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Mutations in the guanine nucleotide exchange factor gene *IQSEC2* cause nonsyndromic intellectual disability

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Abstract

The first family identified as having a nonsyndromic intellectual disability was mapped in 1988. Here we show that a mutation of *IQSEC2*, encoding a guanine nucleotide exchange factor for the ADP-ribosylation factor family of small GTPases, caused this disorder. In addition to MRX1, *IQSEC2* mutations were identified in three other families with X-linked intellectual disability. This discovery was made possible by systematic and unbiased X chromosome exome resequencing.

Intellectual disability is frequent in the general population (~1 in 50 in the population), and genetic causes are highly heterogeneous. Intellectual disability, also referred to as mental retardation, is characterized by substantial limitations both in intellectual functioning and in adaptive behavior and is diagnosed before the age of 18. X chromosome-linked intellectual disability (XLID) is genetically well characterized. Subdividing XLID into syndromic and nonsyndromic forms[1] has facilitated the discovery of associated genes. The first family with nonsyndromic XLID, termed MRX1 (ref. 2), was mapped in 1988 (ref. 3). Even though

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AUTHOR CONTRIBUTIONS

C.S., M.S., F.A., A.G. and J.B. assembled the extended families and confirmed, tracked and analyzed the changes. A.P., H.P. and M.S. performed additional subject and control screening. P.S.T., A.F. and M.R.S. supervised the X-chromosome sequencing, collation and analysis of the sequencing data. A.H., M.F., R.E.S., G.T., C.E.S. and F.L.R. contributed families and clinical data on affected individuals. C.S., S.L.R., J.A.M., R.S.W., R.J.H. and S.R. performed functional assays. C.S. and J.G. conceived and designed the study and wrote the first draft of the manuscript. J.G. directed the study. All authors contributed to discussion of the results and manuscript preparation.

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more than 90 XLID genes have been reported, the underlying defect remains unresolved in over 50% of families with nonsyndromic XLID[4]. Until recently, identification of the causative mutations in XLID has been achieved primarily by positional and functional candidate gene approaches and targeted resequencing of specific regions of the X chromosome[4]. The application of the systematic resequencing of the entire X chromosome exome[5] has removed bias associated with candidate gene selection and revealed previously unidentified XLID-associated genes. However, this approach has also highlighted future challenges in the follow-up and functional analyses linking DNA variation to the pathology of intellectual disability.

As part of a recent X chromosome exome resequencing project[5], we identified four different nonsynonymous SNPs in *IQSEC2* in four separate families with XLID. In addition to moderate to severe intellectual disability in all affected males, seizures, autistic traits, psychiatric problems and delayed early language skills were noted, although none of these additional phenotypes were consistent in all affected individuals in the families (for clinical descriptions, see Supplementary Note). All four nonsynonymous sequence variants were predicted to lead to nonconserved amino acid substitutions. In the MRX1 family (MIM309530)[2,3], we identified a C2587T change in exon 8 leading to a R863W substitution (Fig. 1). In the MRX18 family, we identified a A2402C change in exon 6 leading to a Q801P substitution (Fig. 1). A third, unpublished affected family ascertained in the United States (US166) had a G2273A change in exon 5 leading to a R758Q substitution (Fig. 1). These three changes occur within the Sec7 domain of *IQSEC2* (Fig. 2a). In a fourth, unpublished affected Australian family (AU128), we identified a C1075T change in exon 4 leading to a R359C substitution in the IQ-like domain of IQSEC2 (Fig. 1). We did not detect any other *IQSEC2* sequence variants in the rest of the cohort (comprising 201 male and 6 female probands), which also included 45 affected male sibling pairs[5]. Sequence analysis of the coding region and flanking splice sites in the X chromosomes from 560 control individuals in the MRX1, MRX18 and AU128 families and from 750 controls from the US166 family did not detect any of these or any other variants (data not shown). Each of these changes segregated with the intellectual disability phenotype in the associated family (Fig. 1). The maximum two-point $\log_{10}$ odds scores of 4.61, 2.11, 2.26 and 1.51 were calculated for families MRX1, MRX18, AU128 and USA166, respectively. All four amino acid substitutions identified here are located within key functional domains of IQSEC2: the IQ-like and the Sec7 domains (Fig. 2a). Comparative analysis of the members of the IQSEC protein family shows that all four residues are highly conserved and predicted to be damaging (Supplementary Fig. 1 and Supplementary Tables 1 and **2**). Overall, our data indicated that all four missense changes were highly likely to affect the normal function of IQSEC2.

Much as for other human IQSEC paralogs, there are two isoforms of IQSEC2 (Supplementary Fig. 2) generated by alternative splicing. The shorter isoform of *Iqsec2* is highly expressed in the rodent forebrain, with increasing expression across postnatal life[6]. A second study localized the longer variant of Iqsec2 to excitatory synapses as part of the *N*-methyl-D-aspartate (NMDA) receptor complex, via Iqsec2 interactions with the postsynaptic density proteins DLG1, DLG2, DLG3 and DLG4 (ref. 7). Experimentally, Iqsec2 has been shown to increase GTP binding to ARF GTPases, particularly ARF6 (ref. 6,7). All four nucleotide substitutions identified are within regions common to both IQSEC2 splice isoforms. We show that both isoforms are expressed in fetal and adult human brain (Supplementary Fig. 2c).

Initially, we assessed the effect of the three different Sec7 domain mutations on the ability of IQSEC2 to catalyze GTP binding to the ARF6 GTPase *in vitro* using recombinant glutathione S-transferase-tagged Sec7 domain fusion proteins (Supplementary Methods).

This analysis demonstrated that the Sec7 domain missense changes result in significantly diminished GTP binding to ARF6 as compared to wild-type (Fig. 2b). GTP binding mediated by the Sec7-domain mutants R758Q, Q801P and R863W was similar to that of a control domain having a dominant negative mutation that we engineered by replacing the negatively charged glutamic acid at position E849 with a nonpolar alanine. E849 is critical for proper Sec7 function and is invariant across all ADP-ribosylation factor (ARF) guanine nucleotide exchange factors (GEF)[8]. Subsequently, we tested the GEF activity of full-length IQSEC2 in a cellular model using a pulldown assay with the adaptor protein Golgi-localized, γ ear-containing ARF-binding protein 3 (GGA3). We also used a E849K dominant negative alteration[8] as a control. The GGAs specifically interact with active, GTP-bound ARF but do not interact with inactive ARFs[9]. Co-transfection of ARF6 with wild-type IQSEC2 resulted in an approximately 12-fold increase in GTP-bound ARF6 but only a 6- to 8-fold increase when expressed with IQSEC2 harboring mutations in the Sec7 domain (Fig. 2c). The remaining missense change (R359C) in IQSEC2 occurs in the IQ-like domain. Although the role of the IQ domain in this family of GTP-binding proteins is not well understood, there is evidence that this motif may bind calmodulin and accelerate the exchange activity of some GEFs[10]. In the case of IQSEC2, the IQ-like motif lacks the G and second basic residue of the complete motif (Fig. 2d). Binding of calmodulin by incomplete or IQ-like motifs is calcium dependent[11]. The R359C substitution in the AU128 family disrupts the conserved basic residue in the IQ-like domain consensus motif (FILV)Qxxx(**R**K)xxxxxx and is predicted to result in disrupted calcium-dependant calmodulin binding, leading to an altered exchange of GDP for GTP on ARF6. Consistent with this theory, transfection of HEK293 cells with the IQSEC2 R359C variant and ARF6 also resulted in a decrease in GTP-bound ARF6 in the GGA pull-down assay (Fig. 2c). Neither of the four IQSEC2 variants studied nor the two dominant negative controls seemed to have affected the stability of their respective proteins (Fig. 2).

A consistent phenotype of nonsyndromic XLID is observed in affected individuals regardless of the specific mutation present, suggesting that a loss of GEF activity of IQSEC2 is the likely underlying molecular mechanism of IQSEC2 XLID. Compromised GTP binding activity of IQSEC2 may lead to reduced activation of the ARF6 substrate and may influence the regulation of actin cytoskeleton organization. Iqsec2 mRNA and protein have been identified at dendrites in rodents, suggesting that *Iqsec2* mRNA might be locally translated in an activity-dependent manner, contributing to synaptic plasticity[7]. Taken together, the data strongly suggest that all four missense changes in *IQSEC2* are functionally relevant and are the disease-causing mutations in these families.

IQSEC2 has also been found at the X chromosome breakpoint of an apparently balanced t(X;20) translocation in a female with infantile spasms[12]. However, the molecular mechanism underlying the phenotype of this individual, either through *IQSEC2* or gene(s) on chromosome 20, was not determined[12]. Notably, the human *IQSEC2* gene (unlike its mouse ortholog) escapes X inactivation. Although some carrier females from our four families presented with a mild learning disability (Supplementary Note), this could not be unequivocally attributed to their *IQSEC2* mutation carrier status.

IQSEC2 (also known as BRAG1 or IQ-ARFGEF) is a guanine nucleotide exchange factor for the ARF family of GTP-binding proteins (ARFGEF). The human genome contains 15 ARFGEFs, each with an ~200-amino-acid Sec7 domain responsible for catalyzing nucleotide exchange onto ARF substrates[13]. ARFs are members of the Ras superfamily of small G proteins and have a primary role in the regulation of vesicular transport and organelle structure[13]. In mammals the six ubiquitously expressed genes and paralogs are divided into three classes based on sequence similarity (Class I, ARF1-3; Class II, ARF4-5; Class III, ARF6). Class I and II ARFs are localized to the Golgi and endosomal

compartments, whereas ARF6 is localized to cell periphery in association with the plasma membrane and a subset of endosomes. The ARF proteins switch between GTP-bound active and GDP-bound inactive conformations, with the exchange of GDP for GTP nucleotides mediated by GEFs.

The pivotal role of the actin cytoskeleton in neuronal development, differentiation, and synaptic plasticity and neuronal function has also been highlighted by the identification of several other autosomal and X-chromosome genes that are mutated in individuals with intellectual disability, including *OPHN1* (encoding oligophrenin, a RhoGAP), *PAK3* (encoding p21 protein (Cdc42/Rac)-activated kinase 3), *ARHGEF6* and *ARHGEF9* (RhoGEFs) and *FGD1* (a RhoGEF) (reviewed in ref. 4). Failure to maintain homeostasis within the neural environment has been suggested as a possible, overarching mechanism involved in a broad spectrum of neurocognitive and neuropsychiatric phenotypes[14]. Identification and functional characterization of naturally occurring human mutations resulting in cognitive impairment is essential for better understanding of the brain. The knowledge of these genes and their functions will aid future design of better management strategies, which, together with the recent developments in treatments for diseases such as fragile X[15], provides a much-needed dose of optimism for affected individuals and their families.

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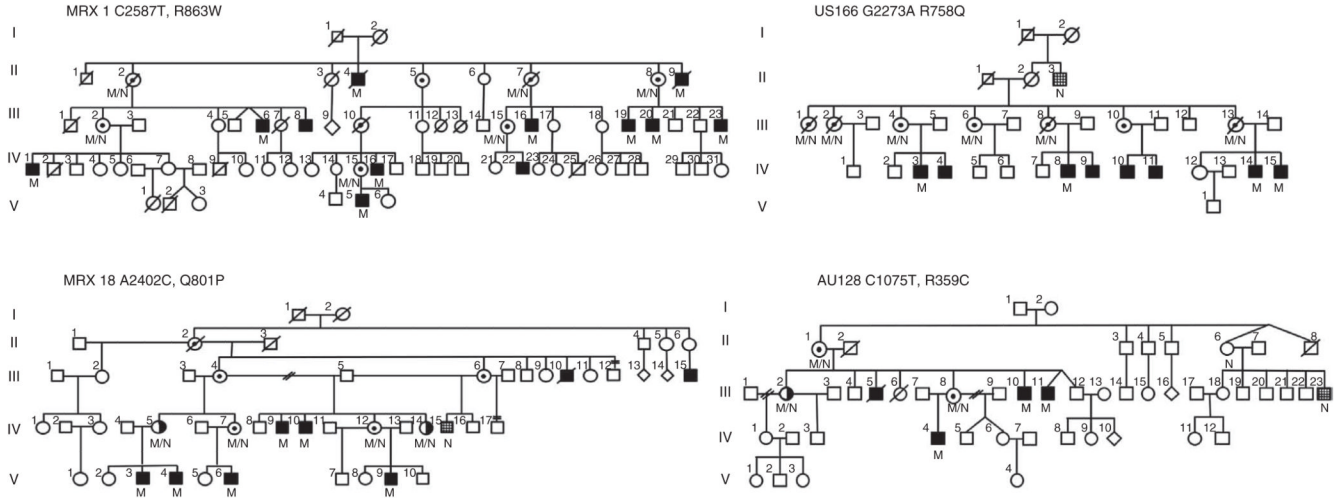

Figure 1.

Identification of *IQSEC2* mutations. cDNA (NM_001111125) and protein (NP_001104595) annotation of individual mutations is shown for each family. Pedigrees of MRX1 and MRX18 families have been updated since their last publication. Open symbols represent normal individuals, filled squares represent affected males, open circles with middle dots represent carrier females and cross-hatched squares represent males with learning problems but without an *IQSEC2* mutation. Two carrier females with some learning problems from the MRX18 and AU128 families are shown with half the circle black. Individual generations are numbered with Roman numerals on the left of each pedigree. Individuals tested for the nucleotide substitution in each family are indicated either M (mutant allele) or M/N (mutant and normal allele). The genotypes of some females tested have not been shown due to privacy issues.

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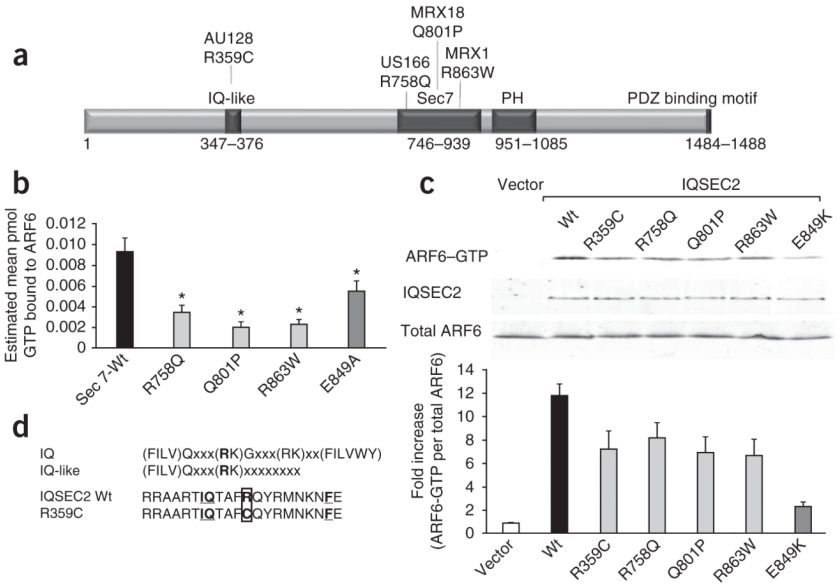

Figure 2.

IQSEC2 structure and mutations. (**a**) Scheme of the human IQSEC2 protein (NP_001104595); shown are the IQ-like domain, Sec7 domain, pleckstrin homology domain (PH) and PDZ binding motif (UniprotKB/Swiss-Prot for Q5J85;1,488-amino-acid protein). Mutations in each family are shown above. (**b**) Missense mutations introduced into the Sec7 domain (light gray) diminish the GTP binding activity compared to the Sec7 wild-type (black) activity to levels seen with the E849A dominant negative mutant (dark gray) predicted to reduce GEF activity of the Sec7 domain. An all-groups comparison of the mean pMol of GTP bound to ARF6 was achieved by linear mixed model analysis. $*P < 0.0001$ compared to Sec7Wt. (**c**) Wild-type (Wt) ARF6-HA and Flag-tagged wild-type or mutant IQSEC2 were transfected into HEK293 cells and lysates were subjected to a pull-down assay with GST:GGA3 to isolate ARF6-GTP. The precipitates (above, top row) and lysates (above, bottom row) were probed with anti-HA to detect ARF6 and anti-Flag to detect expression of IQSEC2 (above, middle row). ARF6-GTP levels were normalized to total ARF6 levels and shown as the fold-increase over ARF6 transfected with empty vector. The E849K dominant negative mutation abolishes GEF activity of the Sec 7 domain[8]. Data are mean ± s.e.m. from three independent experiments (bottom panel). (**d**) The IQ-like motif sequence lacks the G and second basic residue of the IQ motif. Characters within parentheses can substitute for each other. The R359C mutation disrupts the basic (R) residue (in bold and boxed).

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