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ARTICLE

# Identification and characterization of the *TRIP8* and *REEP3* genes on chromosome 10q21.3 as novel candidate genes for autism

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**Autism is a genetic neurodevelopmental disorder of unknown cause and pathogenesis. The identification of genes involved in autism is expected to increase our understanding of its pathogenesis. Infrequently, neurodevelopmental disorders like autism are associated with chromosomal anomalies. To identify candidate genes for autism, we initiated a positional cloning strategy starting from individuals with idiopathic autism carrying a *de novo* chromosomal anomaly. We report on the clinical, cytogenetic and molecular findings in a male person with autism, no physical abnormalities and normal IQ, carrying a *de novo* balanced paracentric inversion 46,XY,inv(10)(q11.1;q21.3). The distal breakpoint disrupts the *TRIP8* gene, which codes for a protein predicted to be a transcriptional regulator associated with nuclear thyroid hormone receptors. However, no link between thyroid gland and autism has been reported so far. In addition, the same breakpoint abolishes expression of a nearby gene, *REEP3*, through a position effect. Receptor Expression-Enhancing Proteins (REEP) 3 is one of the six human homologs of yeast Yop1p, a probable regulator of cellular vesicle trafficking between the endoplasmatic reticulum and the Golgi network. These observations suggest that *TRIP8* and *REEP3* are both positional candidate genes for autism. In addition, our data indicate that in the selection of positional candidate genes when studying chromosomal aberrations, position effects should be taken into account.**
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**Keywords:** autism; positional cloning; paracentric inversion; position effect; thyroid hormone receptor interacting protein 8; receptor expression-enhancing protein 3

## Introduction

Autism is a severe developmental disorder of the central nervous system of unknown pathogenesis. The prevalence of autism is estimated at about 1/1000 to 1/2000 and the prevalence of autism disorders at about 1/160.[1] In 5–10% of individuals with autism, a single underlying cause can be established, such as a chromosomal aberration or a

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monogenic disorder (eg, the Fragile X syndrome, tuberous sclerosis[2,3] or more rarely, mutations in the *NLGN3/4* genes[4] and the *SHANK3* gene[5]). In these instances associated physical anomalies are often present. In contrast, in the majority of individuals the autism is isolated and no identifiable single cause can be identified. In these cases, polygenic inheritance is likely in most of them. However, estimates on the number of interacting genes vary from two to 10,[6] and it is likely that different combinations of genes may be implicated in unrelated individuals.[7]

The diagnosis of autism is achieved on purely clinical grounds, given the lack of diagnostic neurobiological or genetic markers. The identification of genes involved in the origin of autism is expected to increase our understanding of the pathogenesis of this disorder. Genetic studies have yielded suggestive linkage and association to several different chromosomal regions, but up to now, the large number of association studies using a candidate gene approach has had limited success.[8–11] As an alternative approach, the characterization of different types of chromosomal abnormalities could result in the identification of candidate genes for autism.[8,9,12] About 5% of autistic patients have a chromosome abnormality visible with cytogenetic methods.[13] Almost all chromosomes have been involved including translocations and inversions resulting in disruption of genes at the breakpoints (eg,[14,15]), and interstitial or terminal deletions and duplications resulting in gene-dosage effects of 10s of genes.[12,16,17] The most frequently reported imbalances are 15q11–q13 duplication[18] and 2q37 deletion.[19] However most imbalances remain undetectable using routine karyotype analysis. Recent technological developments, such as array-based comparative genomic hybridization (aCGH), allow the investigation of the human genome at a much higher resolution and resulted in the identification of different cryptic imbalances and the fine mapping of the 15q duplication in autism.[13,20,21]

At the Center, we initiated a positional cloning strategy starting form both classical karyotyping[8] and aCGH.[22] As previously reported, in a group of 525 individuals with isolated, sporadic autism, we identified four patients carrying a *de novo* chromosomal aberration using routine karyotyping.[8,14] The incidence of such apparently balanced aberrations in this population of patients with autism is much higher than expected from the low incidence in the normal population (1/10 000 for a paracentric inversion and 1/2000 for a reciprocal translocation[23,24]). Together with the *de novo* origin of the aberrations and negative family histories for autism, this suggests that at least in some of these individuals, there is a causal relationship between the chromosome aberration and the occurrence of autism. Here, we describe the detailed molecular genetic analysis of a boy with autism carrying a *de novo* balanced paracentric inversion 46,XY,inv(10) with breakpoints in chromosome 10q21.3 and the centromere of chromosome 10.

## Materials and methods

### Positional cloning of the inversion breakpoint

Was performed as described before.[14] Electronic-database information and all primers used are provided online as Supplementary Information.

### Southern blot analysis

Genomic DNA of the patient and a control was treated with spermidine, digested with *Eco*RI enzyme and electrophoresed on 0.8% agarose gel. Southern blotting and hybridization were carried out according to the standard protocols.[25] The filter was hybridized with a 301-bp PCR product from intron 1 of the *hTRIP8a-c* gene, purified with QIAquick gel extraction kit (Qiagen, Venlo, The Netherlands) and radiolabelled with $\alpha$-$^{32}$P-dCTP using the Megaprime DNA labelling system (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

### RT-PCR

Reverse transcriptase (RT-PCR) was used (a) to show that both BC068557 and BC018658 mRNAs are corresponding to the same *REEP3* gene, (b) to detect mRNA of all different *TRIP8a-c* transcripts and (c) to study gene expression using real-time PCR and single-nucleotide polymorphisms (SNPs) (see below). mRNA was isolated from Epstein–Barr virus (EBV) transformed leukocytes of the patients and controls using the Rneasy Midi Kit (Qiagen). Next, mRNA was treated with DNaseI (Gibco BRL, Merelbeke, Belgium,) for 15 min followed by cDNA preparation using the Superscript First Stand Synthesis System for RT-PCR (Invitrogen, Merelbeke, Belgium) with random hexamers. Finally, a standard PCR reaction with 25–30 cycles was performed with the cDNA as a template.

### Real-time PCR

Quantitative PCR was performed using qPCR Master Mix for SYBR Green I detection (Eurogentec, Seraign, Belgium) on an ABI PRISM 7000 instrument, in accordance with the manufacturer's guidelines. Primers were developed with Primer Express Software (Applied Biosystems, Foster City, CA, USA). *HPRT* was used for normalization and the relative expression levels were calculated using the delta delta $C_t$ method.[26]

### Study of gene expression using SNPs

To study the expression of genes at or nearby the breakpoints, SNP analysis was performed at the genomic and mRNA level. First, for reported SNPs in the coding sequence of *TRIP8a–c*, *NRBF-2* and *REEP3* we analyzed whether the patient was heterozygous at the genomic level by sequencing the amplified fragment both directly and after subcloning in the pGEM-T easy (Promega, Leiden, The Netherlands). For SNPs for which the patient was heterozygous, the corresponding cDNA sequences were analyzed for the presence of both alleles. As a control, biallelic

expression was tested in heterozygous controls. mRNA was isolated from EBV cell lines of the patient and controls using the Rneasy Midi Kit (Qiagen). cDNA was prepared by RT-PCR as described above. DNA and cDNA fragments were amplified by PCR and sequenced using the DNA Sequencing kit (PE Biosystems, Foster City, CA, USA) and the ABI Prism 3100 Genetic Analyser. Primers used for cDNA amplification were chosen in different exons, except for the primer set used to amplify the cDNA fragment from the 3′UTR of *REEP3* gene.

### Methylation analysis of CpG islands

To investigate the methylation of CpG islands, genomic DNA extracted from peripheral white blood cells of the patient and a control were digested with methylation-independent enzymes cutting at both sides of the island (*Bam*HI for CpG37, *Eco*RI for CpG122 and CpG125), alone or in combination with methylation-dependent restriction enzymes cutting at or nearby the CpG island (*Hpa*II for CpG37, *Not*I for CpG 122 and *Cfr*10I for CpG125). Fragment lengths were analyzed by means of Southern blot analysis and the pattern for patient and control was compared. Probes used for hybridizing the blots were designed in a fragment (a) flanked by restriction sites corresponding with an enzyme independent for methylation, and (b) flanked, at least at one site, by a restriction site for a methylation-dependent enzyme within the fragment obtained after digestion with the methylation independent. Expected lengths for double digestion are: 2.7 kb (methylated) or 1.4 kb (unmethylated) for CpG37, 7.1 kb (methylated) or 4.7 kb (unmethylated) for CpG122, and 9.5 kb (methylated) or 623 bp (unmethylated) for CpG125. As GC% of the CpC122 and CpG125 probes are relatively high (69 and 75%), higher hybridization (57.5°C) and washing temperatures (65°C) were used.

## Results

### Case report

CK is a 9-year-old boy, referred for child psychiatric assessment because of social and communication problems. He is the third child of non-consanguineous parents. There was no family history of developmental disorders. Pregnancy and delivery were uneventful. There were no signs of dysmaturity. Psychomotor milestones were within normal range. Early language and social development were delayed. Development of fantasy and pretend play were delayed and as a preschooler, CK was fascinated by circling objects. He did not socialize with peers at school. Clinical assessment showed a boy with no dysmorphic signs. Clinical neurological examination was normal. He had marked deficits in social reciprocity, and 'active-but-odd' type social interactions. Productive and receptive language were mildly delayed. Non-verbal communication was impaired. He showed perseverative behavior and had problems to adjust to changes. Mood and affect were normal. There were signs of a formal thought disorder. In a special school program, CK had 1-year delay in reading and arithmetics. Wechsler's intelligence scales (WISC revised) showed an uneven intelligence profile within the normal range: performal IQ 106, verbal IQ 93. Theory-of-Mind testing showed marked impairment in social perspective taking. In conclusion, CK fulfilled the criteria for the diagnosis of (high-functioning) autistic disorder according to DSM-IV.[27] His mild formal thought disorder is not a typical characteristic in autism.

### Molecular analysis

In the patient, a chromosomal inversion was detected with karyotype 46,XY,inv(10)(q11.1;q21.3). Karyotype of the parents was normal. Genome-wide aCGH using a 1 Mb aCGH did not detect a chromosomal imbalance (data not shown). Therefore, the chromosomal anomaly in the patient is a *de novo* balanced paracentric inversion. The positions of the breakpoints on chromosome 10q were determined by means of FISH on metaphase chromosomes. Cytogenetically the proximal breakpoint was mapped at 10q11.1. However, the centromere probe of chromosome 10 used as a control probe for FISH gave a split signal on the rearranged chromosome 10 (Figure 1b). This revealed that the proximal breakpoint was located in the repetitive sequence of the centromere of chromosome 10. Therefore, the gene(s) implicated in the phenotype must be located at or near the chromosome 10q21.3 breakpoint.

As shown in Figure 1a, the distal breakpoint at 10q21.3 was flanked by BACs RP11-444I9 (NCBI AL607128) and RP11-439F7 (NCBI AL607062). BAC RP11-351O1 (NCBI AC022022, Figure 1c) and cosmids 87d1 (Figure 1c), 14h8 and 29h9 were found to span the breakpoint. Southern blot analysis with a probe from the overlap region of these cosmids showed a rearranged fragment in the DNA of the patient using the *Eco*RI restriction enzyme (Figure 1d). The breakpoint could thus be localized to a 7.3 kb *Eco*RI restriction fragment (Figure 1a).

### Direct disruption of the *TRIP8* gene on 10q21.3

Comparison of sequences of both the cosmid ends and the probe used in the Southern blot study with the human genome sequence, showed that the 10q21.3 breakpoint is located within the first intron of the *TRIP8* gene (Figure 1a),[28] also known as *JMJD1C* (UCSC Browser).

Using expressed sequencing tag (EST) data, we identified three alternative transcripts of the *TRIP8* gene (Figure 1a and Table 1). The three transcripts use an alternative first exon (see Figure 1a). The existence of all three transcripts of the *TRIP8* gene was confirmed by transcript-specific RT-PCR on RNA extracted from an EBV cell line (Figure 2a – right), which yielded fragments of the expected size. For each transcript, one primer was located in the alternative exon 1, the second primer in exon 3 (numbering according

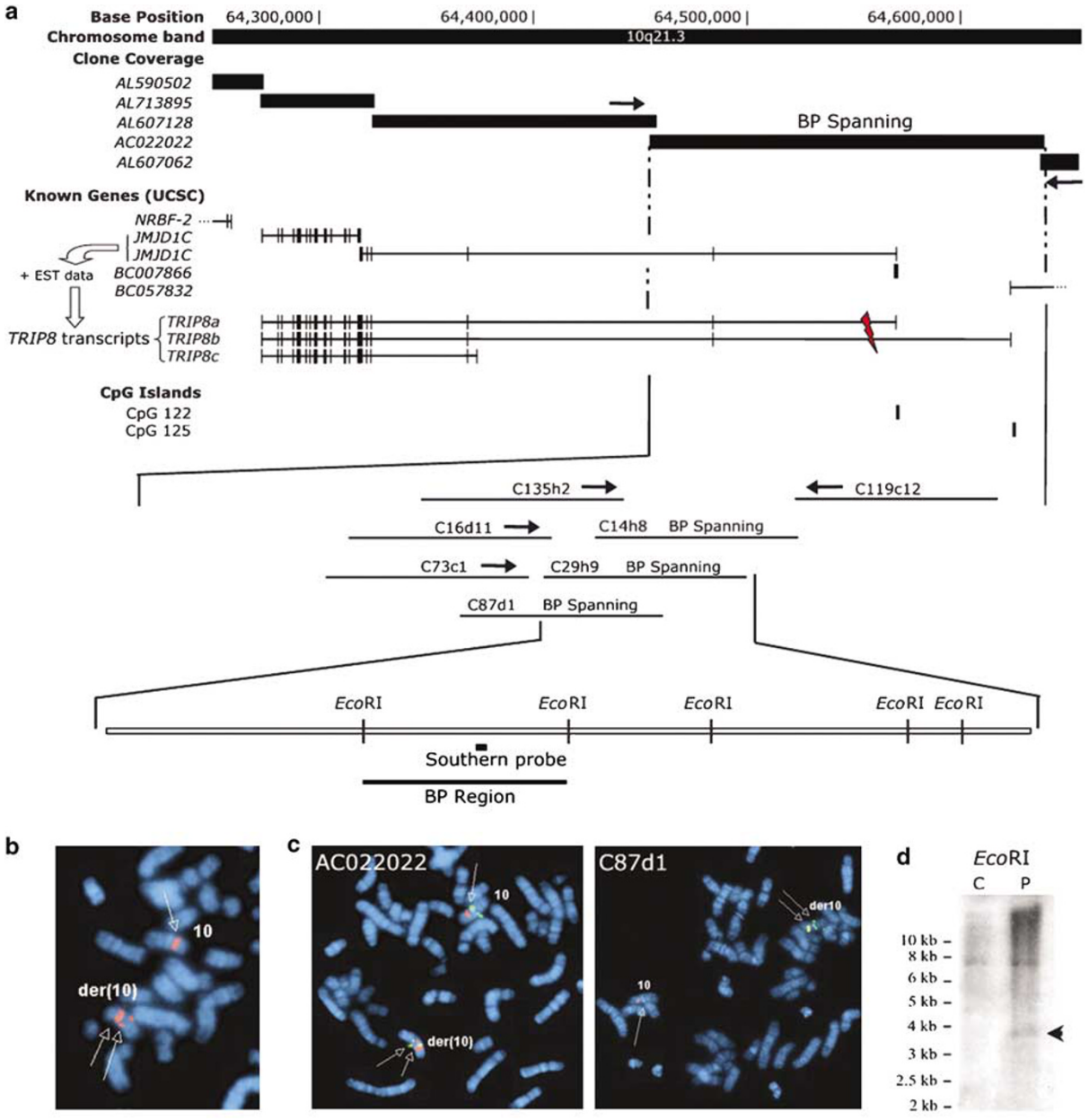



**Figure 1** The *TRIP8* gene on chromosome 10 is directly disrupted by an inversion breakpoint in a patient with idiopathic autism. (**a**) Physical map of 10q21.3 (UCSC Genome Browser, July 2003 version – centromere on the left). BAC AC022022 and cosmids 87d1, 29h9 and 14h8 were found to span the breakpoint. Arrows indicate the position of the inversion breakpoint with regard to the genomic clone (→, distal; ←, proximal). The position of the Southern blot probe used in (**d**) and the 7.3 kb *Eco*RI restriction fragment are indicated on the restriction map of the cosmids spanning the breakpoint. The red lightning depicts the position of the breakpoint in the genomic region 10q21.3. (**b**) FISH analysis on metaphase spread of the patient using the centromere probe of chromosome 10 shows that the proximal breakpoint is located within the centromere. (**c**) FISH analysis showed that BAC AC022022 (B351O1 – left) and cosmid 87d1 (right) span the breakpoint at 10q21.3. (**d**) Southern blot analysis using a probe from intron 1 of the *TRIP8* gene reveals a rearranged fragment (arrow) in the patients genomic DNA (P) compared to a control (**c**).

to *TRIP8a* and *b*). Cloning and sequencing confirmed the identity of the fragments. For both *TRIP8a* and *TRIP8b*, a CpG island was identified (CpG 122 and 125, respectively, Figure 1a). However, we could not identify a CpG island (WebGene) or a putative promotor (Promotor Inspector) in the 280 kb region proximal to the breakpoint that could drive the expression of *TRIP8c* from the derivative chromosome 10q.

From its position in the gene, the chromosomal inversion is expected to disrupt expression of both *TRIP8a* and

**Table 1** Features of the three alternative transcripts of the TRIP8 gene

| *Transcript* | *EST/mRNA* | *CpG* | *Transcript length (kb)* | *Exonic structure* | *Genomic size (kb)* | *Start* | *Predicted protein (AA)* |
|---|---|---|---|---|---|---|---|
| *TRIP8a* [28] | AL027280 | 122 | 9 | 1a – 2→26 | 300 | Exon 1 | 2540 |
| *TRIP8b* | BF515122 | 125 | 8.42 | 1b - 2→26 | 355 | Exon 2 | 2484 |
| *TRIP8c* | BE778964 | — | 8.69 | 1c - 3→26 | 102 | Exon 4 | 2372 |

ESTs (NCBI BF515122 and BE778964) and mRNA (NCBI AL027280) used for *in silico* identification of the transcripts are shown.

*TRIP8b* transcripts, whereas expression of the alternative *TRIP8c* transcript may be preserved. By means of real-time PCR on cDNA from EBV cell lines, expression levels of the different *TRIP8* transcripts were studied in the patient and compared to controls. Interestingly, ddCt, analysis revealed a twofold decrease in expression of both *TRIP8a* and *TRIP8b*, whereas the expression of *TRIP8c* is clearly unaffected by the chromosomal aberration in the patient (Figure 2b). $C_t$ values of the amplification of the different transcripts in the EBV cell line of control C1 indicate that *TRIP8b* is expressed in relative low amounts compared to the other transcripts (Figure 2b), which is consistent with the semiquantitative RT-PCR data (Figure 2a – right).

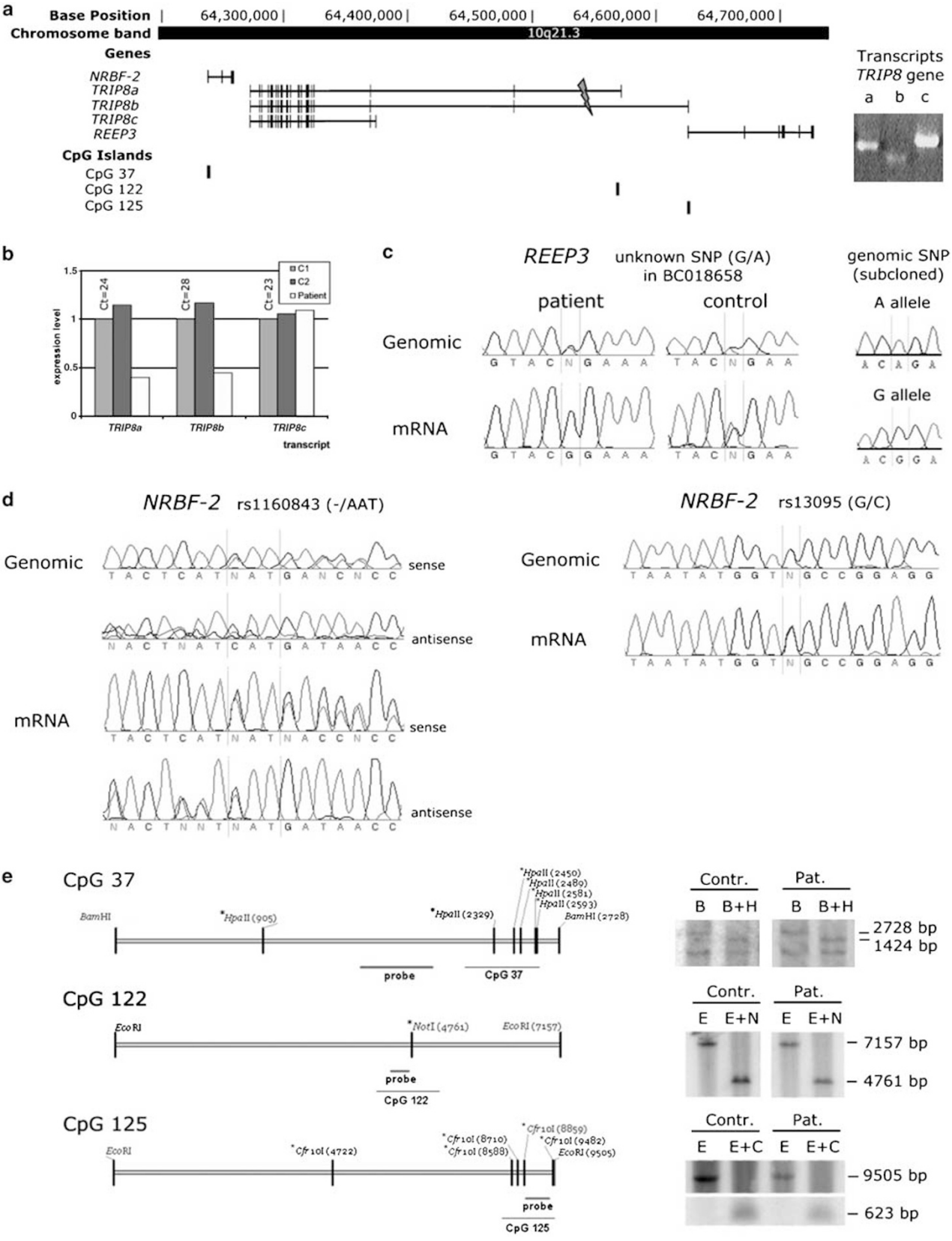
### Breakpoint position effect affecting the expression of the *REEP3* gene, a yeast *Yop1p* homolog

As a result of the inversion, 10q21.3. sequences are now juxtaposed to centromere sequences in this patient, and position effects may have occurred in genes more distant from this breakpoint, both distal and proximal. We investigated the methylation pattern of CpG islands and the expression levels of genes, both proximal and distal to the breakpoint (Figure 2a). Using SNPs, we investigated the expression of the genes *NRBF-2* (NM_030759) 315 kb proximal, and *REEP3* (NM_001001330, formerly known as *C10ORF74*) 43 kb distal to the breakpoint on an EBV cell line from the subject (Figure 2b and c). The next gene telomeric from *REEP3*, *CTNNA3* is located at a distance of more than 2 Mb and was therefore not studied. For two of the five reported exonic SNPs in the 3′UTR of *NRBF-2* (NCBI mRNA AK054957), rs1160843 (−/AAT) and rs13095 (G/C), the patient was heterozygous. Analysis of the corresponding mRNA fragments revealed a normal biallelic expression of this *NRBF-2* gene (Figure 2c). However, for the mRNA BC018658 (corresponding to *REEP3*, see below), the patient was heterozygous for an unreported SNP (G/A) in the 3′ UTR, as shown by sequencing of the amplified DNA containing the polymorphism of interest, both directly and after subcloning (Figure 2c). At the mRNA level only the G-allele was detected, indicating monoallelic expression of this gene (Figure 2b). The possibility of monoallelic expression owing to imprinting of the locus of interest or a preferential amplification of one of the alleles was excluded by studying four heterozygous controls, which showed biallelic expression of the *REEP3* gene (shown for one control, Figure 2b).

As shown in Figure 2e, the methylation of the investigated methylation-dependent restriction sites at or nearby CpG island 37 proximal, and islands 122 and 125 distal to the breakpoint in the patient were unaffected by the vicinity of the centromere sequence as compared to control.

Although the unreported G/A SNP was located in the mRNA BC018658, and the UCSC database shows the non-overlapping mRNA BC068557 1200 bp upstream chromosome 10 as the full-length cDNA sequence of *REEP3*, we showed that both mRNAs correspond to a single gene. First, we identified a cDNA clone (IMAGE562868 (NCBI ESTs AA100605 and AA100609)) containing part of the 3′ end of *REEP3* and the entire BC018658 mRNA. In addition, using a sense primer in BC068557 (*REEP3* up) and antisense primer in BC018658 (*REEP3* low), we were able to amplify a cDNA fragment of the expected size of 1.6 kb, which had the predicted nucleotide sequence, identical to that of the corresponding fragment of the IMAGE562868 clone (data not shown). Interestingly, the transcript was

**Figure 2** TRIP8 and REEP3 genes affected by the inversion breakpoint at 10q21.3. (**a**) Physical map of 10q21.3 (UCSC Genome Browser, July 2003 version – centromere on the left). The breakpoint position is indicated with a lightning. The three *TRIP8* transcripts, and the *NRBF-2* and *REEP3* genes are shown. RT-PCR in EBV cell lines of a control using transcript-specific primers proved the actual existence of all three transcripts, *TRIP8a*, *b* and *c*. CpG islands in the region are shown, and the location of SNP rs3211105 in exon 20 of *TRIP8* is indicated with an asterisk. (**b**) Expression levels of the different *TRIP8* transcripts in EBV cell lines form the patient versus two controls (C1 and C2) by means of real-time PCR. Expression levels are calculated relative to expression in control C1. $C_t$ values for this control are indicated. (**c**) Expression analysis of *REEP3* in the patient. Sequence analysis of amplified DNA fragments, both directly and after subcloning, showed heterozygosity for a previously unreported G/A polymorphism. cDNA analysis of the G/A SNP in *REEP3* revealed expression of the G allele only for this gene. (**d**) Expression analysis of *NRBF-2* in the patient. Sequence analysis showed heterozygosity for rs1160843 (−/AAT) and rs13095 (G/C). For both polymorphisms in *NRBF-2*, both alleles are expressed in the patient mRNA. (**e**) Methylation patterns of CpG37 proximal, and CpG122 and 125 distal to the 10q21.3 breakpoint in the patient (P) compared to control (**c**). Enzymes used are: *Bam*HI (B) and *Hpa*II (H) for CpG37, *Eco*RI (E) and *Not*I (N) for CpG122, and *Eco*RI (E) and *Cfr10*I (C) for CpG125. Schematic view of the restriction sites inside and outside the CpG islands of interest shows positions of CpG islands, probes and restriction sites (methylation-dependent restriction enzymes are depicted with an asterisk*).


a
Base Position
64,300,000
64,400,000
64,500,000
64,600,000
64,700,000
Chromosome band
10q21.3
Genes
NRBF-2
TRIP8a
TRIP8b
TRIP8c
REEP3
CpG Islands
CpG 37
CpG 122
CpG 125
Transcripts
TRIP8 gene
a b c
b
expression level
Ct=24
Ct=28
Ct=23
C1
C2
Patient
TRIP8a
TRIP8b
TRIP8c
transcript
c
REEP3 unknown SNP (G/A)
in BC018658
genomic SNP
(subcloned)
patient
control
Genomic
mRNA
A allele
G allele
d
NRBF-2 rs1160843 (-/AAT)
Genomic
sense
antisense
mRNA
sense
antisense
NRBF-2 rs13095 (G/C)
Genomic
mRNA
e
CpG 37
BamHI
HpaII (905)
HpaII (2329)
HpaII (2450)
HpaII (2480)
HpaII (2581)
HpaII (2593)
BamHI (2728)
probe
CpG 37
CpG 122
EcoRI
NotI (4761)
EcoRI (7157)
probe
CpG 122
CpG 125
EcoRI
Cfr10I (4722)
Cfr10I (8710)
Cfr10I (8588)
Cfr10I (8859)
Cfr10I (9482)
EcoRI (9505)
probe
CpG 125
Contr.
Pat.
B B+H
2728 bp
1424 bp
E E+N
7157 bp
4761 bp
E E+C
9505 bp
623 bp

also found on the AceView database, referred to as TB2_DP1_HVA22.4b. In conclusion, the full-length *REEP3* gene (NM_001001330) consists of eight exons spanning 104 kb of the human genome at 10q21.3. The gene codes for a transcript of 4677 bp, with a coding sequence of 765 bp (255 AA).

Through BLASTP search, proteins homologous to the predicted protein corresponding to human REEP3 (AAH68557) in other species, like mouse (AAH04607, *D10UCla1* gene (NM_178606)), rat (XP_215383, *LOC294375* gene (XM_215383)), zebrafish (AAH45373, *MGC55529* gene (NM_200161)), *Drosophilae* (AAM68228, *CG30193* gene (NM_166571)), *Caenorhabditis elegans* (AAC46595, *T19C3.4* gene (NM_064820)) and *Saccharomyces cerevisiae* (CAA07720, *Yop1p* gene (AJ007902)) were identified. For mouse, rat and zebrafish, we were able to show syntheny between human 10q23.1 and the chromosomal region where the homologous gene was located (chr10 in mouse, chr20 in rat and chr12 in zebrafish). Therefore, the corresponding proteins are true orthologs of the human REEP3 (Supplementary Figure). The yeast *Yop1p* gene has several homologs in human, of which *REEP5/C5orf18* (NM_005669) at 5q22.2 coding for the protein known as TB2/DP1 (AAH65926) is likely to be the true ortholog.[29] Alignment of protein sequences of the orthologs and homologs of REEP3 in other species revealed a conserved N-terminal region, corresponding to the 'TB2/DP1 and HVA22' domain (Pfam PF03134, Supplementary Figure). Northern blot analysis (not shown) revealed wide expression of this gene, including the central nervous system, which is in accordance with data represented in the public databases (eg, UniGene and Genecards).

## Discussion

Progress on the genetic basis of autism has been very slow, despite the numerous linkage and association studies reported over the last 5–10 years.[8–11] Chromosomal abnormalities detected by cytogenetics or aCGH are of major aid to locate relevant genes for any monogenic and polygenic disease.[9,11] Recently, this approach has been successful in the identification of candidate genes for other psychiatric disorders of unknown cause, that is, developmental dyslexia[30] and Tourette syndrome.[31] Therefore, as an alternative way to identify candidate genes for autism, we analyzed the breakpoint regions of *de novo* inversion inv(10)(q11.1;q21.3) in an individual with autism. As the proximal breakpoint was found to be located in the repetitive sequence of the centromere, we focused on the gene(s) located at the chromosome 10q21.3 breakpoint. This 10q21.3 breakpoint was located in the first intron of the *TRIP8* gene, and resulted in the disruption of at least one of the alternative transcripts we identified for this gene. In addition to this, we also found that the inversion breakpoint abolished expression of the *REEP3* gene, located 43 kb distal to the breakpoint. Both disrupted genes are expressed in brain, a requirement for an autism susceptibility gene. However, from a functional point of view, little is known on these two genes.

### Two novel positional candidate genes

The *TRIP8* gene was recently characterized *in silico* as a member of the family of thyroid receptor interacting proteins (TRIPs).[28] For this gene, we identified two unreported alternative transcripts. Expression analysis on an EBV cell line from the subject suggested that only transcripts *TRIP8a* and *b* were disrupted, but not *TRIP8c*. The function of TRIP8 is currently not known. The predicted TRIP8 protein consists of two bipartite nuclear localization signals, TRI8H1 and TRI8H2 and a JMJC domain.[28] Because JMJC domain proteins are implicated in chromatin remodelling,[32] TRIP8 was predicted to be a transcriptional regulator associated with nuclear hormone receptors. Consistent with this is the observation that TRIP8 interacts with T3 receptor $\beta$ in a T3-dependent manner.[33] Our finding therefore implicates a possible role for thyroid hormone signalling in the pathogenesis of the autism seen in the present patient. It is well established that thyroid hormones play a critical role in the regulation of brain development,[34] and therefore, it has been hypothesized that neurobehavioral disabilities of childhood, including autism[35] can be attributed to fetal thyroid endocrine disruption *in utero*. However, studies investing a possible link between autism and thyroid hormone or T3 receptors remained negative.[36–39] Alternatively, it is not excluded that TRIP8 might have an additional function besides its interaction with TRβ. Nearly all TRIPs interact with the retinoid X receptor,[33] and TRIP2 also interacts with other transcription factors, like vitamin D receptor, peroxisome proliferation-activated receptor- alpha and gamma, retinoic acid receptor alpha and, to a lesser extent, estrogen receptor-1.[40]

Likewise, the second candidate gene *REEP3* has not been functionally characterized. It codes for a predicted protein with high homology to proteins in human and several other species. Human REEP3, formerly known as C10ORF74, is a member of the recently described family of Receptor Expression-Enhancing Proteins (REEP).[41] In humans, this family consists of six members, but only for the prototype REEP1 a probable function is known, ie transport of G protein-coupled receptors to the cell surface membrane.[41] REEP proteins share a common domain called 'TB2/DP1 and HVA22', also found in other proteins in several other species. Functional data are available on two homologs, the yeast Yop1p and a plant homolog HVA22, and both reveal a role in cellular vesicle trafficking. Yop1p (Yip one partner) was identified as a probable regulator of cellular vesicle trafficking, as it interacts with Ypt1p (a Rab GTPase required for transport from the

endoplasmatic reticulum (ER) to the Golgi complex), as well as with Yip1p (a Ypt1p-interacting protein).[42] It is thought that these 'Rab effector' complexes serve as cores recruiting different 'effector partner' proteins involved in membrane trafficking to their proper subcellular localization. In agreement with the idea that Rab proteins function in all aspects of vesicular transport, their diverse effectors and effector partners have recently been shown to function in all identified aspects of this process.[43] Overexpression of Yop1p blocks membrane trafficking, resulting in huge swollen cells of aberrant shape.[29] This block was shown to be at the level of the ER, leading to an accumulation of internal membrane structures.[29] Interestingly, this phenotype could be rescued by co-expression of Yip1p, further indicating that Yop1p and Yip1p function in a common transport step.[29]

Expression of the *HVA22* plant gene is induced by environmental stresses, such as dehydration, salinity and extreme temperatures and by abscisic acid stress hormone.[44] Based on observations about the yeast Yop1p homolog, it has been suggested that HVA22 regulates vesicular traffic in stressed cells, either to facilitate membrane turnover, or to decrease unnecessary secretion.[45]

### Chromosomal breakpoint position effects

Positional cloning of translocation or inversion breakpoints has been a successful way of identifying candidate genes for many genetic disorders. The underlying assumption is that a gene disrupted by the breakpoint is causative of the phenotype. However, in many instances, no genes are detected that are directly disrupted by the breakpoints. This suggests either that the chromosomal aberration is unrelated to the phenotype, or, that genes located at a distance of the breakpoint must be implicated.[46] In this study, we show that besides the direct disruption of one gene the expression of another gene, located 43 kb away from the breakpoint, is equally affected. Similarly, genes flanking the 7q11 microdeletion in patients with Williams–Beuren syndrome showed decreased levels of expression.[47] In general, a position effect could occur as a result of the disruption of enhancers, or the physical separation of the gene from more distant enhancers. Alternatively, the present chromosomal aberration is unusual, in the way that it results in juxtaposition of genes to centromere repeat sequences. Centromeres are composed of large repetitive DNA domains packaged into a heterochromatin structure. These structures seem to be able to induce a generalized repression of transcription, a phenomenon called 'position effect variegation' or silencing.[48] The finding of a normal expression of the *NRBF-2* gene, 315 kb proximal to the breakpoint, and the apparently normal methylation pattern of the promoter regions of this gene and of the silenced *REEP3*, *TRIP8a* and *b* genes distal to the breakpoint, argues against a more general silencing effect imposed on the region nearby the breakpoint at 10q21.3. Therefore, the most likely mechanism causing disruption of *REEP3* expression is the loss of regulatory sequences. As in most (but not all) cases of such position effects, the breakpoint is located at the 5′ position of this gene.[49] Nevertheless, as the gene affected by the translocation breakpoint causing the phenotype might be located up to a distance of 1 Mb (eg, *SOX9* in Campomelic dysplasia[50]) additional positional candidate genes might be taken into account. As *CTNNA3*, the gene closest to *REEP3*, is located 2.46 Mb distal to the breakpoint, it is most unlikely that genes distal to the breakpoint are affected by a possible silencing effect caused by the aberration. Moreover, as mentioned earlier, normal expression in the patient of both *NRBF-2* and *TRIP8c* genes argues against a silencing effect affecting genes proximal to the breakpoint. Nevertheless, it has been reported that enhancer elements can regulate the expression of a gene at large distant, even with other uninvolved genes located within this genomic region separating enhancer and the regulated gene (eg, *SHH* at 7q36.3 in preaxial polydactyly[51]). Taken together, also genes more distant to chromosomal breakpoints should be considered as candidate genes, even when they are separated from the aberration by unaffected genes. Therefore, although unlikely, for the two additional genes within the 1 Mb-region proximal to the 10q21.3 breakpoint, *EGR2* (involved in Charcot–Marie–Tooth disease[52]) at 650 kb and *C10orf22* at 655 kb, a silencing effect cannot be completely excluded. Unfortunately, the absence of polymorphisms in de coding sequence of these genes together with low expression levels in EBV cell lines make it currently impossible to investigate this further.

In conclusion, we identified two novel candidate genes, *TRIP8* and *REEP3*, both affected by the 10q21.3 breakpoint of a *de novo* paracentric inversion in a patient with isolated autism. As the *REEP3* gene is not directly disrupted by the aberration, our observation suggests that, when studying chromosomal anomalies, genes located nearby should be considered as positional candidate genes as well. Unfortunately, both the functional data on these genes and our knowledge on the pathogenesis of autism are too limited to allow defining whether or not these two genes are also functional candidate genes for autism. Up to now, the chromosomal region 10q21.3 has not been identified as a candidate region containing susceptibility genes for autism in any of the linkage studies or in chromosomal aberrations.[8]

In the future, besides further functional studies, validation of the two candidate genes identified here will rely on association studies in patients and families with autism.

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