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Mutations in the calcium-related gene *IL1RAPL1* are associated with autism

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In a systematic sequencing screen of synaptic genes on the X chromosome, we have identified an autistic female without mental retardation (MR) who carries a *de novo* frameshift Ile367SerfsX6 mutation in *Interleukin-1 Receptor Accessory Protein-Like 1* (*IL1RAPL1*), a gene implicated in calcium-regulated vesicle release and dendrite differentiation. We showed that the function of the resulting truncated IL1RAPL1 protein is severely altered in hippocampal neurons, by measuring its effect on neurite outgrowth activity. We also sequenced the coding region of the close related member IL1RAPL2 and of NCS-1/FREQ, which physically interacts with IL1RAPL1, in a cohort of subjects with autism. The screening failed to identify non-synonymous variant in *IL1RAPL2*, whereas a rare missense (R102Q) in *NCS-1/FREQ* was identified in one autistic patient. Furthermore, we identified by comparative genomic hybridization a large intragenic deletion of exons 3–7 of *IL1RAPL1* in three brothers with autism and/or MR. This deletion causes a frameshift and the introduction of a premature stop codon, Ala28GlufsX15, at the very beginning of the protein. All together, our results indicate that mutations in *IL1RAPL1* cause a spectrum of neurological impairments ranging from MR to high functioning autism.

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INTRODUCTION

Autistic spectrum disorder (ASD) is a neurodevelopmental disease characterized by stereotyped and repetitive behaviors, impairments in social interaction and in verbal and non-verbal communication. It includes classical autism, Asperger syndrome (AS) and pervasive developmental disorder not otherwise specified (PDD-NOS). Genetics plays an important role in the etiology of ASD, as revealed by twin and familial studies (1,2). Several whole genome linkage studies led to the identification of numerous susceptibility regions, but these regions rarely overlap between studies (3). Over the last few years, direct sequencing of candidate genes has successfully identified genetic factors involved in ASD. Causal mutations in the synaptic genes *NLGN4*, *NLGN3* (4) and *SHANK3* (5) have been identified and independently confirmed by other teams (6–9). However, mutations in these genes account for only a small proportion of ASD families, suggesting strong genetic heterogeneity in ASD which likely explains the limited impact of classical genetic approaches on the identification of causative mutations. Together, these observations suggest that disruption of numerous genes by rare but penetrant mutations, including *de novo* variants, could represent an important cause of ASD. In order to test this hypothesis, we are screening 142 ASD patients for mutations in synaptic genes on the X chromosome, since synaptic dysfunction has been strongly implicated in ASD (10,11) and males are preferentially affected.

One of the genes selected for the sequencing, the *Interleukin-1 Receptor Accessory Protein-Like1* (*IL1RAPL1)*, encodes a transmembrane protein of 696 amino acids that show homology with interleukin-1 receptor accessory protein family (12), but does not seem to be involved in interleukin-1 (IL-1) pathway. It contains a divergent extracellular domain with immunoglobulin motifs, an intracellular Toll/Il-1 Receptor (TIR) domain and a 150 amino acids extension that interacts with the neuronal calcium sensor 1 (NCS-1) protein, also known as frequenin (FREQ) (13). Via this interaction, IL1RAPL1 plays a role in the down-regulation of voltage-dependent calcium channels (VGCC) activity, in calcium-dependent exocytosis and NGF-induced neurite outgrowth in PC-12 cells (14).

In this study, we found two damaging mutations in *IL1RAPL1* in patients with ASD and/or mental retardation (MR). The first one, a *de novo* frameshift mutation in a girl with AS, results in a truncated protein. We have developed an assay that measures the impact of ILRAPL1 activity on neurite outgrowth and found that this mutation dramatically decreases its function. We also identified a large intragenic deletion in *IL1RAPL1* gene in three brothers with ASD and/or MR. All together, these results indicate that disruption of *IL1RAPL1* has the potential of causing a wide spectrum of conditions ranging from MR to high-functioning autism.

RESULTS

Sequencing of the *IL1RAPL1* gene and identification of *de novo* frameshift mutation in one as girl

We sequenced all 10 coding exons of *IL1RAPL1* in a cohort of 142 subjects (20 females and 122 males) with ASD.

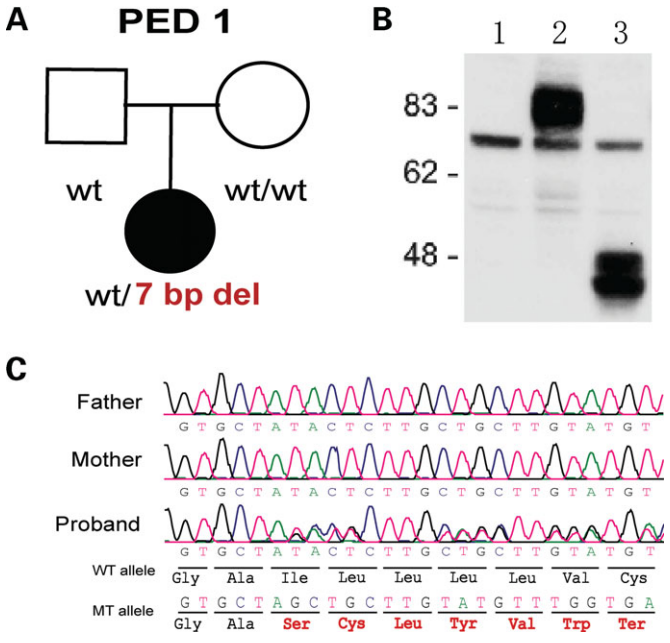

Figure 1. Identification of a *de novo* frameshift mutation in the *IL1RAPL1* gene in a girl with AS. (**A**) Pedigree PED1 of the AS girl with the I367SfsX6 *de novo* mutation. (**B**) Western blot analysis of protein extracts from HEK293 cells transfected with: 1, empty plasmid; 2, WT IL1RAPL1; 3, I367SfsX6 IL1RAPL1 using an anti-FLAG antibody. (**C**) Sequence traces of the seven nucleotide deletion and new frame caused by the mutation (I367SfsX6).

We identified a deletion of seven nucleotides in a French-Canadian girl diagnosed with AS, a form of ASD without language delay (Fig. 1A). The mutation was present in the lymphoblastoid DNA of the proband as well as in the DNA directly extracted from her blood, but was absent in her parents, indicating that it is *de novo*. The patient did not have any siblings and there was no familial history of ASD. This seven nucleotide deletion (c.1730delTACTCTT) occurred in exon 9 of the gene and caused a frameshift at Ile367 with a premature stop codon (TGA) 6 codons downstream (p.Ile367SerfsX6) (Fig. 1C). The resulting truncated protein is predicted to lack part of the transmembrane domain as well as the entire cytoplasmic domain (Fig. 2). Accordingly, transfection of HEK93 cells with the mutant *ILRAPL1* cDNA resulted in the expression of a truncated protein as revealed by western blot analysis (Fig. 1B). The I367SfsX6 mutation is thus likely to affect severely the function of the protein. We sequenced the entire coding region in 276 ethnically matched control chromosomes (87 females and 102 males) and did not identify any frameshift or nonsense variants in *IL1RAPL1*. Several missenses were also identified in the course of this study, including the K379R substitution in one subject of the ASD cohort, the T637S and I643V substitutions in individuals of the control cohort and the Q618H substitution in individuals from both cohorts (Table 1). These variants are unlikely to be pathogenic because they are found in the control cohort and/or they are predicted to be tolerated by the protein according to the different single nucleotide polymorphism prediction software.

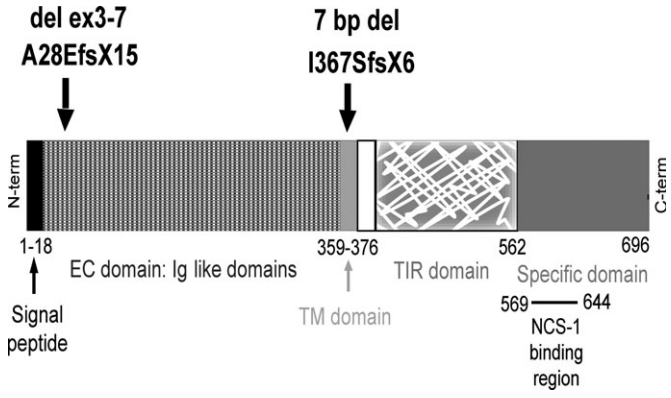

Figure 2. Schematic representation of the IL1RAPL1 protein and localization of the truncation mutation I367SfsX6 (PED1) and A28EfsX15 (PED2). EC domain, extracellular domain; TM domain, transmembrane domain; TIR domain, toll/Il-1 receptor domain.

Functional impact of the *de novo* frameshift mutation I367SfsX6 on neurite outgrowth regulation in hippocampal neurons in culture

We next sought to characterize the functional impact of the I367SfsX6 mutation on IL1RAPL1 activity. Expression studies of the homologous mouse *Il1rapl1* gene found the highest level of expression in the developing and post-natal hippocampus (15). In cultures of rat hippocampal neurons, we observed by immunocytochemistry that rat *Il1rapl1* endogenous protein is present in the cell body but also in growth cones of short, dendrite-like processes (Fig. 3A). To determine the effect of the I367SfsX6 mutation on ILRAPL1 function in HEK293 cells, we first knocked down expression of the endogenous ILRAPL1 using specific miRNAs. As shown in Figure 3B, transfection of hippocampal neurons with a miRNA directed against endogenous *IL1RAPL1* resulted in a dramatic decrease of IL1RAPL1 protein levels. Knock-down of IL1RAPL1 lead to a significant increase in both the number and the length of neurites (Fig. 4). Cotransfection of the anti-endogenous IL1RAPL1 miRNA together with the full-length wild-type (WT) IL1RAPL1 cDNA restored the expression of IL1RAPL1 (Fig. 3C) and rescued the neurite outgrowth activity to control levels (Fig. 4). In contrast, cotransfection of the miRNA together with the IL1RAPL1 I367SfsX6 mutant cDNA resulted in cells with high neurite number and length, similar to cells transfected with the miRNA alone. Therefore, the mutated IL1RAPL1 protein was unable to rescue ILRAPL1 knockdown. This observation shows that the 7 bp deletion results in a loss of IL1RAPL1 function. Moreover, immunofluorescence studies using an antibody directed against IL1RAPL1 protein revealed a cytoplasmic pool of mutant protein but did not detect its presence at the cell surface, suggesting that the truncated form of IL1RAPL1 is mislocalized (Fig. 3D). Interestingly, transfection of rat hippocampal neurons with either the human WT or the mutant *IL1RAPL1* cDNA alone did not affect neurite outgrowth (Fig. 4).

Sequencing of the coding regions of the *IL1RAPL2* and *NCS-1/FREQ* genes

IL1RAPL1 has a close homolog, *IL1RAPL2*, which is also located on the X chromosome. In view of its homology with *IL1RAPL1*, we explored the possibility that it could also be involved in ASD by sequencing its coding region (for primers, see Table 2) in our cohort of patients, but no non-synonymous variants were found (Table 1). We also sequenced the coding region of the *NCS-1/FREQ* gene and identified one rare missense R102Q in one boy, transmitted by his mother. This variant was not found in the control population from French Canadian origin. As the patient had a Jewish Sefarade origin and we did not have ethnically matched controls, we cannot exclude that this could be a polymorphism in this particular population.

Identification of an intragenic deletion of *IL1RAPL1* in three brothers with MR and/or ASD

In the course of this work, we identified another family not included in the ASD cohort that was sequenced, in which disruption of *IL1RAPL1* is associated with ASD (Fig. 5A). The proband (II-3), an 8-year-old boy, has MR and exhibits some autistic signs, but is too severely affected to be formally tested for the presence of ASD. Comparative genomic hybridization (CGH) using a custom-designed array that targets several MR genes detected a 730 kb deletion within the *IL1RAPL1* gene of patient II-3 (Fig. 5B). The last non-deleted probe was located at position 29104527–29104586 and the first deleted one at 29135420–29135479, indicating that the deletion occurred in intron 2 of the *IL1RAPL1* gene. At the 3′ side of the deletion, the last deleted probe was located at position 29838694–29838753 and the first non-deleted one at position 29856482–29856541, showing that the end of the deletion was located somewhere between introns 6 and 8 of the *IL1RAPL1* gene. PCR were performed in patient II-3 and confirmed that exons 3, 4, 5, 6 and 7 were absent in the proband (Fig. 5C). Exons 2, 8 and 9, however, could be amplified by PCR. Additional primer pairs were used to define more precisely the border of this deletion (Table 2) and showed that the end of the deletion is located in intron 7, between the forward primers Intron7a_F and Intron7b_F (Fig. 5C). The genomic deletion observed in patient II-3 thus results in the deletion of exons 3–7 of the *IL1RAPL1* gene (Fig. 5E). This deletion is predicted to cause a frameshift at alanine 28 with a premature stop codon 15 codons downstream (Ala28GlufsX15), thus truncating the majority of the IL1RAPL1 protein (Fig. 2). The proband (II-3) has two older brothers. One of them (II-2) has PDDNOS, according to Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview (ADI) criteria, whereas the other one (II-1) shows mild MR with repetitive behaviors, but no other autistic signs. Quantitative PCR targeting intron 5 indicated that the two brothers and their mother, who does not show any cognitive or behavioral abnormality, also carried the deletion (Fig. 5D).

DISCUSSION

We have identified two damaging mutations, I367SfsX6 and A28EfsX15, in the *IL1RAPL1* gene of individuals with ASD, indicating that this gene is involved in the pathogenesis of ASD. Mutations in *IL1RAPL1* are not a frequent cause

Table 1. Summary of the non-synonymous variants found in the sequencing of the coding regions of *IL1RAPL1*, *IL1RAPL2* and *NCS-1/FREQ*

Gene	Exon	Amino acid change	Cohort	ASD	CONT	Gender	Ethnicity	Transmission/family
IL1RAPL1	9	I367SfsX6	ASD	1/142	0/190	Female	French-Canadian	*de novo*
	9	K379R	ASD	1/142	0/190	Male	Anglo-Saxon	Mother (mut/+)
	12	Q618H	Both	1/142		Male	French-Canadian	Mother (mut/+), one maternal uncle AS (not tested)
					1/190	Male	French-Canadian	Mother (mut/+)
	12	T637S	CONT	0/142	1/190	Female	French-Canadian	Mother (mut/+)
	12	I643V	CONT	0/142	1/190	Male	French-Canadian	Mother (mut/+)
IL1RAPL2		No non-synonymous variant found						
NCS-1/FREQ	4	R102Q	ASD	1/142	0/190[a]	Male	Jewish sefarade	Mother (mut/+)

[a]Only the fragment corresponding to exon 4 was sequenced in control individuals (CONT).

of ASD. This observation is consistent with an emerging model suggesting that a fraction of ASD cases is explained by a great number of rare but penetrant genes. The mutations described here were found by two different approaches, namely the screening for point mutations by direct sequencing of candidate genes, and the whole-genome search of copy number variants by CGH. Our work thus demonstrates the relevance of using these two complementary approaches for the genetic exploration of common neurodevelopmental disease, such as ASD.

We demonstrated that the mutation I367SfsX6, found in one AS girl, leads to a loss of function of IL1RAPL1. Indeed, the resulting truncated protein is not able to control neurite outgrowth in hippocampal neurons. The second mutation, A28EfsX15, is also likely to cause a loss-of-function of the corresponding protein since it is predicted to contain only eight amino acids in addition to the signal peptide. Two nonsense mutations in *IL1RAPL1*, Y459X and W487X, were previously described in families with X-linked MR (15,16). In one of these two families, two of the five female carriers showed learning impairment but the others did not show any neurological phenotype. Deletions or duplications of the region that includes the *IL1RAPL1* gene were also described in patients with MR (17,18). Recently, a balanced pericentromeric inversion with one breakpoint located in the *IL1RAPL1* gene was found in a boy and his mother who both were diagnosed with ASD and MR (19). Moreover, a copy number variant encompassing the *IL1RAPL1* gene was associated with ASD in one family (20). Our discovery of *IL1RAPL1* frameshift mutations in ASD patients therefore provides a definitive documentation of the involvement of this gene in the pathogenesis of ASD. Moreover, we found that disruption of *IL1RAPL1* can cause ASD with or without MR. The spectrum of phenotypes caused by a loss-of-function mutation of the *IL1RAPL1* gene thus varies in females from no obvious neurological impairment to learning disabilities or ASD with no or mild MR, which could be partially explained by the X inactivation phenomenon. In males, these mutations cause also a spectrum of phenotypes, with mild to severe MR, ASD features or both. It is interesting that a single mutation can lead to these different phenotypes within the same family (PED2, Fig. 5A). This was already observed for mutations in *NLGN4*, a gene for which nonsense mutations or deletion of several exons have been described in MR, AS, autism and recently in Tourette syndrome with hyperactivity disorder (4,6,7).

IL1RAPL1 is highly expressed in the developing hippocampus, a structure involved in memory and shown to be altered in ASD patients (21). We showed here that this protein is located at the synaptic sites and particularly in growth cones of dendrites of cultured hippocampal neurons. Gambino *et al*. (14) reported that the expression of IL1RAPL1 in PC12 cells, a neuron-like cell line in which it is not normally expressed, decreases neurite outgrowth by inhibiting N-type voltage-gated calcium channels via its physical interaction with NCS-1/FREQ. In contrast, overexpression in hippocampal neurons did not affect neurite outgrowth. Unlike PC12 cells, hippocampal neurons produce high level of IL1RAPL1, possibly explaining why IL1RAPL1 overexpression has no effect on hippocampal neurite outgrowth while knock-down dramatically increases it.

Human IL1RAPL1 participates in the regulation of neurite outgrowth and exocytosis, via its interaction with NCS-1/FREQ and the down-regulation of calcium channels. It was recently demonstrated that Il1rapl1b, the zebrafish orthologue of mammalian protein IL1RAPL1, plays an important role in presynaptic differentiation during synapse formation, via the regulation of synaptic vesicle accumulation and subsequent morphological remodeling (22). IL1RAPL1 protein thus appears to be involved in both synapse formation during development and modulation of synaptic transmission. The alteration of these activities caused by loss-of-function mutations in *IL1RAPL1* gene may explain the cognitive impairments observed in MR and ASD patients.

IL1RAPL1 exerts these synaptic functions presumably via its action on neuronal type-N calcium channels and calcium levels (23). Recently, a link between the regulation of these calcium levels and ASD has been suggested (24). Indeed, mutations associated with autistic phenotypes have been found in the genes encoding calcium channel subunits CACNA1H (25), CACNA1F (26) and CACNA1C (27) and the calcium-dependent potassium channel BKca (28). We can also note that neuroligins and *SHANK3*, the other genes for which mutations in AUT have been identified, are linked to this calcium signaling. Indeed, neuroligins possess Ca_2^+-binding EF-hand domains that regulate its activity (29) and SHANK proteins are able to interacts with CACNA1D calcium channel (30).

In conclusion, our discovery that disruptions of *IL1RAPL1* cause not only MR but also ASD provides a new framework for the further dissection of pathways involved in this neurodevelopmental disease.

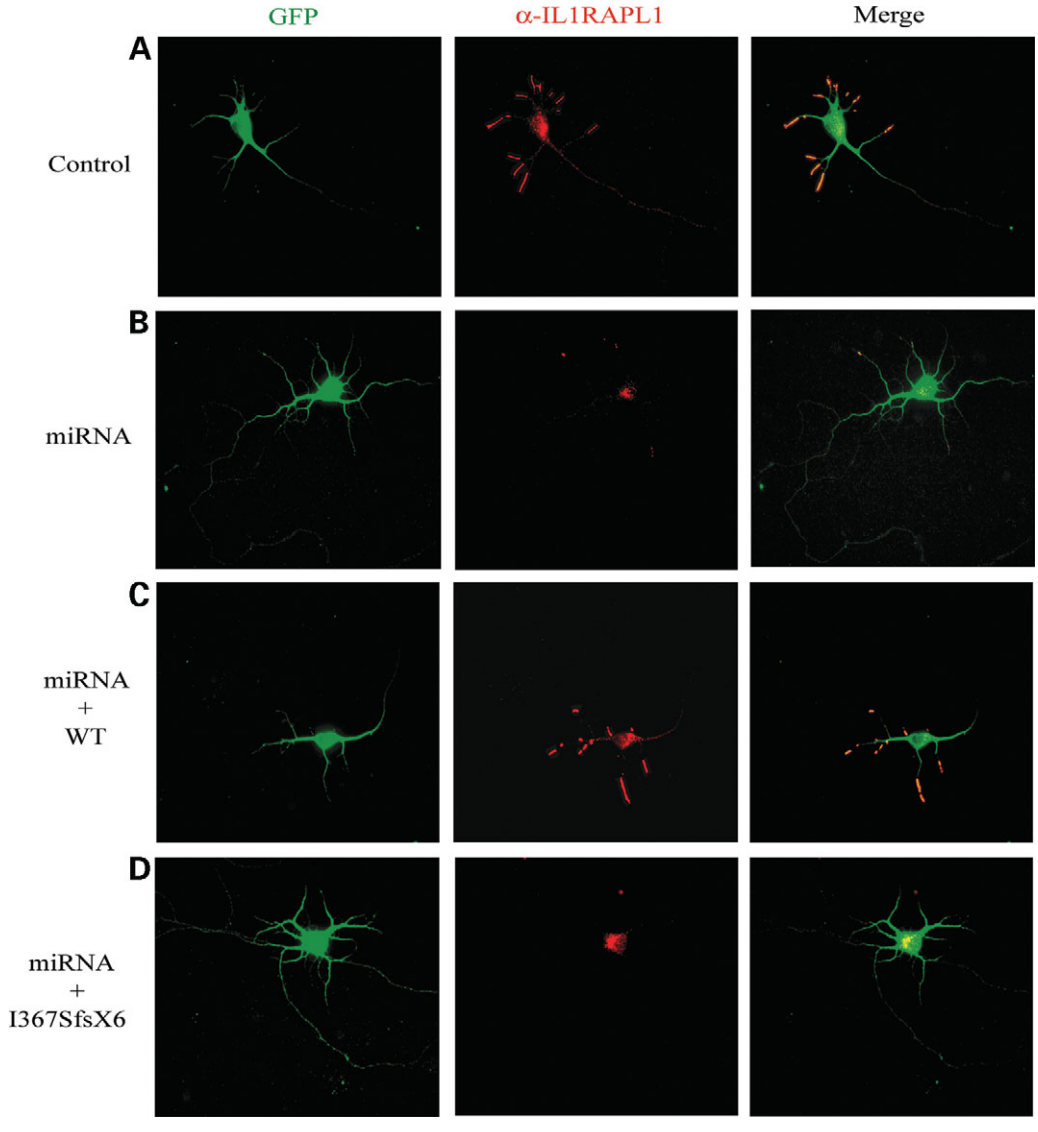

Figure 3. Quantification of IL1RAPL1 expression in hippocampal neurons reveals an absence of IL1RAPL1 I367SfsX6 at the dendrites. (**A**–**D**) Hippocampal neurons were transfected with GFP, miRNA against IL1RAPL1 and/or cDNA coding for human WT or I367SfsX6 IL1RAPL1. It is visualized by direct fluorescence (green/left), by immunofluorescence with an anti-IL1RAPL1 antibody (red/middle) or both (merge/right). miRNA against IL1RAPL1 (B) reduces expression of the endogenous protein (A) level that is rescued with the full-length WT (C) but not by the I367SfsX6 cDNA (D).

MATERIALS AND METHODS

Patients

The cohort of patients used for the sequencing included 142 unrelated ASD patients already described (31). All patients were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders criteria. Depending on the recruitment site, Autism Diagnostic Interview-Revised and the ADOS were used. In addition, the Autism Screening Questionnaire was also completed for all the subjects. We excluded patients with an estimated mental age <18 months, a diagnosis of Rett syndrome or Childhood Disintegrative Disorder and patients with evidence of any psychiatric and neurological conditions including: birth anoxia, rubella during pregnancy, fragile-X disorder, encephalitis, phenylketonuria, tuberous sclerosis, Tourette and West syndromes. DNA from patients and their parents were extracted from blood lymphocytes and lymphoblastoid cell lines. The biological relatedness of the parents to each proband was confirmed using a panel of highly polymorphic markers (D15S659, D4S3351, D8S1179, D3S1754 and D6S1043). Our control group included 87 females and 102 males (276 control chromosomes) of French-Canadian origin.

The proband of PED1 is a girl functioning at the borderline range of intelligence (fifth centile rank on the Wechsler Intelligence Scale for Children), with a level of fluid intelligence around the 25th centile rank (Raven Matrices Test) and an adaptation level below the second centile rank (Vinland Adaptive Behavior Scale). She is positive at the ADI and ADOS criteria for autism, but received a diagnosis of Asperger syndrome, as she did not have a language delay. However, her language is simplified, without intonation, and is unaccompanied by gestures. At the social level, she is devoid of inhibition and lacks

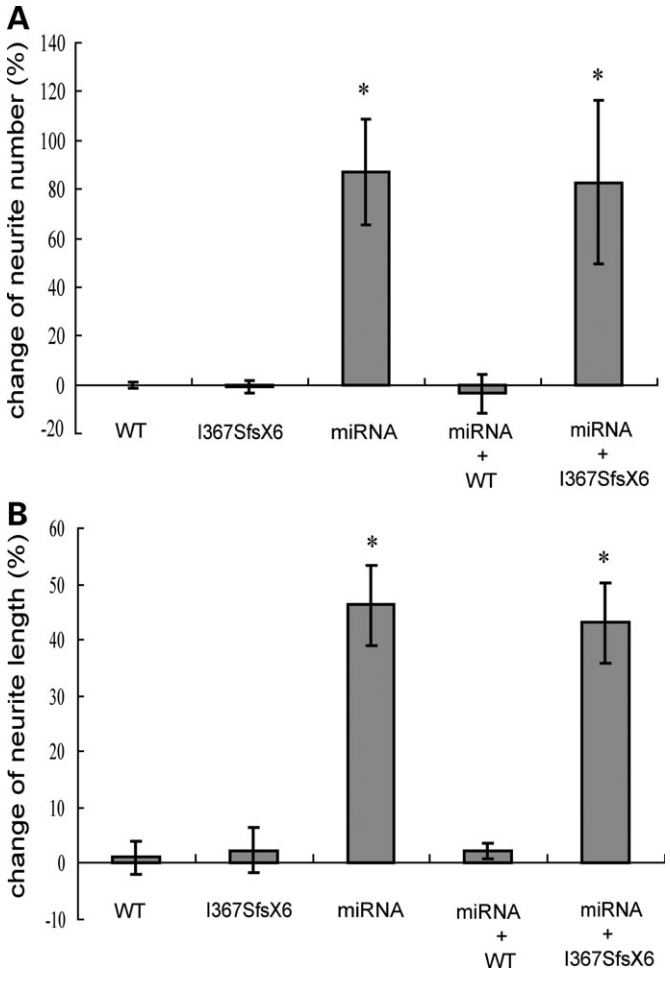

Figure 4. Characterization of the effect of the I367SfsX6 mutation on IL1RAPL1 function by measurement of neurite outgrowth activity. (**A**–**B**) Rat hippocampal neurons were transfected with miRNA against IL1RAPL1 and/or cDNA coding for human WT or I367SfsX6 IL1RAPL1, and effects on neurite number (A) and length (B) were quantitated. $^{*}P < 0.05$ (Student's *t*-test).

social initiatives. Her main social difficulties are related to a marked anxiety. She shows restricted interest in clothes and asks repetitive questions when her daily routines are modified. As is frequent in Asperger syndrome, she does not show the visuo-spatial peaks of ability that are usual in autism.

In the second family (PED2), all three siblings were born with a normal weight and showed normal growth, without any dysmorphic features. They each showed a distinct pattern of cognitive and behavioral abnormalities. II-3 is an 8 years old non-verbal child with profound MR. He started to walk at 54 months of age. He spends most of his time rocking his head and slapping his legs in association with laughs. He shows a marked intolerance for noises, especially voices. Language is limited to 'no' and to his name. His profound level of MR prevents the straightforward attribution of his autistic symptoms in the communication (hand-leading), the social (absence of direct gaze and of socially oriented behaviors) and in the restricted interest and repetitive domain (self injury, repetitive flapping of objects) to a diagnosis of autism. Karyotyping (at the 500 band resolution) and testing for the common expansion associated with the Fragile X syndrome were negative. Brain MRI and spectroscopy, performed at 6 years of age, did not show any abnormalities. II-2 is a 10 years old verbal boy functioning at the borderline level of intelligence, with ASD. At age 3, he was positive at the ADI and ADOS criteria for autism. Major autistic symptoms in the communication domain were an important language delay, with first one word sentence uttered at 48 months and two-word sentences at 60 months, immediate and delayed echolalia when entering in speech, followed by stereotyped language. Most of his current verbal production is response to questions or non-reciprocal monologue. His repetitive behaviors and restricted interests are prolonged observations of spinning objects, atypical exploratory behaviors (prolonged holding of objects close to the eyes), marked obsession for films, washing and drying machines, hand flapping, fist-clenching and stereotyped jumps. The social area is less markedly impaired, with absence of direct gaze and negative reaction to other people's overtures. This area dramatically normalized after 6 years of age, resulting in a diagnosis of PDD-NOS rather than autism based on the ADI. Karyotyping (at the 500 band resolution) and testing for the common expansion associated with the Fragile X syndrome were negative. II-1 is an 11 years old verbal child with mild MR and attention deficit hyperactivity disorder. Developmental milestones were markedly delayed: he walked at 24 months, uttered his first words at 24 months and two-word sentences at 30 months of age. An ADI performed at 11 years of age indicated an absence of autistic symptoms in the communication and social domain, and an unremarkable variety of play behavior. In contrast, he showed repetitive behaviors of an autistic type, like hand flapping, between 15 months and 7 years, strong restricted interest for keys and fans, prolonged fixation for rotator objects and avoidance behavior for vacuum cleaners.

IL1RAPL1, *IL1RAPL2* and *NCS-1/FREQ* mutation screening

Primers for the amplification and the sequencing of coding regions of the *IL1RAPL1, IL1RAPL2* and *NCS-1/FREQ* genes were designed using NCBI Probe (http://www.ncbi.nlm.nih.gov/sites/entrez?db=probe) and Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 2). Only 88.4% of the *NCS-1/FREQ* gene was sequenced since it was not possible to amplify the first coding exon. The sequencing was then performed at the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada) and genomic variations were identified using Mutation Surveyor® v3.10 (SoftGenetics, State College, PA, USA) software. The prediction of the effect of missense variants on the protein function was performed using three different software: Polyphen (http://genetics.bwh.harvard.edu/pph/), SIFT (http://blocks.fhcrc.org/sift/SIFT_BLink_submit.html) and PANTHER (http://www.pantherdb.org/tools/csnpScoreForm.jsp).

IL1RAPL1 cDNA constructs

The full-length human IL1RAPL1 cDNA (Image ID 8992061) was obtained from Open Biosystems (Huntsville, AL, Canada). The wild-type (WT) and the I367SfsX6 deletion

Table 2. Primers for the amplification of *IL1RAPL1*, *IL1RAPL2* and *NCS-1/FREQ* coding sequence and for the characterization of the 3′ border of the ∼730 kb deletion in the *IL1RAPL1* gene

Amplicon	Foward primer sequence	Reverse primer sequence
IL1RAPL1 (NM_014271.2) sequencing		
Exon 2	5′-gggtgagggctatggaggca-3′	5′-aattccctatgagtgtgctccacc-3′
Exon 3a	5′-acagcagaagcagactcagtgaagaa-3′	5′-gcctgtgctttctttgattcagcag-3′
Exon 3b	5′-cccttgcccaaagtgctgga-3′	5′-tgtctgcgaagggcaatagca-3′
Exon 4	5′-ggtagcaagagagaaccctgtg-3′	5′-ttttctatccaatttgtcccag-3′
Exon 5	5′-aatgaccagtgcaacttgccatatt-3′	5′-tggacttcagtggaagatgattcaaa-3′
Exon 6	5′-agcttgcctgaaggtgttatctgc-3′	5′-gggtcctaaggcacgcactg-3′
Exon 7	5′-gccatgttgtcgaaagcacaca-3′	5′-tctttgcaaatggcattcatgtt-3′
Exon 8	5′-cagtgtgattgtcctgctaaggga-3′	5′-tgcctcggtggctctaatgc-3′
Exon 9	5′-tgcaagctaccaaccaaggttca-3′	5′-tgctgccattgattatgcacg-3′
Exon 10	5′-tcagaaatgggacatttggagacg-3′	5′-tggtttctttcaatgtcaaggctca-3′
Exon 11	5′-ccaaatgagagatttgtaccgggaa-3′	5′-ccatggaaatggcgagaca-3′
IL1RAPL2 (NM_017416) sequencing		
Exon 2	5′-aagatctgtggttcaaactttatatcc-3′	5′-acaggtgccctctgggtg-3′
Exon 3	5′-aagattgaatgaagtaatctatgtgg-3′	5′-tgcaacctttcaagaacatatcc-3′
Exon 4	5′-tcctaaatggaaaagccaatactc-3′	5′-atctcccttcctcatcccc-3′
Exon 5	5′-agtactaacttgctggtactgagatg-3′	5′-aaaactcttgcttcccaaacc-3′
Exon 6	5′-tggatagagtaacaacccaagtagg-3′	5′-tggcaataaacctttcctgg-3′
Exon 7	5′-aaatggtcgcttcctgtctc-3′	5′-aatggaatgatttggagttgc-3′
Exon 8	5′-ccatctgtagtcttggcatgg-3′	5′-cactacatgataaaataaagccagg-3′
Exon 9	5′-gaattttctggaaaggaaaacag-3′	5′-ttgccatagccctttaccc-3′
Exon 10	5′-ttgtgctattcctgcctcttag-3′	5′-ttggctctgatgtttgaagc-3′
Exon 11a	5′-ttcagttttaaggaggggaatg-3′	5′-tcagcctgagatgacaccag-3′
Exon 11b	5′-gcacttagtatatgaaatgcccatc-3′	5′-cacaacgttattaggtgtactttatgc-3′
NCS-1/FREQ (NM_014286.2) sequencing		
Exon 2	5′-agcgaatgtctgcccatc-3′	5′-actgctgtccccatcagc-3′
Exon 3	5′-gatgttggtggtgggtgg -3′	5′-ctagaagggaacatcggcag-3′
Exon 4	5′-cccgtcccgaggttcag -3′	5′-caccctgcctggtgag-3′
Exon 5	5′-gtacagagaggaggggcgag -3′	5′-ggctgcacaagtgctctctc-3′
Exon 6	5′-aaactgccaggtctgggg -3′	5′-gatccagggactccaattcc-3′
Exon 7	5′-ctgccatgagtgccgag -3′	5′-gagctctgaccaatttgaagg-3′
Characterization of the deletion 3′ border		
Intron 6	5′-ccatgttgtcgaaagcacac-3′	5′-aggcaaactgacaggtaacattt-3′
Internal exon 7	5′-acctgcagagctttctttgg-3′	5′-cccaaactcgattttcatcc-3′
Intron 7a	5′-gtgaatagaaagtaagctgaaagga-3′	5′-tgcaaatggcattcatgttt-3′
Intron 7b	5′-cggatcacctgaggtcaaga-3′	5′-cccgagtagctgggactaca-3′
Intron 7c	5′-cataatgcctaagccccttttt-3′	5′-aaaaataagtatctatgccaaagca-3′

forms were amplified by PCR using the following primers: forward 5′-CCG GGG TAC CGG AAG ACT GTT GTG GGG AAC-3′, wt reverse 5′-ATA CCG CTC GAG GGA CGG GAT GTC CCT TGC TTT TCT-3′ and I367SfsX6 reverse 5′-ATC CGC TCG AGT CAC CAA ACA TAC AAG CAG CTA GCA CCA AGG CCT CCA GCA AG-3′. The PCR products were digested with KpnI and XhoI, and subcloned first into pEntr2B before being transferred in PDEST 12.2 vector (Invitrogen, San Diego, CA, USA), introducing a FLAG sequence just after the signal peptide (between amino acids 23 and 24).

HEK293 transfection and expression of WT and I367SfsX6 forms of IL1RAPL1

HEK293T cells were transfected with a control plasmid or with plasmids encoding the WT and I367SfsX6 forms of IL1RAPL1 using standard calcium phosphate transfection methods. After cell lysis, total proteins were subjected to electrophoresis on a polyacrylamide slab gel, electroblotted overnight onto Hybond Enhanced Chemiluminescence nitrocellulose membranes (Amersham, Buckinghamshire, UK) and incubated with an anti-FLAG M2 antibody (Sigma, Saint-Louis, MO, USA).

Hippocampal neuronal culture, immunofluorescence and quantification of neurite outgrowth

Hippocampal cultures were prepared from embryonic rats (E18) using previously described methods (32). Dissociated neurons were transfected with GFP, miRNA and/or IL1RAPL1 constructs by electroporation using a Nucleofector Kit (Amaxa Inc., Gaithersburg, MD, USA) at a density of $2-3 \times 10^7$ cells/ml. The transfected neurons were plated at 4000 cells/cm^2 on poly-L-lysine-coated glass coverslips. The neurons were allowed to attach in Minimal Essential Medium with 10% fetal bovine serum and N2 supplement for 4 h, then the medium was switched to serum-free neuron culture media. Cultures (3–5 days old) were fixed for 15 min with 4% paraformaldehyde 4% sucrose dissolved in PBS. Fixed cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min and incubated for 1 h in blocking

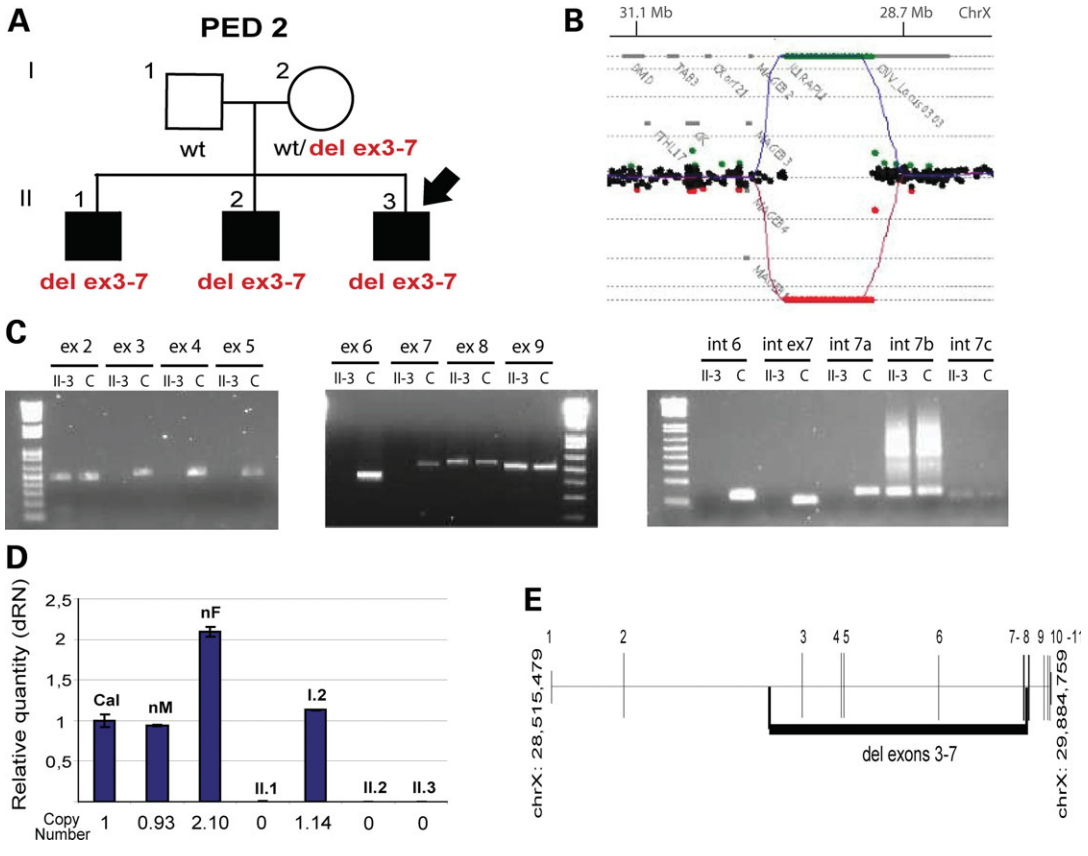

Figure 5. Identification of an intragenic deletion of one part of the *IL1RAPL1* gene in three brothers with MR and/OR ASD. (**A**) Pedigree PED2 composed of three male siblings affected by mild to severe MR and/or ASD (II-1, II-2 and II-3) that carry a deletion in *IL1RAPL1*, transmitted by their mother. (**B**) CGH results performed in patient II-3 (PED2) for the region Xp21.1–Xp21.2 reveal a large deletion of ~730 kb. DNA from patient II-3 and a sex-matched reference DNA were labeled with Cy5 (red) and Cy3 (green), respectively. (**C**) PCR amplification from genomic DNA of the proband II-3 (II-3) and an unrelated control (C). The fragments cover *IL1RAPL1* coding exons 2–9 and the region around exon 7: intron 6 (int 6), internal exon 7 (int ex 7), intron 7 fragment a (int 7a), intron 7 fragment b (int7b) and intron 7 fragment c (int 7c). (**D**) *IL1RAPL1* gene dosage by quantitative PCR targeting intron 5 in a control calibrator individual (Cal), a normal male (nM), a normal female (nF), the three brothers II-1, -2 and -3 and their mother I-2. (**E**) Genomic structure of the *IL1RAPL1* gene, showing the deletion encompassing exons 3–7. Coordinates are based on NCBI Build 36.1 of the human reference sequence, and exons are numbered 1 through 11.

solution (10% bovine serum albumin in PBS). They were then incubated with a monoclonal antibody against IL1RAPL1 (1:500, Abnova Corporation, Taipei, Taiwan) followed by a fluorescently labeled donkey anti-mouse IgG1 (1:1500; Molecular Probes, Eugene, OR, USA). Images were taken of transfected or control neurons with Zeiss Axioskop microscope. Neurites were counted and their lengths measured using Northern Eclipse Version 7.0 image analysis software (Empix Imaging, Mississauga, ON, Canada). Neurite length and number was quantified relative to untransfected cells in sister cultures. To quantify the intensity of IL1RAPL1, labeling fluorescence intensity was determined by grey density using Northern Eclipse Version 7.0 image analysis software. Statistical analysis of the results was carried out using a Student's *t*-test with $P < 0.05$ as the criterion for significance.

Comparative genomic hybridation (CGH) on PED2 proband II-3 and PCR analysis of the *IL1RAPL1* deletion

For the CGH, a half microgram each of patient DNA and sex-matched reference DNA was labeled with Cy5 (red) and Cy3 (green), respectively, using a commercially available random primer labeling kit (Enzo Life Sciences, NY, USA). The labeled DNA samples were purified on a QiaQuick purification kit (Qiagen, Dusseldorf, Germany), combined, and hybridized to a custom-designed 105K oligonucleotide array (Agilent Technologies, Santa Clara, CA, USA) at 60°C for 40 h. The array was designed to specifically target with high density a large set of MR genes. The *IL1RAPL1* region was covered by 95 probes between chrX:28515602 and chrX:29883938. The arrays were washed in commercially available solutions and scanned. The resulting TIFF images were analyzed with Feature Extraction software and output from this software was imported into CGH Analytics software for final analysis. The data were analyzed using a z-score algorithm with a 0.25 cut-off threshold. The wash buffers, scanner and all software were available from Agilent Technologies.

Confirmation of the deletion in *IL1RAPL1* gene and characterization of its 3′ border

PCR with the primers used for the sequencing of *IL1RAPL1* was performed for all the coding exons of *IL1RAPL1*. All

the amplifications were done in the same time on DNA from the proband II-3 and from an unrelated control individual. Additional primers were designed to amplify different ~150 bp fragments between the end of intron 6 and intron 7 (Table 2).

Segregation analysis and confirmation of *IL1RAPL1* deletion by quantitative PCR

Quantitative PCR targeting *IL1RAPL1* intron 5 was used to confirm the deletion in patient II-3 and to test the other family members. *IL1RAPL1* gene dosage was performed by genomic real-time PCR using TaqMan probes chemistry. FAM-labeled probes from the Universal Probe Library (Roche Applied Science, Basel, Switzerland) were selected to assay fragments amplified from intron 5 of the *IL1RAPL1* gene. The *SOD1* gene was used for assay normalization. DNA samples obtained from three normal controls (one is randomly assigned to be a calibrator) and from the patients II-1, -2, -3 and their mother I-2 were run in triplicate on the Mx3000P QPCR instrument (Stratagene). Calculation of gene copy number used the relative quantification method in which the amount of the target gene is measured in test samples (the unknown) and compared to a reference sample (the calibrator), both normalized to the control gene. The amplicon was generated using primers IL1RAPL1int5Q77F (5′-TGT TGT AAC ATT ATG CCG TCA A-3′) and IL1RAPL1int5Q77R (5′-AAC GGT TTT GTG GTT CTC CA-3′).

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REFERENCES

1. Lauritsen, M. and Ewald, H. (2001) The genetics of autism. *Acta Psychiatr. Scand.*, **103**, 411–427.
2. Bailey, A., Le Couteur, A., Gottesman, I., Bolton, P., Simonoff, E., Yuzda, E. and Rutter, M. (1995) Autism as a strongly genetic disorder: evidence from a British twin study. *Psychol. Med.*, **25**, 63–77.
3. Yang, M.S. and Gill, M. (2007) A review of gene linkage, association and expression studies in autism and an assessment of convergent evidence. *Int. J. Dev. Neurosci.*, **25**, 69–85.
4. Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I.C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C. *et al.* (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat. Genet.*, **34**, 27–29.
5. Durand, C.M., Betancur, C., Boeckers, T.M., Bockmann, J., Chaste, P., Fauchereau, F., Nygren, G., Rastam, M., Gillberg, I.C., Anckarsater, H. *et al.* (2007) Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat. Genet.*, **39**, 25–27.
6. Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M.P., Raynaud, M., Ronce, N., Lemonnier, E., Calvas, P. *et al.* (2004) X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am. J. Hum. Genet.*, **74**, 552–557.
7. Lawson-Yuen, A., Saldivar, J.S., Sommer, S. and Picker, J. (2008) Familial deletion within NLGN4 associated with autism and Tourette syndrome. *Eur. J. Hum. Genet.*, **16**, 614–618.
8. Moessner, R., Marshall, C.R., Sutcliffe, J.S., Skaug, J., Pinto, D., Vincent, J., Zwaigenbaum, L., Fernandez, B., Roberts, W., Szatmari, P. *et al.* (2007) Contribution of SHANK3 mutations to autism spectrum disorder. *Am. J. Hum. Genet.*, **81**, 1289–1297.
9. Gauthier, J., Spiegelman, D., Piton, A., Lafreniere, R.G., Laurent, S., St-Onge, J., Lapointe, L., Hamdan, F.F., Cossette, P., Mottron, L. *et al.* (2008) Novel de novo SHANK3 mutation in autistic patients. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* [Epub ahead of print July 9, 2008].
10. Zoghbi, H.Y. (2003) Postnatal neurodevelopmental disorders: meeting at the synapse? *Science*, **302**, 826–830.
11. Garber, K. (2007) Neuroscience. Autism's cause may reside in abnormalities at the synapse. *Science*, **317**, 190–191.
12. Born, T.L., Smith, D.E., Garka, K.E., Renshaw, B.R., Bertles, J.S. and Sims, J.E. (2000) Identification and characterization of two members of a novel class of the interleukin-1 receptor (IL-1R) family. Delineation of a new class of IL-1R-related proteins based on signaling. *J. Biol. Chem.*, **275**, 29946–29954.
13. Bahi, N., Friocourt, G., Carrie, A., Graham, M.E., Weiss, J.L., Chafey, P., Fauchereau, F., Burgoyne, R.D. and Chelly, J. (2003) IL1 receptor accessory protein like, a protein involved in X-linked mental retardation, interacts with Neuronal Calcium Sensor-1 and regulates exocytosis. *Hum. Mol. Genet.*, **12**, 1415–1425.
14. Gambino, F., Pavlowsky, A., Begle, A., Dupont, J.L., Bahi, N., Courjaret, R., Gardette, R., Hadjkacem, H., Skala, H., Poulain, B. *et al.* (2007) IL1-receptor accessory protein-like 1 (IL1RAPL1), a protein involved in cognitive functions, regulates N-type Ca2+-channel and neurite elongation. *Proc. Natl. Acad. Sci. USA*, **104**, 9063–9068.
15. Carrie, A., Jun, L., Bienvenu, T., Vinet, M.C., McDonell, N., Couvert, P., Zemni, R., Cardona, A., Van Buggenhout, G., Frints, S. *et al.* (1999) A new member of the IL-1 receptor family highly expressed in hippocampus and involved in X-linked mental retardation. *Nat. Genet.*, **23**, 25–31.
16. Tabolacci, E., Pomponi, M.G., Pietrobono, R., Terracciano, A., Chiurazzi, P. and Neri, G. (2006) A truncating mutation in the IL1RAPL1 gene is responsible for X-linked mental retardation in the MRX21 family. *Am. J. Med. Genet. A*, **140**, 482–487.
17. Froyen, G., Van Esch, H., Bauters, M., Hollanders, K., Frints, S.G., Vermeesch, J.R., Devriendt, K., Fryns, J.P. and Marynen, P. (2007) Detection of genomic copy number changes in patients with idiopathic mental retardation by high-resolution X-array-CGH: important role for increased gene dosage of XLMR genes. *Hum. Mutat.*, **28**, 1034–1042.
18. Menten, B., Maas, N., Thienpont, B., Buysse, K., Vandesompele, J., Melotte, C., de Ravel, T., Van Vooren, S., Balikova, I., Backx, L. *et al.* (2006) Emerging patterns of cryptic chromosomal imbalance in patients with idiopathic mental retardation and multiple congenital anomalies: a new series of 140 patients and review of published reports. *J. Med. Genet.*, **43**, 625–633.
19. Bhat, S.S., Ladd, S., Grass, F., Spence, J.E., Brasington, C.K., Simensen, R.J., Schwartz, C.E., Dupont, B.R., Stevenson, R.E. and Srivastava, A.K. (2008) Disruption of the IL1RAPL1 gene associated with a pericentromeric

inversion of the X chromosome in a patient with mental retardation and autism. *Clin. Genet.*, **73**, 94–96.
20. Marshall, C.R., Noor, A., Vincent, J.B., Lionel, A.C., Feuk, L., Skaug, J., Shago, M., Moessner, R., Pinto, D., Ren, Y. *et al.* (2008) Structural variation of chromosomes in autism spectrum disorder. *Am. J. Hum. Genet.*, **82**, 477–488.
21. Cody, H., Pelphrey, K. and Piven, J. (2002) Structural and functional magnetic resonance imaging of autism. *Int. J. Dev. Neurosci.*, **20**, 421–438.
22. Yoshida, T. and Mishina, M. (2008) Zebrafish orthologue of mental retardation protein IL1RAPL1 regulates presynaptic differentiation. *Mol. Cell. Neurosci.* [Epub ahead of print July 1].
23. Zheng, J.Q. and Poo, M.M. (2007) Calcium signaling in neuronal motility. *Annu. Rev. Cell. Dev. Biol.*, **23**, 375–404.
24. Krey, J.F. and Dolmetsch, R.E. (2007) Molecular mechanisms of autism: a possible role for Ca2+ signaling. *Curr. Opin. Neurobiol.*, **17**, 112–119.
25. Splawski, I., Yoo, D.S., Stotz, S.C., Cherry, A., Clapham, D.E. and Keating, M.T. (2006) CACNA1H mutations in autism spectrum disorders. *J. Biol. Chem.*, **281**, 22085–22091.
26. Hope, C.I., Sharp, D.M., Hemara-Wahanui, A., Sissingh, J.I., Lundon, P., Mitchell, E.A., Maw, M.A. and Clover, G.M. (2005) Clinical manifestations of a unique X-linked retinal disorder in a large New Zealand family with a novel mutation in CACNA1F, the gene responsible for CSNB2. *Clin. Exp. Ophthalmol.*, **33**, 129–136.
27. Splawski, I., Timothy, K.W., Sharpe, L.M., Decher, N., Kumar, P., Bloise, R., Napolitano, C., Schwartz, P.J., Joseph, R.M., Condouris, K. *et al.* (2004) Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell*, **119**, 19–31.
28. Laumonnier, F., Roger, S., Guerin, P., Molinari, F., M'Rad, R., Cahard, D., Belhadj, A., Halayem, M., Persico, A.M., Elia, M. *et al.* (2006) Association of a functional deficit of the BKCa channel, a synaptic regulator of neuronal excitability, with autism and mental retardation. *Am. J. Psychiatry*, **163**, 1622–1629.
29. Tsigelny, I., Shindyalov, I.N., Bourne, P.E., Sudhof, T.C. and Taylor, P. (2000) Common EF-hand motifs in cholinesterases and neuroligins suggest a role for Ca2+ binding in cell surface associations. *Protein Sci.*, **9**, 180–185.
30. Zhang, H., Maximov, A., Fu, Y., Xu, F., Tang, T.S., Tkatch, T., Surmeier, D.J. and Bezprozvanny, I. (2005) Association of CaV1.3 L-type calcium channels with Shank. *J. Neurosci.*, **25**, 1037–1049.
31. Gauthier, J., Bonnel, A., St-Onge, J., Karemera, L., Laurent, S., Mottron, L., Fombonne, E., Joober, R. and Rouleau, G.A. (2005) NLGN3/NLGN4 gene mutations are not responsible for autism in the Quebec population. *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **132B**, 74–75.
32. Goslin, K. *et al.* (1998) *Culturing Nerve Cells*. MIT Press, Cambridge, pp. 339–370.