Convergent evidence identifying MAP/microtubule affinity-regulating kinase 1 (MARK1) as a susceptibility gene for autism

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Abstract

Autism spectrum disorders (ASDs) are common, heritable, but genetically heterogeneous neurodevelopmental conditions. We recently defined a susceptibility locus for ASDs on chromosome 1q41-q42. High-resolution single-nucleotide polymorphisms (126 SNPs) genotyping across the chromosome 1q41-q42 region, followed by a MARK1 tagged-SNP association study in 276 families with autism from the Autism Genetic Research Exchange, showed that several SNPs within the MARK1 gene were significantly associated with ASDs by transmission disequilibrium tests. Haplotype rs12740310*C-rs3737296*G-rs12410279*A was overtransmitted ($p_{corrected}=0.0016$), with a relative risk for autism of 1.8 in homozygous carriers. Furthermore, ASD-associated SNP rs12410279 modulates the level of transcription of MARK1. We found that MARK1 was overexpressed in the prefrontal cortex (BA46) but not in cerebellar granule cells, on postmortem brain tissues from patients. MARK1 displayed an accelerated evolution along the lineage leading to humans suggesting possible involvement of this gene in cognition. MARK1 encodes a kinase regulating microtubule-dependent transport in axons and dendrites. Both overexpression and silencing of MARK1 resulted in significantly shorter dendrite length in mouse neocortical neurons and modified dendritic transport speed. As expected for a gene encoding a key polarity determinant Par-1 protein kinase, MARK1 is involved in axon-dendrite specification. Thus, MARK1 overexpression in humans may be responsible for subtle changes in dendritic functioning.
Introduction

Autism and autism spectrum disorders (ASDs; MIM 209850) are severe neurodevelopmental disorders characterized by three core symptoms: impairment of verbal and non-verbal communication, impairment of reciprocal social interaction and a restricted repertoire of activities and interests, generally diagnosed before the age of three years. The identification of potential genetic determinants of ASDs has been hampered by the genetic and phenotypic heterogeneity of these disorders (1-4). Recent studies have indicated that ASDs result from a combined effect of multiple genetic changes, including common single nucleotide polymorphisms and copy number variations, interacting with environmental factors (5-7). The high prevalence of ASDs, estimated at 1/150 (8), makes it possible to analyze subgroups of homogeneous phenotypes, thereby facilitating genetic analyses of this heterogeneous condition (9). Several genome-wide scans with microsatellites have been carried out in multiplex families affected by autism (2). We previously described a direct physical mapping approach based on identity-by-descent in 116 families in the Autism Genetic Research Exchange (AGRE), and the use of a BAC-derived DNA chip covering the human genome (10). This new technique made it possible to confirm linkage to autism loci, including a region on chromosome 1q41-q42. In a subset of 35 autism-affected families, severe obsessive and compulsive behavior has been shown to be linked to a region of about 35 Mb on chromosome 1q41-q42.2, between markers D1S346 and D1S547 (11).
Here, we refined the chromosome 1q41-q42 locus of susceptibility to autism to a 7.3 Mb region. High-resolution SNP genotyping showed that microtubule affinity-regulating kinase 1 (MARK1) SNPs and haplotypes were significantly associated with autism. Furthermore, we demonstrated that ASD-associated SNP rs12410279 modulates the level of transcription of MARK1 in a reporter gene assay.

We studied two complementary aspects of this MARK1 gene, (i) primate-specific acceleration of its evolution and (ii) an analysis of deregulation of its expression in cortical neurons. Genes displaying primate-specific acceleration during their evolution are thought to have been involved in the evolution of cognitive functions in primates (12). We showed that MARK1 presented human-specific evolutionary acceleration. MARK1 is expressed in the brain and its product is thought to have an important neuronal function, like other MARK proteins. These kinases phosphorylate microtubule-associated proteins, trigger microtubule disruption and regulate mitochondrial trafficking along microtubules, either in axons or in dendrites (13-15). The dendritic trafficking of mitochondria is a key process in synaptic function and plasticity (16-18). Furthermore, it was recently demonstrated that MARK2 is involved in regulating neuron polarity, as would be expected for a protein containing motifs conserved in invertebrate PAR proteins (19, 20, 21). We analyzed the expression levels of MARK1 in postmortem brain tissues from individuals with ASDs and compared them with those in control samples. Analyses of postmortem brain subregions of this type, focusing on protein levels and/or transcript levels, as determined by quantitative
RT-PCR, have proved useful in studies of autism and neuropsychiatric diseases (21-24). We found that *MARK1* was overexpressed in a prefrontal region (BA46) in the brains of patients with ASDs. MARK1 encodes a kinase regulating microtubule-dependent transport in axons and dendrites. Both overexpression and silencing of *MARK1* modified dendrite length and the velocity of mitochondrial transport along microtubules, in primary cultures of mouse cortical neurons. The overexpression of *MARK1* may therefore cause subtle changes in synaptic function.

**Results**

**SNPs and haplotypes of *MARK1* associated with ASDs**

Fig. 1

Supplementary Table 1

Supplementary Table 2

We previously refined the chromosome 1q41-q42 autism locus to a 7.3 Mb interval, by a direct physical mapping approach on 116 families with autism from the AGRE Study (10). In a two-stage association study, we first genotyped 126 SNPs covering the candidate region in 116 families with two sib affected by narrow autism using both strict diagnostic criteria as defined by the ADI-R and speech delay beyond the age of 36 months (Supplementary Table 1). Five SNPs showed evidence for association with autism at a nominal P-value of 5%, within
or in the flanking regions of the \textit{MARK1} gene. The most significantly associated SNPs, \texttt{rs3753873} ($P_{\text{nominal}}=0.0064$, \texttt{Z-score}=2.724) and \texttt{rs12410279} ($P_{\text{nominal}}=0.008$, \texttt{Z-score}=2.408), were flanked, respectively, 2.5 kb from the 5' region and 14 kb from the 3' region of the \textit{MARK1} gene. The other three SNPs, \texttt{rs4846655}, \texttt{rs3737296} and \texttt{rs6695583}, were located within \textit{MARK1} (Fig. 1).

In a second stage, we refine our genetic study by genotyping 34 tagged-SNPs (including 9 tSNPs from stage I) of the ~180 kb region that includes the 137 kb of \textit{MARK1} gene, in 276 families from the AGRE Study (Supplementary Table 2). This larger sample includes the 116 sib-pairs families of stage I and an additional 160 sib-pair families with the same inclusion criteria to narrow autism. Three main haplotypic blocks were covered (Fig.1B). Block 2 contains the \textit{MARK1} gene and its promoter region. Blocks 1 and 3 are intergenic regions located in 5' and 3' of \textit{MARK1}, respectively. Five tSNPs were found to be associated with autism. Four are located within the gene (\texttt{rs17584152}, $p=0.041$; \texttt{rs11590946}, $p=0.038$; \texttt{rs12740310}, $p=0.016$ and \texttt{rs3737296}, $p=0.04$) whereas the fifth, \texttt{rs12410279} ($p=0.04$), is about 14 kb downstream from the 3'UTR. High pairwise LD values were obtained for \texttt{rs17584152}, \texttt{rs12740310} and \texttt{rs3737296} ($D'\geq0.81$) which are located in block 2. For \texttt{rs12410279} located in block 3, the LD was lower (Fig. 1C & Supplementary Table 2). The most frequent 3-tSNP haplotype in our cohort ($F=0.66$) was C-G-A for \texttt{rs12740310-rs3737296-rs12410279}. This haplotype was associated with autism ($p_{\text{nominal}}=0.0005$) and this association remained significant after corrections ($p_{\text{corrected}}=0.0016$). The relative risk associated with this
haplotype was 1.4 ([1.1-1.6], 95% confidence interval) for heterozygous carriers and 1.8 ([1.3-2.6], 95% confidence interval) for homozygous carriers.

**ASD-associated SNP rs12410279 modulates the level of expression of MARK1**

Fig. 2A

The expression of *MARK1* might be regulated by polymorphisms located in the 5’ promoter region and in the 3’ intergenic region. The associated 3-tSNP risk haplotype covers the 3’ region of *MARK1* gene with 2 tSNPs located in introns and one, rs12410279, located in the 3’ intergenic region (Fig. 1). We used a luciferase reporter gene expression assay driven by 881 bp of *MARK1* promoter and 2.2 kb of the intergenic region that contains rs12410279 (Fig. 2A1). We first demonstrated that 2.2 kb of this 3’ region was able to repress the expression of a reporter luciferase gene compared to the vector containing the 881bp *MARK1* promoter region only (Fig. 2A2). Furthermore, we found a significant difference in the transcription regulated by the A versus G alleles of rs12410279 (Fig. 2A2). We further analyzed the specificity of this effect by quantifying expression under the regulation of another tSNP not associated to autism and within the 2.2 kb intergenic region, rs7539344 (Supplementary Table 2). We did not found any difference of expression (p=0.43) between constructions that contain the allele A (40.00±1.06) or the allele G (38.59±1.34) for rs7539344. Thus, we demonstrate that a tSNP of the autism risk haplotype modulates the level of transcription of *MARK1*. 
**MARK1** is overexpressed in the postmortem prefrontal cortex of autistic subjects

Fig. 2B

Supplementary Table 3

Supplementary Table 4

We used postmortem human brain subregions from patients and controls to investigate if **MARK1** is overexpressed or downregulated in autism. Quantitative real-time RT-PCR (QRT-PCR) was performed on samples from a frontal cortical region (BA46), and on the granule cells of cerebellum hemisphere lobule VI, and normalized with respect to **NFL**, a gene with *bona fide* neuron-specific expression (Fig. 2B & Supplementary Table 3). Relative transcript levels were compared between nine autistic subjects and eight controls. **MARK1** was expressed at a significantly higher level (p=0.018) in the frontal cortex of autistic subjects (0.457±0.056) than in controls (0.284±0.031). No difference in the level of **MARK1** expression in the granule cells of cerebellum lobule VI was found between autistic subjects (10.881±3.126) and controls (7.057±2.608) (Fig. 2B; Supplementary Table 4). The two groups differed significantly in age distribution, but regression analysis showed that the level of expression was independent of age, sex and postmortem interval. Thus, **MARK1** is upregulated in a specific area of the brain in subjects with autism.
As we found that ASD-associated SNP rs12410279 modulates the level of transcription of MARK1, we attempted to identify a specific genotype associated with the overexpression of MARK1 in ASD BA46 samples. We genotyped 7 SNPs of MARK1 associated with ASD in the postmortem sample. Due to the small sample size of ASD and control subjects, we were not able to observe a significant association but evidenced a trend (p=0.08) of overexpression associated with the AA genotype of rs12410279 in ASDs compared to controls (data not shown).

**A human-specific acceleration of the evolution of MARK1 gene**

Fig. 3
Supplementary Fig. S1
Supplementary Fig. S2
Supplementary Fig. S3
Supplementary Table 5

We analyzed the primate-specific evolution of the genes within this autism locus on chromosome 1. This region contains 45 genes, 38 have been completely sequenced in human, chimpanzee, macaque, rat and mouse. For these 38 genes, we compared the non synonymous (Ka) to synonymous (Ks) substitution rates of protein-coding sequences between primates and rodents and between humans and chimpanzee or macaque, macaque and mouse sequences,
respectively, as outgroups (25-27). A high Ka/Ks ratio at a given locus is a potential signature of adaptive evolution. We found a significant difference (p=0.038) in the mean Ka/Ks ratio of the 38 genes between primates (0.251±0.028) and rodents (0.176±0.021) (Supplementary Table 5; Fig 3; Fig S1). We used a phylogenetic approach to calculate the Ka/Ks ratio of each gene in the primate lineage (human versus chimpanzee comparison, with macaque as the outgroup). These 38 genes had high Ka/Ks values in the primate and rodent groups, as shown by comparison with the Ka/Ks values of the neuronal gene repertoires studied by Dorus et al. (2004) (28). The mean Ka/Ks values obtained for the 38 genes from the ADS-associated locus in primates (0.251±0.028) and rodents (0.176±0.021) were significantly higher (primates: p<0.005, rodents: p<0.0001) than the mean values for primates (0.148±0.024) and rodents (0.080±0.015) obtained with the 53 neuronal genes studied by Dorus et al. (2004) in the developmental gene class. Furthermore, no significant difference in primate mean Ka/Ks ratio was observed between the 38 chromosome 1 locus genes and the primate mean value (0.306±0.046) reported by Dorus et al. (2004) for the subgroup of 24 genes identified by these authors as nervous system genes showing significantly faster evolution in primates. We identified three genes, MARK1, SLC30A10 and KIAA1822L, with high Ka/Ks values (2.11, 1.97 and 1.59, respectively) that displayed a particular evolutionary pattern from primate ancestors to the human species (Fig. 3). We measured the level of expression of these genes in both BA46 and the cerebellum, and found no significant differences (Supplementary Tables 3 & 4).
The *MARK1* gene has the highest protein evolution index. Microtubule affinity-regulating kinases (MARKs) are probably involved in axonal and dendritic trafficking (13-15). Human MARK1 and MARK2 have 795 and 788 amino acids, respectively, and are 66% identical and 78% similar (Fig. S2). Mouse Mark1 and Mark2 have 778 and 776 amino acids, respectively, and are 66% identical and 79% similar. MARK proteins have a kinase domain at their N-terminus, a divergent intermediate domain and a conserved carboxyl-terminal kinase-associated domain 1 (KA1) (Fig. S3B). We constructed a phylogenetic tree with MARK1 sequences from human, chimpanzee and macaque (Fig. S3A). We found a prominent trend of accelerated protein evolution along the lineage from simian progenitors to humans and chimpanzees, with a Ka/Ks ratio greater than 1, the signature of adaptive evolution (25). We then compared the human/chimpanzee ancestral MARK1 sequence with the human, chimpanzee, macaque, rat and mouse sequences, using a sliding-window Ka/Ks profile (26-27) (Fig. S3B). A high Ka/Ks peak corresponding to the intermediate domain of MARK1 protein was identified, suggesting that this domain is under positive selection. Troughs (Ka/Ks<<1), which corresponded to functional domains (kinase and KA1 domains), were indicative of strong selective constraint (Fig. S3B).
**MARK1 overexpression and silencing decreases the length of cortical neuron dendrites**

Fig. 4

Supplementary Fig. S4

MARK proteins are involved in cargo (i.e. mitochondria, RNA granules) trafficking along microtubules in neurites (14-15). We studied the effect of MARK1 gene dosage on the development and/or function of neurites, by overexpressing either Mark1-EGFP or Mark1-ΔC-EGFP, in which the KA1 domain is truncated, and by silencing Mark1 in neurons derived from E14 embryonic mice. Primary cultures of cortical neurons can be classified as pyramidal and non pyramidal, based on morphological criteria: the apical dendrite is longer than the basal dendrites in pyramidal cells (29). We therefore focused our analysis on the mean dendrite outgrowth of pyramidal neurons (Fig. 4A-C). Cortical neurons were plated on embryonic day 14 (E14), transfected on day 1 in culture (DIC1) and analyzed on DIC3. We analyzed the effects of overexpression (Fig. 4A) or silencing (Fig. 4B-C). Neurons overexpressing Mark1-ΔC-EGFP and Mark1-EGFP had significantly (p<0.001) shorter dendrites (35.59±3.24%, n=24, and 35.37±4.33%, n=19, respectively) than neurons transfected with the control EGFP vector (taken as 100%; 383.14±40.46 μm, n=25) (Fig. 4D). For silencing experiments, silencing efficiency among three sh-RNA plasmids (Sh1-Mark1, Sh2-Mark1 and Sh3-Mark1) was measured by quantifying the MARK1 expression level in primary cortical neurons, selected on the basis of their fluorescence, using Fluorescence-activated cell sorting, 48h after transfection of both Sh-RNA and fluorescent
reporter plasmids (Fig. S4). Neurons silenced with Sh1-Mark1 had significantly (p<0.001) shorter dendrites (29.58±3.88%, n=10) than neurons transfected with the control scrambled shRNA (taken as 100%, 500.34±38.72 µm, n=12) (Fig. 4B-D). We found no effect of Sh2-Mark1 on dendritic length (93.01±5.35%, n=8) (data not shown).

**Mark1 overexpression and silencing modifies mitochondrial trafficking in cortical dendrites**

Fig. 5

Changes in dendritic length induced by an imbalance in Mark1 gene dosage were used to study the possible effects of Mark1-EGFP on mitochondrial trafficking in the dendrites of neocortical neurons in primary culture. Mark1-EGFP fully colocalized with DsRedMito, a marker targeted to the mitochondrial matrix (Pearson’s coefficient Rr=0.995±0.02 for DsRedMito versus Mark1-EGFP, with n=8; Rr=0.972±0.03 for DsRedMito versus Mark1-ΔC-EGFP, with n=15; p<0.05).

In primary cultures of cortical neurons, mitochondria displayed processive and oscillatory movements in both directions in dendrites, at a speed of 6.5±0.5 nm/s, as previously reported (18). The mobility of mitochondria stained with dsRedMito after the dsRedMito/p-EGFP control vector transfection was 6.80±0.46 nm/s 8±1.3 (n=21) and that after dsRedMito/Mark1-ΔC-EGFP cotransfection was 6.84±0.44 nm/s (n=21).

*Mark1-EGFP* overexpression significantly (p=0.024) decreased the velocity of mitochondrial trafficking (80.6±4.7%; n=16) (Fig. 5A-B; Fig. 5F). In contrast,
silencing of Mark1 induced a significant increase of the mitochondria velocity along dendrites (144.4±15.7, n=9, and 175.1±14.9, n=10, for Sh1-Mark1 and Sh2-Mark2, respectively) (Fig. 5C-F).

**MARK1 is involved in the specification of neuronal polarity**

Fig. 6.

MARK proteins are involved in the regulation of central neuron polarity (19,20,21). The ectopic expression of mouse Mark2 causes neuron to lose both axons and polarity, whereas the abolition of Mark2 expression promotes axonal outgrowth and the formation of multiple axons (20).

We characterized the effects of Mark1 on neuronal polarity, by expressing wild-type Mark1 in mouse E14 cortical neurons. After transfection with Mark1-EGFP on DIC10, neurons were analyzed on DIC11, using Map2 antibodies to stain dendrites specifically (Fig. 6). Transfected neurons displayed non uniform staining for MAP2. The length of dendritic fragments not stained with Map2 antibodies was significantly greater for neurons overexpressing Mark1-EGFP than for control neurons (18.22±1.62 µm, n=7, versus 10.58±1.73 µm, n=6, p<0.01) (Fig. 6A-B; Fig 6E). Similarly, silencing of Mark1 induced longer dendritic fragments without MAP2 expression (23.13±1.07 µm, n=9, and 16.34±1.55 µm, n=8, for Sh1-Mark1 and Sh2-Mark1, respectively, compared to 10.69±1.66 µm, n=14, for Sh-scramble, p<0.05 and p<0.001) (Fig. 6C-D; Fig 6E). Altogether, these results indicate that Mark1 is involved in the specification of neuronal polarity.
Discussion

This work focuses on a genomic region on chromosome 1q41-q42 identified as linked to ASDs in two independent studies (10, 11). Narrow ASD phenotypes were used in both studies. Narrow definition of phenotypes can reduce genetic heterogeneity in the sample and has been suggested to increase the power to detect linkage (30, 31). We refined the autism locus to a 7.3 Mb region. Using high-resolution SNP analysis, we showed that SNPs and haplotypes within the 

MARK1

gene are associated with ASDs. However, we did not found evidence for genetic association with autism using the 160 families added in the stage II. Further studies based on AGRE-independent cohorts should be carried out in order to obtain replication data for the genetic associations.

In this study, the most significant 3-SNP haplotype rs12740310*C-rs3737296*G-rs12410279*A was found to carry a relative risk of 1.8 for homozygous individuals. Direct sequencing of the complete coding sequence of the gene showed no mutations resulting in amino-acid changes (data not shown). Using expression assays, we demonstrated that the ASD-associated SNP rs12410279 modulates the transcription of MARK1 gene. Analysis of MARK1 transcript levels in brain subregions on postmortem examinations of subjects with ASD and controls showed MARK1 to be upregulated in BA46 in the autistic subjects.

In addition to the experimental data we show that MARK1 displays a human-specific acceleration in evolution greater than that for the entire set of 38 genes in the 1q41-linked region analyzed. These data are consistent with a recent
report of widespread accelerated sequence evolution for genes functioning in the nervous system during human evolution (28).

Members of the KIN1/PAR-1/MARK kinase family are conserved from yeast to humans and have a similar primary structure organization (19). Their functions are at the crossroads of various key biological mechanisms, such as cell polarity, cell cycle control and microtubule stability (32). MARKs phosphorylate microtubule-associated proteins (MAPs), such as MAP2, in dendrites, and tau protein in axons, via tubulin binding sites, resulting in the detachment of these proteins from the microtubules (13-15). During the course of this work, it was reported that murine Mark2 specifically regulates development of dendrites in hippocampal neurons (32). MARK overproduction in non neuronal cells disrupts the cellular microtubule network, leading to immediate cell death (13). We found that Mark1 overexpression in mouse cortical neurons gave a milder phenotype. We were able to detect a statistically significant decrease in both dendrite length and the velocity of mitochondrial trafficking along dendrites. The absence of effect on dendrite length obtained for sh2-Mark1 may be attributed to the death of neurons that integrated high levels of the plasmid, with the survival of neurons transfected with lower doses of sh2-mark1 plasmid.

With SLC25A12, another susceptibility gene associated with ASDs and overexpressed in BA46 in autistic subjects (34), we observed a similar but slightly different phenotype in transfected cortical neurons, which had short dendrites but displayed an increase in the velocity of mitochondrial trafficking (23). Mark1 overexpression also deregulated the polarity of neurons with
dendrites that do not express the specific dendritic marker MAP2, as recently reported for Mark2 overexpression (20). However, both the overexpression and silencing of Mark1 induced the death of migrating granule cells of the cerebellum in an *ex vivo* migration assay after electroporation of the explant (data not shown). It was not possible to determine the level of overexpression in any of these experiments, but it would be expected to be much greater than the doubling observed in the B46 subregion of subjects with ASDs. These results suggest that such a doubling in MARK1 expression in the brains of subjects with ADSs may be sufficient to generate subtle changes in dendritic mitochondrial trafficking, potentially modifying synaptic plasticity, as recently reported (18).

Furthermore, several genes involved in the dendritic trafficking pathway were recently identified through genome-wide expression profiling of lymphoblastoid cell lines from patients with autistic features due to a fragile X mutation (FMR1-FM) or a 15q11-q13 duplication (dup(15q)) and comparison with cells from non autistic controls (35). The PRKCB1 gene, which we had previously identified as a susceptibility gene for ASDs (10), was identified as involved in the dendritic trafficking pathway in this way. This gene encodes a protein that interacts with microtubules, as shown in non neuronal cells (36). PRKCB1 binds to a microtubule associated kinase, MAPK6, which phosphorylates MAP2 (37-39). It is tempting to suggest that the effects of PRKCB1 on synaptic plasticity (40) may be a consequence of changes in dendritic trafficking. FMR1-interacting protein 1 (CYFIP1) (41) has been found to be deregulated in both FMR1-FM and dup(15q)
patients, linking the FMRP-containing RNA granule pathway (42) to autism pathophysiology.

Mutations in genes encoding proteins involved in central neuron excitatory synaptic function, such as neuroligins, neurexins and Shank3, have led to the identification of glutamate-related genes as promising candidates for involvement in ASDs (43, 5, 44). Our results extend this model to genes encoding proteins involved in upstream pathways, such as the dendritic trafficking required for the plasticity of glutamatergic synapses. This control of dendritic trafficking allows the local translation of key proteins in dendritic spines (42, 45). We suggest that candidate susceptibility genes for ASDs may encode proteins directly linked to the trafficking machinery, such as MARK1 or PRKCB1, or proteins of FMRP-RNA granules, such as CYFIP1, or mRNAs transported towards dendritic spines and necessary for synaptic plasticity.

In conclusion, our data show that MARK1, a haplotype of which was found to be associated with ASDs, is overexpressed in the prefrontal cortex of subjects with ASDs. This overexpression induces changes in the function of cortical neuron dendrites. We suggest that MARK1 overexpression in subjects with ASDs may be responsible for subtle changes in synaptic plasticity linked to dendritic trafficking.
Methods

Plasmids, immunohistochemistry and the validation of Sh-RNAs are described in the Supplementary Methods.

Families with autism

The sample consists of 276 nuclear families from the AGRE repository, consisting of two parents and at least one child with strict autism, based on the diagnostic criteria defined by the Autism Diagnostic Interview-Revised (ADI-R) (46). Our sample includes the 116 AGRE autism sib-pair families for stage I and an additional 160 families in stage II, as described in a previous study (10). In total, 536 children with autism were included, 78% of whom were male, close to the 4:1 sex ratio generally observed (47).

Two-step association study

An initial association analysis was performed by genotyping 126 SNPs covering the 7.3 Mb of chromosome 1q41-q42 in a set of 116 families with autism described elsewhere (10). The second step involved genotyping 34 tagged SNPs covering the MARK1 gene in our whole cohort of 276 families. We used data from the International HapMap Project (data release #21a phase II) and the Tagger program (de Bakker et al., 2005) to select tagged SNPs (tSNPs). Tagger makes use of the pairwise $r^2$ to define the best set of SNPs covering the region. We defined the set of tSNPs such that all known common SNPs had an estimated $r^2$ greater than 0.8 with at least one of the tSNPs. All SNPs were
genotyped using SNPllex oligoligation assays from Applied Biosystems, according to the manufacturer's instructions.

Pairwise linkage disequilibrium and the Hardy-Weinberg equilibrium (HWE) were analyzed with Haploview software (48). The results of the association study were analyzed with Family-Based Association Test (FBAT) software version 1.7.3, using the empirical variance option to test for association in the presence of linkage (49, 50). We did not correct for multiple testing in the first step, as this step was used to identify candidate genes potentially associated with the disease. However, the second association analysis was corrected for multiple testing, using the false discovery rate (FDR) approach described in (51). This method is conservative, due to the non independence of the tests (but less so than Bonferroni correction) and gives a lower limit for statistical significance.

Genotype and haplotype relative risks were estimated as described in a previous study (52). For each haplotype allele studied, estimates were obtained for heterozygous and homozygous carriers, using "homozygous non carrier" as the reference genotype and assuming an additive model.

**N18 Cell line culture, transfection and luciferase assay**

The mouse neuroblastoma N18 cell line was cultured in DMEM supplemented with 10% of foetal calf serum in 35 mm dishes. N18 cells were transfected with (i) 600 ng of *Mark1*-EGFP construct or (ii) co-transfected with 600 ng of *Mark1*-EGFP construct and 2400 ng of Sh-RNA Mark1 vector or (iii) co-transfected with 200ng of pRLSV40 vector and 800 ng of pGL3 construct described above.

For luciferase assay, cells were washed two times in PBS. Proteins were extracted and luciferase activities were measured using a dual-luciferase reporter
assay system (Promega) as described by the manufacturer. Luciferase activities were quantified using a luminometer Lumat LB 9501 (Berthold). Constructs used in these assays are described in SI.

**Human brain postmortem samples**

Frozen postmortem samples of human frontal cortex (BA46) and of cerebellum (lobule 6) from nine autistic patients and eight controls were provided by the Autism Tissue Program. The age, sex and post-mortem interval of individuals are presented in SI Tables 4 & 5. RNA extraction and Q-PCR analysis are described in SI.

**Efficiency test of Sh-RNA plasmids**

To test the efficiency of Sh-RNA plasmids, primary cortical neurons were cultured in 100 mm dishes as described below. They were transfected with 4800ng of Sh-RNA and 1200 ng of pEGFP empty vector. Cell culture was interrupted 48 hours after transfection. Cells were washed with HBSS medium. Dissociation was performed using a trypsin-EDTA solution (0.05% trypsin-0.05mM EDTA) and re-suspended in DMEM (Invitrogen) supplemented with 10% fetal calf serum. GFP positive cells were sorted using a Influx 500 (Cytopeia, Seattle) cell sorter. GFP positive populations were characterized (i) by their forward scatter score (FSC) (between 111 and 219) (ii) by their side scatter score (SSC) (between 112 and 218) (iii) by their fluorescence (between 16.73 and 6720). Cells were collected in XB buffer (Alphesys) for RNA extraction as described by the manufacturer. RNA quality was estimated using RNA pico 6000 Agilent chips. Mark1 expression level was determined by Q-PCR.
Primary neuron culture, transfection and electroporation

E14 mouse telencephalic neurons were dissociated enzymatically (0.25% trypsin, DNase), mechanically triturated with a flamed Pasteur pipette, and plated on 35 mm dishes \((8 \times 10^5 \text{ cells per dish})\) coated with poly-DL-ornithine (Sigma), in DMEM (Invitrogen) supplemented with 10% fetal calf serum. Four hours after plating, DMEM was replaced with Neurobasal® medium (Invitrogen) supplemented with 2 mM glutamine and 2% B27 (Invitrogen). Primary neuronal cultures were transfected various times after plating. Cells were transfected with the constructs described above, using Lipofectamine (Invitrogen), as described by the manufacturer. Neurite analysis was carried out with ImageJ software (Wayne Rasband, NIH).

We dissected the cerebellum from P5 mice. Fast Green-tagged DNA at a concentration of 4µg/µl was injected into the cerebellum. The cerebellum was electroporated (conditions: voltage: 50V; duration of pulse: 50 ms; delay between pulses: 450 ms; number of pulses: 5; Nepagene electroporator). Microexplant cultures were prepared as previously described (53, 54). EGL explants were cultured on polylysine \((0.2 \text{ mg.ml}^{-1}, \text{Sigma})\), laminin \((20 \mu\text{g/ml}, \text{Sigma})\). Cultures were fixed by incubation with 4% PFA and 0.33 M sucrose and counterstained with Hoechst 33342 \((10 \mu\text{g/ml}, \text{Sigma})\).
Time-lapse video microscopy: image acquisition and quantification of mitochondrial movements

Mitochondrial movements were analyzed with mouse cortical neurons, cultured as described above. Cell images were taken with a Cool Snap HQ (Roper Scientific) CCD camera mounted on the Leica DM IRE2 microscope and a 100× oil immersion objective lens (N.A. 1.4, Zeiss). The cells were illuminated with a DG4 shutter. Cells were then maintained at 37°C in an incubator containing 5% CO₂ (Saur), on the stage of a Leica inverted microscope. Fluorescent images were captured every 5 s for 15 min with the CCD camera. The movement of each mitochondrion was analyzed manually with the SpotTracker plug-in of ImageJ software (Wayne Rasband, NIH).

Statistical Analysis

Results from quantitative real-time PCR were analyzed using Student's t tests, ANOVA and linear regression tests.

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References


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Figure 1. Genomic organization of the *MARK1* gene and SNPs associated with ASDs
(A): Genomic structure of the *MARK1* gene (the bars indicate exons and arrows the direction of transcription) and positions of SNPs (blue triangles for SNPs genotyped in the stage I of the association study; red triangles for tSNPs genotyped in the stage II).
(B). Linkage disequilibrium (LD) structure of autism-associated SNPs in the *MARK1* gene.
(C). P values of family-based association test (FBAT) for SNPs and autism in the two-step association studies (with blue text for the first step and red text for the second step). F is frequency of the associated haplotype. SNPs significantly associated in the two stages are boxed.

Figure 2. Modulation of level of transcription of *MARK1* gene
A Effect of the ASD- associated SNP rs12410279 on transcription regulation of *MARK1* in neuroblastoma cells.
(A1) Constructs containing luciferase reporter gene and either a 881bp *MARK1* promoter region alone, or associated with a 2.2kb of *MARK1* 3’ region containing rs12410279 with different alleles, were used to determine the effect of associated rs12410279 on transcription regulation.
(A2) The 2.2kb 3’region of *MARK1* combined with *MARK1* promoter decreases luciferase activity. *MARK1* promoter combined with rs12410279*G polymorphism
generate luciferase activity significantly higher than *MARK1* promoter combined with rs12410279*A. (***, p<0.001).

(A3) rs12410279 alleles validated by sequencing of the DNA fragment inserted in the plasmid.

(B) *MARK1* mRNA levels in postmortem human prefrontal cortex (BA46) and cerebellum (lobule VI) granule cell specimens from autistic patients and controls. mRNA levels were quantified by quantitative real-time RT-PCR (QRT-PCR). Granule cells were microdissected from lobule 6, as described in materials and methods. BA46 prefrontal cortex of subjects with autism (n=9) and controls (n=8). Lobule VI granule cells of subjects with autism (n=9) and controls (n=7).

(*, p<0.05).

**Figure 3.** *MARK1* as an autism susceptibility candidate gene

Ka/Ks ratio of the 38 genes of the chromosomal locus 1q41 associated with susceptibility to autism

**Figure 4.** Effect of *Mark1* overexpression and silencing on dendrite length at prenatal stages

(A-D) Dendrite outgrowth after overexpression or silencing of *Mark1*.

Dissociated neocortical neurons from embryonic day 14 embryos plated in culture, were transfected on day 1 in culture (DIC1) and fixed on DIC3 Representative pyramidal neurons transfected with EGFP (A1-A3), Mark1ΔC-EGFP (A4-A6), Mark1-EGFP (A7-A9), Sh-scrambled (B), Sh1-Mark1 (C). GFP staining (A1, A4, A7), MAP2 staining (A2,
A5, A8) and merged images (A3, A6, A9) for transfections with EGFP, Mark1ΔC-EGFP and Mark1-EGFP, respectively.

(D) Quantitative analysis of mean dendrite length for the different conditions. (***, p<0.001).

Figure 5. Mark1-EGFP overexpression and Mark1 silencing alter mitochondrial dendritic transport velocity in cortical dendrites

(A-B) Neocortical neurons were cotransfected with EGFP (A) or MARK1-EGFP (B) and MitoDSred. Scale bar: 10 μm.

(C-E) Neocortical neurons were cotransfected with Sh-Scramble (C) or Sh1-Mark1 (D) or Sh2-Mark1 plasmid and MitoDSred. Scale bar: 10 μm. Merged image of p-EGFP control vector (green) and DsRedMito (red) (A1), Mark1-EGFP vector (green) and DsRedMito (red) (B1) cotransfected neurons, respectively. Scale bar: 10 μm. Time-lapse movies of the rectangular areas indicated in A1 and B1, illustrating examples of the movement of the mitochondria for 240 s, with frames taken at 20-second intervals. Scale bars: 1 μm.

Image of DsRedMito/Sh-Scramble (C1) or DsRedMito/Sh1-Mark1 (D1) or DsRedMito/Sh2-Mark1 (E1) co-transfected neurons, respectively. Scale bar: 10 μm. Time-lapse movies of the rectangular areas indicated in C1, D1 and E1, illustrating examples of the movement of the mitochondria for 60 s, with frames taken at 5-second intervals. Scale bars: 1 μm.

(F) Quantitative analysis of mitochondrial transport velocity for Mark1-ovverexpression and silencing (*; p<0.05; ***,p<0.001).
Figure 6. The role of MARK1 in neuronal polarity

Dissociated neocortical neurons from embryonic day 14 embryos were transfected after ten days in culture (DIC10) and fixed on DIC11.

(A,B). Neurons transfected with EGFP (A1-A4), or EGFP-MARK1 (B1-B4) were visualized for the detection of GFP (A1, B1) and stained with antibodies against Map2 (A2, B2), with merged images shown in A3 and B3, respectively.

(C-E). Neurons co-transfected with EGFP and Sh-Scramble (C1-C4) or with EGFP and Sh1-Mark1 (D1-D4) or with EGFP and Sh2-Mark1 (E1-E4) and stained with antibodies against Map2 (C2, D2, E2), with merged images shown in C3, D3 and E3, respectively.

(F) Quantitative analysis of neuronal polarity, based on measurements of dendrite segments without Map2 expression (Map2-). Insets (A4) and (B4) illustrate examples of dendrite staining for neurons transfected with EGFP and Mark1- EGFP, respectively. Insets (C4, D4 and E4) illustrate examples of dendrite staining for neurons co-transfected with EGFP and Sh-Scramble, or with EGFP and Sh1-Mark1, or with EGFP and Sh2-Mark1 respectively.