Increased Dosage of RAB39B Affects Neuronal Development and Could Explain the Cognitive Impairment in Male Patients with Distal Xq28 Copy Number Gains

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ABSTRACT: Copy number gains at Xq28 are a frequent cause of X-linked intellectual disability (XLID). Here, we report on a recurrent 0.5 Mb tandem copy number gain at distal Xq28 not including MECP2, in four male patients with nonsyndromic mild ID and behavioral problems. The affected region harbors eight genes of which RAB39B encoding a small GTPase, was the prime candidate since loss-of-function mutations in Rab39b, in mouse primary hippocampal neurons demonstrated a significant decrease in neuronal branching as well as in the number of synapses. Taken together, we provide evidence that the increased dosage of RAB39B causes a disturbed neuronal development leading to cognitive impairment in patients with this recurrent copy number gain. Hum Mutat 00:1–8, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: intellectual disability; copy number gain; neuronal branching; RAB39B; Xq28

Introduction

Intellectual disability (ID) results in a significant loss of adaptive behavior and affects about 2% of the population [Lubs et al., 2012; Ropers 2010]. So far, about a hundred genes on the X chromosome have already been implicated in X chromosome-linked ID (XLID) [Lubs et al., 2012]. The most common genetic defects leading to XLID are loss-of-function mutations including nonsense, frameshift and missense mutations, microdeletions, and translocations. However, it is now generally accepted that an increased gene dosage due to a microduplication comprising whole genes can result in XLID as well. Especially the Xq28 region is prone to copy number variations (CNVs), including gains. The most prevalent copy number gain on the X chromosome causes the MECP2 duplication syndrome (MIM #300260), a microduplication of variable size, from 0.2 to about 2.0 Mb, but always harboring MECP2 (MIM #300005; 153.3 Mb; UCSC Hg19). The increased dosage of MECP2 leads to a severe syndromic ID phenotype in male patients [Meins et al., 2005; Van Esch et al., 2005]. The causal role for MECP2 was clearly demonstrated in a mouse model showing that a twofold increase in Mecp2 is sufficient to induce symptoms resembling the human situation [Collins et al., 2004; Luikenhuis et al., 2004]. A second ID-associated copy number gain at Xq28 is a recurrent 0.3 Mb copy number gain distal to MECP2. The affected region (153.5–153.8 Mb) includes 11 genes, of which the increased level of GDI1 (MIM #300104) was proposed to cause the patients’ cognitive impairment [Vandewalle et al., 2009]. This hypothesis was based on the fact that loss-of-function mutations in GDI1 lead to nonsyndromic ID [D’Adamo et al., 2002] and on a striking GDI1 dosage-dependent correlation with the ID severity [Vandewalle et al., 2009]. However, no cellular or animal models have proven this hypothesis so far. A third recurrent duplicated region at Xq28, located even more distal to GDI1, was described in patients with mild ID and behavioral problems [El-Hattab et al., 2011]. Four patients harbored an apparently identical 0.5 Mb copy number gain (154.1–154.6 Mb) that includes eight protein coding genes. No candidate gene was proposed for the ID in these patients. Here, we report on four additional male patients with the same 0.5 Mb copy number gain and also presenting with mild to moderate ID and behavioral problems. Based on expression analyses, we proposed RAB39B...
(MIM #300774) as the most likely candidate for this disorder. Indeed, overexpression of Rab39b in primary neurons showed reduced neuronal branching and number of synapses. Together, we propose that increased dosage of the small GTPase RAB39B results in the cognitive defects but also the behavioral abnormalities observed in all eight affected patients.

Material and Methods

Array-CGH and Duplication Mapping

The proband of T61 (II.1) and patient AV1 were analyzed for copy number alterations by an X chromosome tiling-path BAC array, as previously described [Froyen et al., 2007]. Patient KM1 was analyzed on Illumina HumanCNV370 Bead Chips according to the recommended procedures [Illumina Inc., San Diego, CA]. Confirmation and analysis of the extent of the duplication was done by real time relative quantitation (qPCR) using SYBRgreen, on an LC480 instrument (Roche, Basel, Switzerland). qPCR primers in and around this CNV were designed with the LightCycler Probe Design2 software (Roche) and are provided in Supp. Table S1. All samples were run in duplicate and data were analyzed with the software of the instrument and further analyzed in Excel using the comparative ΔΔCt method (Lifetechnologies, Foster City, CA).

qPCR Expression Analysis and X Inactivation

Total RNA was extracted from blood lymphocytes or Epstein–Barr Virus-transformed peripheral blood lymphocytes (EBV-PBLs) and cDNA prepared with random hexamer primers (Lifetechnologies) as described elsewhere [Van Esch et al., 2005]. qPCR was performed on cDNA with primers in different exons as described earlier [Vandewalle et al., 2009]. The housekeeping genes GUSB and Hprt1 were used for normalization. Each 15 µl reaction well contained 0.5 µM of each primer and 1× SYBRgreen PCR Master Mix (Roche). For quantitation of the mouse homologs, total RNA was extracted from mouse tissues with TRIzol (Lifetechnologies) as described elsewhere [Froyen et al., 2008]. qPCR expression analysis was then performed using Gusb and Hprt as normalizers as described above. Primer sequences can be found in Supp. Table S1.

For X chromosome inactivation, genomic DNA extracted directly from lymphocytes of patients and controls was subjected to the standard androgen receptor (AR) methylation assay. Fragment analysis was performed on an ABI3500xL sequence analyzer (Lifetechnologies) as described elsewhere [Froyen et al. 2008]. qPCR expression analysis was then performed using Gusb and Hprt as normalizers as described above. Primer sequences can be found in Supp. Table S1.

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Breakpoint Mapping and Analysis

The sequence of the Xq28 region (154.2–154.7 Mb; Hg19) was imported and analyzed in CLC DNA workbench 6.5 (CLC Bio, Aarhus, Denmark). Primer sequences and PCR conditions, to amplify the int22h1, int22h2, and int22h2/1 LCRs, were taken from Liu and colleagues [Liu et al., 1998] using ELT-PCR (Roche) reagents. Primer pairs Q-P and B-A were first applied on control DNA to optimize the conditions. Then, the primer pair B-P was used with the aim to amplify over the recombined int22h2/int22h1 LCR. As our attempts were unsuccessful, we also produced alternative B’ and P’ primers (own set). Primer sequences can be found in Supp. Table S1.

Neuronal Culture, Transduction, and Analyses

Primary cultures of mouse hippocampal neurons at embryonic day E18.5 were prepared as described earlier [Giannandrea et al., 2010]. The mouse Rab39b cDNA was cloned into a lentiviral vector to yield the pcCL-Rab39b plasmid as described previously [Giannandrea et al., 2010]. Transduction was performed immediately after plating of the neuronal cells at an MOI of 1. Western blot was performed using the in-house developed polyclonal anti-RAB39B and anti-Synapsin Abs, and the commercially available anti-Gapdh Ab (MAP374; Millipore, Billerica, MA). Immunofluorescence, Sholl, and statistical analyses were performed as previously reported [Giannandrea et al., 2010].

Results

Patients

Family T61 consists of two affected brothers from Caucasian unrelated parents (Supp. Fig. S1A). The proband II.1 is reported in DECIPHER (http://decipher.sanger.ac.uk/) as patient 248532. This male was born at term with a weight of 3,690 g, length of 50 cm (both at the 50th centile), and head circumference of 32.5 cm (5th centile). At the age of 6.5 years, he presented with mild ID and speech impairment. His weight was 32.2 kg, height was 131.54 cm (both >97th), and head circumference was 51 cm (50th centile). Mild facial dysmorphic features include a high forehead, frontal cow-lick, large ears, auricular pits, hypertelorism, antverted nares, enlarged philtrum, thin upper lip, and spaced central incisors. At that age, he exhibited moderate ID and aggressive behavior. High-resolution karyotype was normal. His younger brother (II.2), at the age of 3 years, presented with mild ID, speech impairment, mild craniofacial anomalies, cow-lick, and large ears. Their mother had learning difficulties but no other anomalies.

Patient AV1 is the second child of Belgian unrelated and healthy parents, born after an uneventful pregnancy. He was briefly described previously as patient 18 [Isrie et al., 2012]. He has one healthy older brother and family history is negative for ID or congenital anomalies. Orchidopexy was undertaken at the age of 7 years and he was diagnosed with a unilateral dysplasia of the hip. Learning difficulties were present and special education was necessary throughout schooling. Because of severe behavior difficulties, he was admitted to a boarding school for intellectually disabled children at the age of 13 years. When he was 15, he was referred to a psychiatrist by whom he was diagnosed with paranoid ideas and treated with pimozide. Around this time, he presented fugues and verbal aggression. At the age of 22, he was admitted to the psychiatric unit for the intellectually disabled because of severe automutilation. He was anxious and had paranoid ideas, telling the nurses that someone tried to kill him. During admission, he avoided contact with other patients and showed poor hygiene. Cognitive testing using the WAIS (Wechsler Adult Intelligence Scale) showed a mild ID with a TIQ of 61, a PIQ of 64, and a VIQ of 68. A DSM-IV diagnosis of Schizophrenia of the paranoid type was made. When he was 29 years old, he was readmitted to the same psychiatric unit with similar psychiatric problems for which clozapine, zuclopenthixol, and valproic acid were prescribed. During his hospitalization, he was seen by a clinical geneticist. On physical examination, the patient’s height was 179 cm (50th centile), weight was 126.3 kg (97th centile = 106 kg), BMI was 39.42 and OFC was 59 cm (97th centile). Facial features included a long face with a high forehead and ears with simple helices (Supp. Fig. S1B). Brain MRI showed no abnormalities.
Patient KM1 is from Estonia. He is the only child of nonconsanguineous parents (Supp. Fig. S1C). He was born at term with birth weight 3,400 g. His early development was normal; he started to walk at 13 months. At the age of 6.5 years, he was investigated for the first time due to developmental delay. His growth was normal—height 116 cm (0 SD), weight 20.5 kg (−0.5 SD), and head circumference 51 cm (−1 SD). He had mildly feminine body composition—wide hips with fat distribution. His gait was clumsy and spastic. He had a mildly dysmorphic face—deep set eyes and a high nasal bridge. His feet had a sandal gap, short T2, and partial syndactyly of T2–3. Wechsler Intelligence Scale for Children (WISCI) showed an IQ of 60 (verbal < nonverbal; 61 and 67, respectively), his speech was dysarthric and he had difficulties with socialization and was diagnosed with mild ID. Brain MRI and EEG were normal. He had frequent bronchitis and allergy episodes, and asthma was later diagnosed. His half-brother has mild ID as well and his half-sister suffers from epilepsy. His mother had early menopause. She was not cooperative and refused blood sampling.

Identification of a Recurrent 0.5 Mb Duplication at Xq28

X chromosome-specific array-CGH performed on DNA of the proband of family T61 showed increased ratios for BAC clones RP11–296N8 (154.13–154.30 Mb), RP11–402H20 (154.20–154.38 Mb), RP11–207O16 (154.24–154.38 Mb), and RP11–143H17 (154.34–154.50 Mb) with a mean ratio of 0.68 (± 0.08) pointing to a duplication (positions based on UCSC hg19, build 37). Subsequently, array-CGH analysis performed in a cohort of 2,222 sporadic male patients with ID referred for diagnosis in Leuven (Belgium) detected one additional ID patient (AV1, or patient 18 in [Isrie et al., 2012]) with apparently the same copy number gain. A similar screen in Tartu (Estonia) revealed another positive sample (KM1) out of a screen of 750 ID patients. Therefore, we estimate the frequency of this aberration at about 1 in 1,000 sporadic male ID patients. Moreover, this aberration was not detected by oligo-array in more than 7,000 controls. Fine-mapping of the copy number gains by qPCR revealed a recurrent duplication of about 300 kb in size, flanked proximally by the 8 kb long int22h1 LCR (chrX:154,109,090–154,118,602) and distally by the int22h2 LCR (chrX:154,606,155–154,615,708) (Fig. 1). Primer sequences for qPCR can be found in Supp. Table S1. The region in between both int22h LCRs harbors eight protein coding genes: F8 (partially), FUNDG2, CMG4, MTCP1, BRCC3, VBP1, RAB39B, and CLIC2. Both int22h segments also contain the genes H2AFB1–3, H2AFB2, H2AFB3, F8A1, F8A2, F8A3, and the microRNAs MIR1184–1, MIR1184–2, and MIR1184–3, all of which are also present in the int22h3 LCR (chrX:154,684,312–154,693,869) not included in this copy number gain. Because each of the sequences of the H2AFB1–3, F8A1–3, and MIR1184–1–3 are virtually identical, it is expected that they have the same function, if any. Therefore, in our male patients, the genes located in the intermediate region, except for F8, are all duplicated while the paralogous genes within the int22h LCRs are increased from 9 to 12 copies.

X-Inactivation in Female Carriers

DNA of two carrier mothers was subjected to the AR X-inactivation assay. The mother (I.2) of family T61 was heterozygous (320/341) and showed a random X-inactivation pattern (68/32). Both sons inherited the X chromosome with the 320 allele that was mostly inactivated in their mother. Patient AV1 inherited the X chromosome with the 317 allele that was predominantly inactivated in the blood cells of his carrier mother, who showed a skewed inactivation ratio of 11/89 for the 314/317 alleles. No DNA was available from the mother of KM1.

Copy Number and FISH Analysis

For copy number analysis, we quantified the CNV by qPCR using at least three primer pairs located between int22h1 and int22h2 in the affected boys and their mothers. A copy number of about 2.0 was found for T61–II.1, T61–II.2, and AV1 (Fig. 2). Unexpectedly, a higher copy number (mean 3.58) was consistently found for patient KM1 indicating the presence of three or four copies in this boy. Both carrier mothers tested, T61-I.2 and AV1-m, showed ratios of about 1.5 (Fig. 2) proving the presence of three copies instead of the normal two. FISH was then performed to assess whether the extra copy had been inserted in cis or in trans. BAC

Figure 1. Schematic representation of the distal part of the Xq28 region showing some of the genes as well as the three most common copy number gains identified in multiple unrelated ID patients. The nonrecurrent MECP2 duplication (striped box) can range from 0.2 to 2.0 Mb but always includes MECP2. The recurrent 0.3 Mb copy number gain (black box) containing GDI1 as the most likely dosage sensitive ID gene, and the more distal recurrent 0.5 Mb copy number gain (gray box) that we identified in the four patients. The latter region is enlarged at the bottom on which the genes are indicated as well as the positions of the LCRs. The directly oriented LCRs int22h1 and int22h2 mediated the NAHR event. On top, the locations of the qPCR primer sets used for quantitation and mapping are shown. The genes within the int22h repeats are not shown.
DNA RP11–143H17 (ChrX:154.4 Mb; labeled in red), which locates within the copy number gain, was cohybridized with RP11–373B10 (ChrX:148.1 Mb labeled in green) on interphase chromosomes of patients T61-II.1 and AV1. For both patients, the two red signals were in close proximity to each other (Supp. Fig. S2) strongly suggesting that both copies are next to each other as is expected for a nonallelic homologous recombination (NAHR) event.

Increased Expression of Duplicated Genes

We reasoned that if a twofold increase in expression of a candidate gene would have a detrimental effect on normal physiological conditions, its expression in controls should be highly stable. Therefore, we first analyzed the relative mRNA expression levels of four selected brain-expressed genes, BRCC3, VBP1, RAB39B, and CLIC2, in five EBV-PBL cell lines derived from control individuals. All four genes were expressed in the PBL cells at a level that allows reliable comparison of relative transcript levels. The normalized mRNA levels of three of these genes were all highly similar (1.00 ± 0.23) in these control samples (Supp. Fig. S3A) indicating a tightly regulated expression of these genes in this cell type. CLIC2 shows a more variable expression (1.00 ± 0.4). Next, we quantified the mRNA levels of the corresponding mouse genes (except for Clic2 that does not exist in the mouse) in the cortex and hippocampus of three 6 weeks old male mice. Again, a stable expression was noted for each of these genes in both brain regions (Supp. Fig. S3B). Finally, we analyzed the expression of the genes in the EBV-PBL cell line derived from two patients and five controls. In the proband of T61 (II.1) as well as in patient AV1, a significantly increased mRNA expression was found for BRCC3 (2.0-fold), VBP1 (2.5-fold), and RAB39B (1.5-fold), whereas CLIC2 mRNA levels were not increased in AV1 making a dosage-sensitive role for this gene unlikely (Fig. 3).

Study of the Recombination Mechanism

Because of the presence of directly oriented 9.5 kb long int22h LCRs flanking the microduplication (proximally int22h1 and distally int22h2 having > 95% identity), the recurrent copy number gain is likely due to NAHR (Supp. Fig. S4). This mechanism of rearrangement was also proposed by El-Hattab et al. (2011) but no direct molecular proof was provided. We performed several attempts for long distance ELT-PCR on control DNA using the primer combinations B-A and Q-P (Supp. Fig. S4) but were unable to generate any PCR products of about 12 kb. The large size of this LCR as well as potential secondary structures could have caused this inability. Not surprisingly, the B-P primer set did not reveal a PCR band of the presumed junction in our patients either. Testing of alternative primers, conditions and combinations were unsuccessful so we could therefore not formally prove the mechanism of NAHR between both int22h LCRs. Nevertheless, the 0.5 Mb recurrent copy number gain in Xq28 involves ChrX: 154,109,090–154,615,708 (Hg19).

Increased Rab39b Levels Alters Neuronal Differentiation

Previously, analysis of neuronal differentiation of primary neuronal cells after downregulation of Rab39b with shRNA showed a significant decrease in the numbers of neuronal branches and presynaptic terminals. Similarly, we now tested the effect of an increased Rab39b dosage by transducing primary neuronal cells with a pCCL-Rab39b lentivirus to mimic the duplication in our patients. The primary neuronal cells were transduced at a MOI of 1. After 3 days in culture, Western blot quantification of four independent neuronal cultures revealed on average an eightfold increase of Rab39b (Fig. 4A). To evaluate the morphology and growth of the neurons, we stained the cells with β-tubulin and used Sholl analysis to count the number of times neurites cross a radial segment of 10 μm (Fig. 4B). Sholl analysis of 278 control- and 305 Rab39b-infected primary neuronal cells of four independent neuronal preparations revealed a significant decrease in the number of interceptions (n = 4; ANOVA: P = 0.0004). Factorial ANOVA for each Sholl category separately gave significant P values for distances of 10 μm (P = 0.0004), 20 μm (P = 0.0001), 30 μm (P = 0.0001), and 40 μm (P = 0.01) from the cell body (Fig. 4C). Moreover, a significant decrease of neuronal branching was observed in Rab39b-overexpressing neurons with significant P values after factorial ANOVA; 10 μm (P = 0.0001), 20 μm (P = 0.0001), 30 μm (P = 0.0001), 40 μm (P = 0.004), and 50 μm (P = 0.0001) (Fig. 4D). No differences were seen however, in the amount of neurites that leave the cell body (Fig. 4E).

Next, we analyzed the presynaptic terminals with an anti-Synapsin Ab in Rab39b-transduced DIV11 neuronal cells (Fig. 5A). At this later stage, Rab39b protein was still significantly overexpressed as shown by Western blot (Fig. 5B). Counting of
Figure 4. Decrease of branching in Rab39b-overexpressing primary neuronal cells. A: Immunoblot analysis, performed on control and Rab39b-infected DIV3 primary neuronal cell extracts, showing an increase in Rab39b levels. Quantification was done on four independent assays analyzed with ImageJ and normalized to Gapdh. B: Immunostaining of DIV3 primary neuronal cells with β-tubulin. C: For Sholl analysis a mask of concentric circles that are 10 μm apart from each other was superimposed on the neuronal cells. Counting the interceptions at DIV3 of control and Rab39b-infected primary neuronal cells revealed a significant reduction in the interceptions of Rab39b-infected neurons. Mean ± SE. D: The amount of branches at each specific distance was significantly reduced in Rab39b-infected primary neurons at DIV3 in comparison with the controls. Mean ± SE. E: The amount of neurites that leave the cell body did not change if Rab39b is overexpressed in primary neuronal cells. Mean ± SE.

Discussion

Subtle copy number gains at distal Xq28 are rather frequent due to the high GC content and relative large number of LCRs, which predispose this chromosomal end to genomic rearrangements [del Gaudio et al., 2006; Bauters et al., 2008; Vandewalle et al., 2009]. The high number of LCRs in this region already predicted genomic disorders because of deletion events resulting in the removal of genes in males. The introduction of array-CGH however, also demonstrated a significant number of disease-associated duplications at Xq28 [Van Esch et al., 2005; Vandewalle et al., 2009]. Here, we report on four nonsyndromic ID patients carrying the most distal recurrent CNV reported so far. This 0.5 Mb duplication (154.10–154.60 Mb) was previously reported in patients with ID and behavioral problems but no candidate gene was proposed [El-Hattab et al., 2011]. Similar to the reported patients, the affected male patients in our families present with mild to moderate ID, minor facial features, and behavioral problems such as aggressiveness and difficulty in socialization. From the characteristic facial features described by El-Hattab and colleagues (high forehead, upper eyelid fullness, broad nasal bridge, and thick lower lip), a high forehead is the only feature reported in at least three of our patients (T61-II.1, T61-II.2, and AV1). However, behavioral problems seem to be a strikingly consistent factor in these patients carrying this recurrent 0.5 Mb copy number gain in Xq28. Patients T61-II.1 and AV1 show aggressive behavior, the latter having serious psychiatric symptoms that include paranoia and automutilation. Patient KM1 was reported with difficulties in socializing but no formal tests had been done on him. These features correspond well with those observed by El-Hattab and colleagues who reported abnormal aggressiveness and hyperactivity as the most striking behavioral problems in their four affected males. It is worth noting that these features might only appear at later ages as the 3 years old boy of their family 1 did not present with any of these behavioral abnormalities [El-Hattab et al., 2011].

X inactivation was skewed in the mother of patient AV1 (11/89) while random in the mother (I.2) of T61 (68/32), which could be in agreement with her mild learning problems. If no recombination had occurred between the AR locus and the duplication at Xq28, both mothers mainly inactivate their X chromosome that carries the distal Xq duplication, which is inherited by their affected sons. These data suggest that overexpression of one of the genes in the interval has a negative effect on cell survival in early development, at least of the hematopoietic cell lineage. Skewing of X inactivation in blood cells is often found in XLID gene mutation female carriers. In two of the three families reported by El-Hattab et al. (2011), however, the authors concluded that both carrier mothers have their normal X chromosome predominantly inactivated (89% and 88%), which is unexpected. They quantified the X inactivation ratio based on both AR and FMR1 loci and assumed that the recombination frequency between FMR1 (at 147.0 Mb) and the duplication (at 154.1 Mb) would be less than 1%. However, as the distance between both loci is 7 Mb (not 0.8 Mb as stated) the recombination frequency is 5%. Even though their carrier mothers show learning
Rab39b is involved in synaptic formation. A: Immunostaining of DIV11 primary neuronal cells with Synapsin. B: Immunoblot analysis performed on control and Rab39b-infected primary neuronal cell extract still shows a strong increase in Rab39b levels at DIV11. No difference in the amount of Synapsin could be detected between both samples. C: Synapsin immunostaining and quantification by ImageJ shows a 30% reduction of Synapsin in the presynaptic compartments in neurons infected with Rab39b. Mean ± SE.

difficulties they could still have inactivated the mutant X chromosome, which is in agreement with the data on the carrier mother of the third family who also presents with learning problems and skewing (6/94) [El-Hattab et al., 2011]. Our data support the fact that the duplication-carrying X chromosome is preferentially inactivated in female carriers. However, the inactivation pattern in brain could well be different from those measured in blood, and a more random inactivation ratio, as observed in the mother of T61, could explain their common learning difficulties.

The 500 kb large duplication harbors eight protein coding genes. F8 encoding factor VIII is only partially affected as int22h1 locates in intron 22 of this gene. The function of factor VIII could not be disturbed as the patients in this study do not suffer from hemophilia A (MIM #306700). Of the remaining seven genes RAB39B is the most likely candidate because loss-of-function mutations have been detected in two XLID families [Giannandrea et al., 2010]. These authors demonstrated that Rab39b shows the highest expression in fetal and adult brain of mouse and man, with the highest levels in the mouse hippocampus. Rab39b is specifically expressed in mouse neuronal precursor cells as well as neurons, with very low to no expression in glial cells. Rab39b localizes to the Golgi [Giannandrea et al., 2010], which is in line with the role of small RAB GTPases in vesicular trafficking [Pfeffer 2012]. Finally, knockdown of Rab39b in primary hippocampal neurons affects different aspects of neuronal differentiation and maturation. The number of growth cones is significantly reduced, the branching is decreased and presynaptic terminals are diminished by almost 50% [Giannandrea et al., 2010]. All these features strongly suggest a key role for RAB39B in normal neuronal functioning. From the other six duplicated genes loss of the deubiquitinating enzyme BRCC3 results in the Moyamoya disease type 4 (MIM #300845), which affects blood vessels leading to cardiomyopathy and cerebral infarcts. Other features include short stature, hypergonadotropic hypogonadism, and facial dysmorphism [Miskinyte et al., 2011]. One missense mutation in the chloride intracellular channel 2 (CLIC2) has recently been detected in two brothers [Takano et al., 2012]. Functional analysis suggested it to be involved in a new channelpathy including ID, seizures and cardiac problems. However, as ID was also present in their sister who did not carry the mutation, its correlation with ID is not yet convincing. No diseases have been associated with FUNDC2, CMC4, MTCPIP1, or VBP1 so far. They all show fairly ubiquitous tissue expression according to Unigene (NCBI) but no functional information is available for any of these (predicted) proteins. Moreover, we demonstrated stable expression of BRCC3 and RAB39B in EBV-PBLs from controls and importantly a 1.5- to 2.5-fold increase in expression in cell lines derived from patients T61-II.1 and AV1. Finally, since this affected region maps close to the pseudoautosomal region 2, an effect on this telomeric end, which harbors SPRY3 and VAMP7, cannot be excluded.

Even though an effect of other genes cannot be ignored, RAB39B remains the most promising candidate ID gene for this recurrent 0.5 Mb copy number gain. To test whether an increased dosage of RAB39B would affect neuronal development or connectivity, we studied the overexpression of Rab39b in mouse primary hippocampal neurons. At DIV3, Sholl analysis of Rab39b-overexpressing cells demonstrated a significant decrease in the number of intersections when compared with control neurons. Notably, these data were comparable with what has been observed after downregulation of Rab39b [Giannandrea et al., 2010]. However, the distance to the soma at which the number of intersections was reduced, was different for both, that is, closer to the soma (10–30 μm) with Rab39b overexpression, whereas more to the end of the neurites (30–40 μm) with downregulation of Rab39b. Although the reason for the difference in physical location of the altered differentiation is currently unknown both increase and decrease of Rab39b levels result in a reduced neuronal differentiation. The differential dosage effect on neurons has been studied for two other X-linked genes for which deletion or duplication has been related to disease. Pelizaeus-Merzbacher disease (PMD; MIM #312080) is caused by deletion, mutation, or duplication of the PLP1 gene located at Xq22.2. The place and grade of neuronal loss in the brains of PMD patients was reported to be dependent on the kind of mutation, with deletions being most severe [Sima et al., 2009]. Secondly, deletion or mutation, and duplication of MECP2 at Xq28 results in Rett syndrome (RTT; MIM #312750) and the MECP2 duplication syndrome (MRXSL; MIM #300260), respectively. Analysis of brains of knock-in and transgenic Mecp2 mice showed a reduced dendritic complexity and soma size in Mecp2 loss-of-function mice while neither of these phenotypes were detected in the Mecp2 duplication mouse [Wang et al., 2013]. In our in vitro system, we proposed that the reduced neuronal differentiation after increased Rab39b dosage was due to a reduced number of branches observed at the respective distances from the soma. Analysis of number of branches was not investigated after Rab39b downregulation. However, similar to the data of downregulation of Rab39b [Giannandrea et al., 2010], the number of presynaptic terminals at DIV11 was significantly reduced in Rab39b-overexpressing neurons. The observed 30% decrease, based on Synapsin staining, was however, somewhat less than seen for the Rab39b downregulation (50%). The amount of Synapsin was not detectably changed in the whole cell lysate, which could be due to the lower sensitivity of Western blot. These data are in line with the more severe ID phenotype.
of patients with loss-of-function mutations (moderate to severe ID), versus those with a duplication (mild ID). To conclude, we demonstrated a reduced differentiation, most likely through reduced branching, of the primary neurons with increased levels of Rab39b. Together with the lower number of presynaptic terminals, these alterations are in line with what has been seen in Rab39b downregulation.

In conclusion, we report on four additional male ID patients carrying the recurrent 0.5 Mb duplication at Xq28. Based on expression studies, we propose Rab39b as the most likely candidate gene for which an increased dosage would disturb normal neuronal development or function. We provide functional evidence that increased dosage of Rab39b could be the causal factor for the ID phenotype and possibly the behavioral problems observed in all reported patients with this recurrent 0.5 Mb copy number gain in Xq28.

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