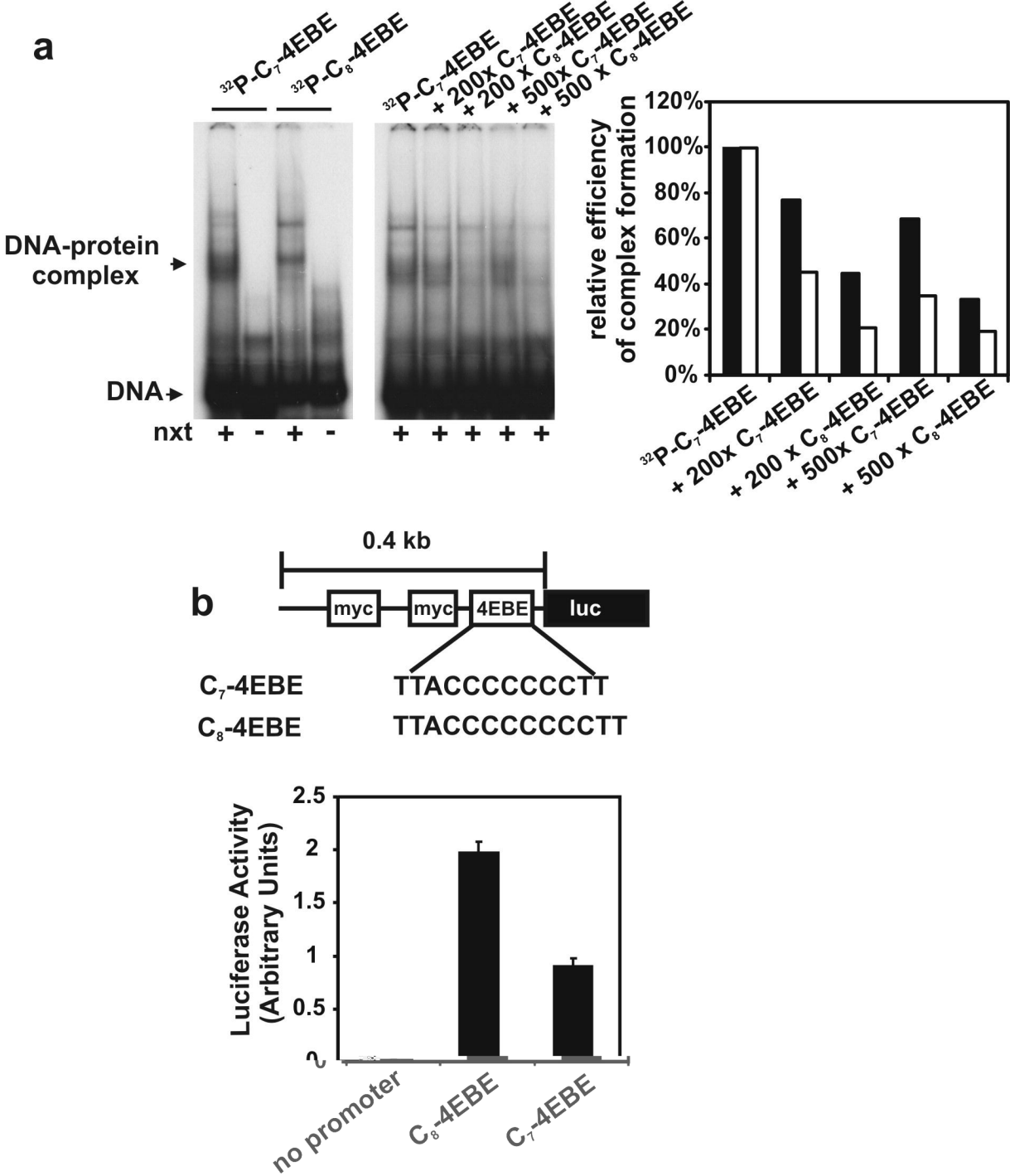
simplicity). Shared exon sequences are shown as black boxes; unique exons are shown in white. Additional downstream exons of the alternative transcript are also shown as white boxes. The translocation breakpoint disrupts the alternative transcript between two of these exons. Note that intron and exon sequences are not represented to scale.



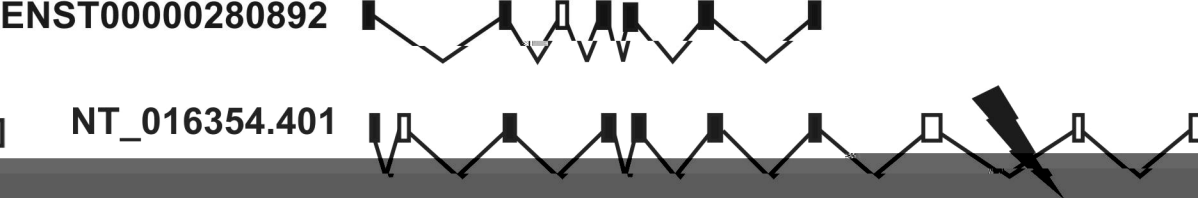

```
Chr 4 (99963962-99964001)    aggtatctttagccagtaagtaaatctcttattggcagca
der(4)                       aggtatctttagccagtTGGAACCCTGTGGCAGTTCCAAC
der(5)                       AATGTCCTTGTGGGGACAC-Aaaatctcttattggcagca
Chr 5 (142824032-142824067)  AATGTCCTTGTGGGGACAC----CCTGTGGCAGTTCCAAC
```

Neves-Pereira et al., Figure 1


a
4EBE
Father
C7/C8



Neves-Pereira et al., Figure 3



Neves-Pereira et al., Figure 4

Table S1: Sequence variations identified by direct sequencing in autism families.

NM_001968*/ NT_016354.401	**Observations in 120 cases**	**Confirmation**	**Location on chr 4**	**SNP id (dbSNP)**	
5'UTR/exon 2	1	HapMap	100070056	rs17028283	non-synonymous in NT_016354.401
-/3'exon2	4	Seen in 2 / 100 controls	100011889	rs7674319	non-synonymous in NT_016354.401

* *EIF4E* mRNA reference sequence

.