Combination of Linkage Mapping and Microarray-Expression Analysis Identifies NF-κB Signaling Defect as a Cause of Autosomal-Recessive Mental Retardation

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Autosomal-recessive inheritance accounts for nearly 25% of nonsyndromic mental retardation (MR), but the extreme heterogeneity of such conditions markedly hampers gene identification. Combining autozygosity mapping and RNA expression profiling in a consanguineous Tunisian family of three MR children with mild microcephaly and white-matter abnormalities identified the *TRAPPC9* gene, which encodes a NF-κB-inducing kinase (NIK) and IκB kinase complex β (IKK-β) binding protein, as a likely candidate. Sequencing analysis revealed a nonsense variant (c.1708C>T [p.R570X]) within exon 9 of this gene that is responsible for an undetectable level of TRAPPC9 protein in patient skin fibroblasts. Moreover, TNF-α stimulation assays showed a defect in IkBα degradation, suggesting impaired NF-κB signaling in patient cells. This study provides evidence of an NF-κB signaling defect in isolated MR.

Mental retardation (MR) is characterized by a broad range of cognitive-function deficits that significantly hamper adaptive capacities required for daily living, communication, social interaction and integration, self-direction, and work.[1] It is the most frequent handicap in children and young adults, affecting 2%–3% of the general population.[2] The severity of MR is commonly classified on the basis of the intelligence quotient (IQ), although other criteria have also been used. With the assumption of a population mean of 100 and a standard deviation of 15, MR is usually classified as "mild" when the IQ value ranges between 50 and 70 and as "severe" when the IQ value is below 50. Finally, MR is also subdivided into syndromic and nonsyndromic forms, depending on whether additional abnormalities are found on physical examination, laboratory investigation, or brain imaging.

These definitions hide a much more complex situation, and little is known about the biological bases of MR. Indeed, despite recent advances in cytogenetic and molecular genetic technologies, the cause of the mental handicap remains unexplained in 40% of the cases, leaving families with neither accurate diagnosis nor genetic counseling. Understanding the biological bases of these disorders is therefore a major challenge for the next years.

An autosomal-recessive mode of inheritance accounts for largely 25% of unexplained MR. Although X-linked MR has been extensively studied in the last decade, very little is known about the autosomal-recessive form of MR, which is far more common than X-linked forms. The broad genetic heterogeneity of autosomal-recessive MR (ARMR) has prevented researchers from pooling families, and the scarcity of large pedigrees has hitherto hampered the identification of disease genes. Yet, autozygosity mapping in large consanguineous families has led to the identification of five genes responsible for nonsyndromic ARMR: *PRSS12*, on chromosome 4q26 (MIM 606709);[3] *CRBN*, on chromosome 3p26 (MIM 607417);[4] *CC2D1A*, on chromosome 19p13.12 (MIM 608443);[5] *GRIK2*, on chromosome 6q21 (MIM 611092);[6] and *TUSC3*, on chromosome 8p22 (MIM 611093).[7,8] Moreover, ten loci have been mapped, but each of these genes or loci accounts for only one or very few families, suggesting that many disease-causing genes remain to be characterized.[9,10]

We ascertained a sibship of three affected boys and one healthy boy born to healthy Tunisian parents who are first cousins (Figure 1A). Institutional ethical approval for research and written consent were obtained for all participants in the study. All patients were born within normal growth parameters after an unremarkable pregnancy and delivery. The neonatal period was uncomplicated. Developmental delay became obvious by the end of the first year, and a significant speech delay was noted thereafter. The older affected brother, patient V-1, was first examined at the age of 10 yrs. He was a friendly, severely mentally retarded patient with a developmental IQ estimated by standardized tests to be 34. Physical examination revealed a mild microcephaly (occipitofrontal circumference [OFC] 49.5 cm, −2 standard deviations [SD]), truncular obesity, hypertelorism, short neck, and prominent upper central incisors. At 19 yrs of age, his height was 167 cm (−1 SD) and OFC was 53 cm (−2 SD). He showed mildly dysmorphic facial features, with short and smooth philtrum. Chromosome analysis, array-CGH testing at 1 Mb resolution, metabolic screening, and molecular testing for fragile X and FRAXE syndromes were all normal. Brain MRI was performed and showed unusual white-matter

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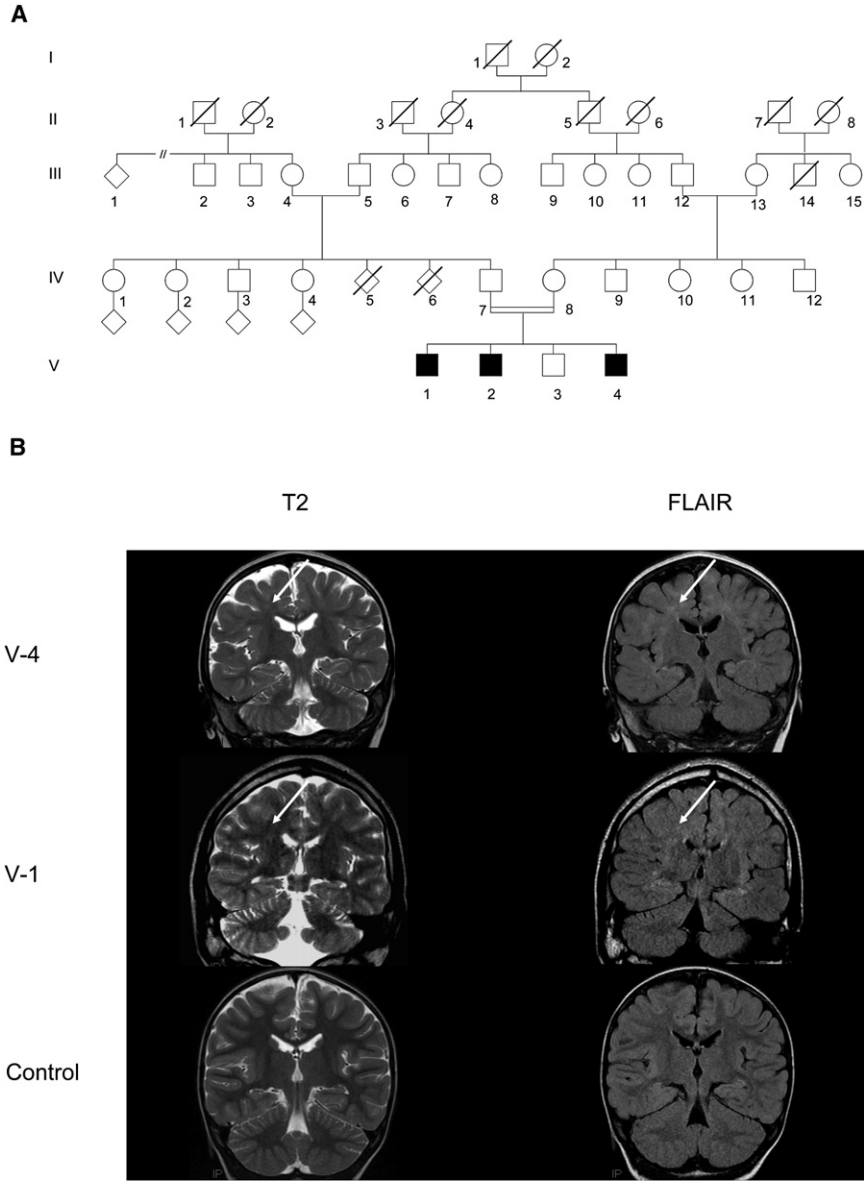

Figure 1. Pedigree of the Family and Brain MRI Features
(A) Pedigree of the family. Shaded symbols indicate individuals presenting with MR.
(B) Discordance between T2 and FLAIR sequences in patients V-1 and V-4. Coronal T2 weighted image (left) and coronal FLAIR image (right) of patient V-4 (upper panels) and patient V-1 (middle panel) at the level of the third ventricle demonstrate normal myelination on T2 sequence. By contrast, FLAIR sequence shows important white-matter abnormalities (white arrow) at the sus tentorial level compared to a normal control (lower panels).

abnormalities with discordance between T2 and FLAIR sequences (Figure 1B). Indeed, whereas normal myelination was observed on T2 sequence, FLAIR sequence showed important white-matter abnormalities when compared to a control. Patient V-2 had surgery for unilateral labial cleft. He walked at 17 mo of age. His speech was delayed, his first words being spoken at age 3. At the age of 7 yrs, his height was 122 cm (+0.5 SD), weight was 30 kg (+3 SD), and OFC was 49 cm (−1 SD). He presented with truncular obesity and midly dysmorphic features including hypotelorism, short neck, and prominent upper central incisors. Happy disposition and hyperactivity were noted. When examined at age 15, he was observed to be a friendly, moderately mentally retarded patient with a slender posture. His height was 172 cm (+1 SD), weight was 50 kg (−1 SD), and OFC was 50 cm (< −3 SD). He had mildly dysmorphic facial features, with short and smooth philtrum and long and thin fingers. He understood simple commands and concrete tasks and communicated verbally, with a low vocabulary and simple sentence structures. The third affected child (patient V-4) had global developmental delay but was less severely affected than his brothers. When examined at the age of 4 yrs, he was able to communicate verbally with about twenty words, understood commands, and was able to performed simple tasks. A hyperactive behavior was noted. His height was 102 cm, weight was 22 kg (with truncular obesity), and OFC was 48.5 cm (−2 SD). Brain MRI showed white-matter abnormalities similar to those observed for patient V-1, with discordance between T2 and FLAIR sequences (Figure 1B). Finally, we investigated the X chromosome inactivation profile in the mother by methyl-sensitive PCR analysis and observed that she showed a random X chromosome inactivation profile (58/42).

In conclusion, MR associated with mild microcephaly, myelination defect, and truncular obesity in the first year were consistent features in the three affected siblings.

Blood samples were collected from all affected and unaffected siblings and both parents, and skin fibroblasts were cultured from patient V-1. For genome-wide autozygosity screen, genomic DNA was extracted by standard methods and analyzed with the Affymetrix GeneChip Mapping 10K Array. Multipoint linkage analysis resulted in two significant linkage peaks on chromosomes 8 and X, respectively (Figure 2A). Further genotype and haplotype analyses confirmed homozygosity by descent and defined a 12 Mb critical interval between markers rs2395855 and rs1397380 on 8q24 (LOD scores 3.13) and a 30 Mb critical interval between markers rs982833 and rs750841 on Xq25-q28 (LOD scores 0.9) (Figure S1, available online).

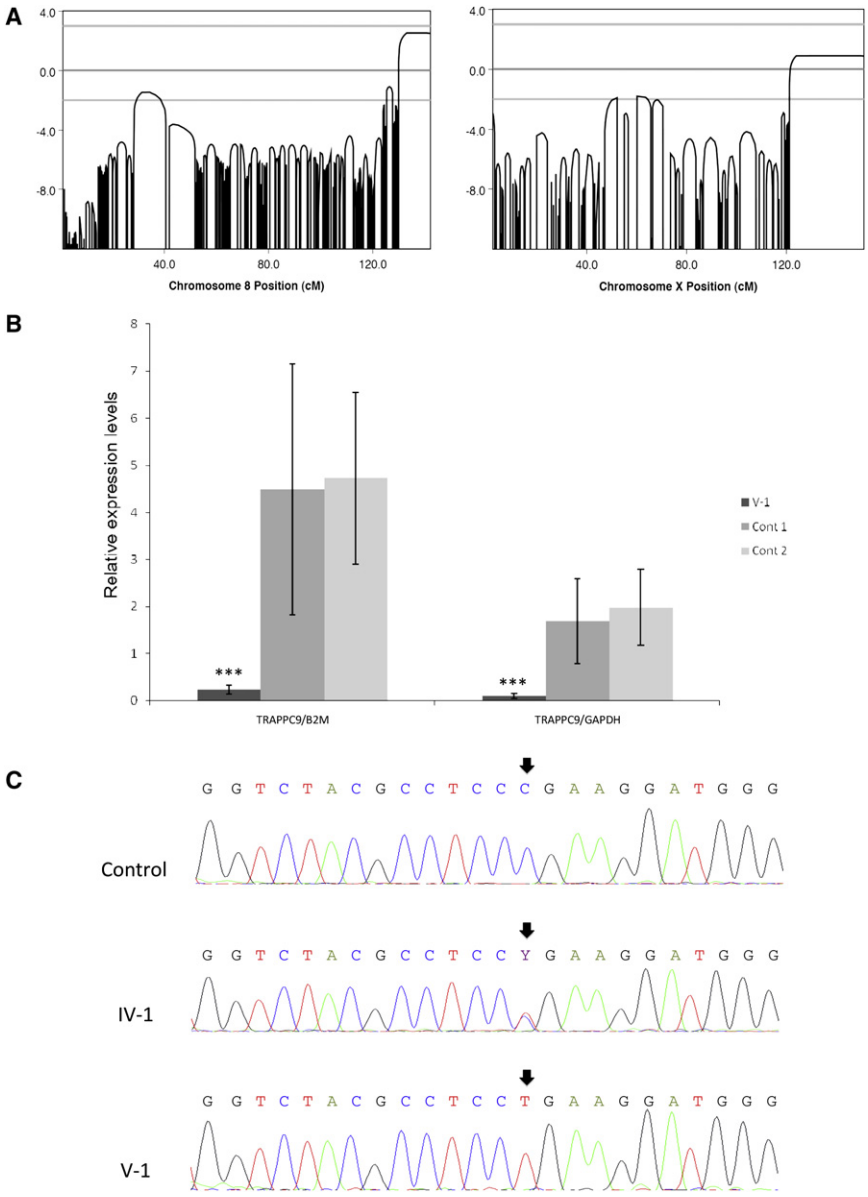

Figure 2. Genetic Analysis of the Family
(A) Results of the linkage analysis with the Merlin software. The y axis represents the LOD score and the x axis represents the genetic distance.
(B) Quantitative PCR analysis of *TRAPPC9* mRNA. *TRAPPC9* expression in fibroblast cells from two controls (black and gray bars) and patient V-1 is shown. Data are normalized to beta2 microglobuline (*B2M*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Means ± standard deviation are given (n = 5 independent experiments). ***Significance of difference with control values (Student's test), $p < 0.01$.
(C) Electrophoregrams illustrating the c.1708C>T variant in the *TRAPPC9* gene. Data are shown for a control, an affected child (V-1), and a healthy heterozygote parent (IV-1). The position of the single-nucleotide change is shown by a black arrow.

No other genomic regions were consistent with linkage. Altogether these two regions encompassed 172 known genes (NCBI build 36.3), the majority of which are expressed in the brain. The coding sequence of 14 candidate genes on 8q24 and three candidate genes on Xq25-q28 (Table S1) was searched for variants at DNA and/or RNA levels, but no causative mutation was identified in any of these genes. Thus, to prioritize the remaining candidate genes for sequencing, we hypothesized that the disease-gene transcript could be downregulated in affected subjects. RNA was extracted from skin fibroblasts derived from patient V-1 and from three healthy gender-matched control fibroblasts and analyzed with Affymetrix U133 Plus2 array. Microarray analysis was performed with bioconductor packages for the R programming environment. Background subtraction, normalization, and probe summarization were done with GeneChip robust multiarray averaging (gcrma), and tests of significance were conducted with the local pooled error (LPE) test.[11]

Analysis was confined to the probe sets mapping to the two regions defined by genetic linkage analyses, and *TRAPPC9* (also known as *NIBP*, NIK, and IKK-β Binding Protein, MIM 611966), located on chromosome 8q24, appeared as a good candidate. Indeed, it was significantly downregulated (40%) in the proband compared to controls ($p = 4.0 \times 10^{-14}$). A 90% *TRAPPC9* mRNA decay was confirmed by quantitative RT-PCR (Figure 2B), and subsequent mutational analyses identified a homozygote nonsense variant (c.1708C>T, p.R570X) within exon 9 of the gene (Figure 2C). This variant cosegregated with the disease and was not found in 1120 control chromosomes, including 196 chromosomes from individuals of Tunisian origin, supporting the hypothesis that the p.R570X change is the disease-causing mutation. Homozygosity at the *TRAPPC9* locus was tested by genotyping with Affymetrix GeneChip Mapping 250K array in thirteen independent consanguineous MR patients. However, no patient was found to be compatible with linkage to this locus.

For the evaluation of the consequence of the c.1708C>T mutation on protein expression, immunoblots were then prepared with fibroblast cell lysates from the affected sib and two healthy controls and probed with a polyclonal anti-TRAPPC9 antibody raised against the complete protein. Whereas TRAPPC9 was clearly detectable in cell lysates from healthy controls as a single band, neither normal nor shortened protein was found in fibroblast cell

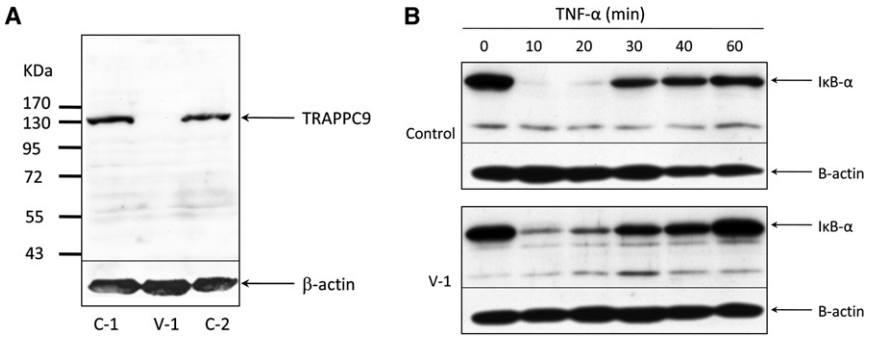

Figure 3. Functional Consequences of the TRAPPC9 Mutation

(A) Immunoblot analysis of TRAPPC9 protein. Lysates from controls (C1-2) and patient (V-1) fibroblasts were subjected to immunoblotting with a polyclonal anti-β-actin antibody as a loading control (lower panel) and with a rabbit polyclonal anti-TRAPPC9 antibody (upper panel). Molecular weights are indicated on the left. TRAPPC9, shown as a 140 kD band in controls, is absent in the patient.

(B) Time-course analysis of TNF-α-induced IκB-α degradation as detected by immunoblot. Lysates were subjected to immunoblotting with a polyclonal anti-β-actin antibody as a loading control (lower panel) and with a rabbit polyclonal anti-IKB-α antibody (upper panel). IKB-α is shown as a 37 kD band. Results from one representative experiment are shown.

extracts from the patient (Figure 3A). Taken together, these results suggest that the mutation causes a nonsense-mediated mRNA decay.

TRAPPC9 has been originally identified as a protein that interacts with the nuclear factor kappaB (NF-κB)-inducing kinase (NIK) and IκB kinase complex β (IKK-β).[12] The NF-κB transcription factor (TF) is a ubiquitously expressed dimeric molecule that regulates the expression of a variety of genes and plays a key role in a number of cellular processes such as innate and adaptive immunity, cellular proliferation, apoptosis, and development. In mammals, the NF-κB family is composed of five related transcription factors: p50, p52, RelA, c-Rel, and RelB.[13] These proteins form homo- or heterodimers that are retained in the cytoplasm by interaction with inhibitory molecules (IκBs).[14] Activation of NF-κB is mediated by both canonical and noncanonical signaling pathways.[15–17] The canonical pathway signals through activation of an IkB kinase (IKK) complex, composed of two catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, NF-κB essential modulator (NEMO)/IKK-γ. Upon stimulation, this complex triggers phosphorylation of the IkBs, leading to their ubiquitination and degradation through the proteasome pathway. Freed p50-RelA and p50-cRel dimers then migrate to the nucleus. The noncanonical pathway involves phosphorylation of IKK-α by NIK, which then triggers inducible processing of the p100, causing the release of a p52/RelB active heterodimer TF. On the basis of its interaction with NIK and IKK-β proteins, a role in both canonical and noncanonical NF-κB pathways was proposed for TRAPPC9.[12]

To further evaluate the functional consequences of the p.R570X mutation on NF-κB signaling pathway, we analyzed the response of cultured skin fibroblasts of patient V-1 to TNF-α, a powerful activator of the NF-κB system. Cultured fibroblasts from controls and a patient were stimulated for various times with 10 ng/ml of TNF-α. The time course of degradation of the inhibitory protein IκB-α was then analyzed by immunoblotting. Figure 3B shows a slight decrease in the level of IκB-α after 10 and 20 min of TNF-α stimulation in patient skin fibroblasts, whereas IκB-α almost completely disappeared in fibroblasts from two independent healthy controls. These results support the hypothesis of an impaired of NF-κB signaling in cells carrying the p.R570X mutation.

NF-κB activity and expression of several NF-κB target genes are altered in many chronic neurodegenerative diseases such as Alzheimer's disease,[18] Parkinson's disease, and Huntington's disease.[19,20] To our knowledge, this study provides the first evidence of a NF-κB signaling defect in an isolated cognitive deficit. How a TRAPPC9 mutation caused isolated MR remains questionable. Previous studies have demonstrated that knocking down *TRAPPC9* expression prevented nerve growth factor-induced neuronal differentiation and suggested that TRAPPC9 might function as a downstream component of the NGF pathway.[12] Yet how absence of TRAPPC9 protein led to MR in our patient remains unexplained.

Cognitive dysfunction is generally regarded as the consequence of a defect in synapse formation and plasticity. Along these lines, it is worth remembering that subcellular distribution, DNA binding activity, and transcription of NF-κB are regulated by various forms of synaptic activity and that this TF plays an important role in the induction of synaptic plasticity and long-term-memory formation.[21,22] Inhibition of NF-κB blocked induction of long-term potentiation (LTP) in the hippocampus and amygdala.[23,24] Moreover, exposure to exogenous TNF-α inhibited induction of LTP, and the knockout of TNF receptors inhibited induction of long-term depression (LTD).[23,25] Finally, recent studies based on cell-restricted ablation of NF-κB subunits demonstrated the prominent role of neuronal NF-κB in memory and cognition and indicated that NF-κB activation is essential for long-term-memory formation, especially when hippocampus is involved.[26]

Impaired NF-κB signaling in glial cells may also play an important role in the pathogenesis of the disease, given that recent data suggest an active role of astrocytes and oligodendrocytes in synaptic transmission.[27] Constitutive TNF-α release by glial cells promoted upregulation of AMPA receptors and internalization of inhibitory GABA receptors in hippocampal neurons, thereby increasing synaptic strength.[28,29]

Finally, our study may shed light on the role of NF-κB in the myelination process in the CNS. Although NF-κB has been shown to orchestrate the axon myelination of peripheral neurons by Schwann cells,[30] whether it plays a similar critical role in the differentiation of oligodendrocytes remains to be establish. The brain MRI observation of a myelination defect in our patients may provide further support to this hypothesis.

In conclusion, our study provides evidence that a NF-κB signaling defect may cause isolated cognitive deficit, expanding therefore the field of the pathophysiology of MR.

Supplemental Data

Supplemental Data include two figures and one table and can be found with this article online at http://www.cell.com/AJHG.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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