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Mutations of *CASK* cause an X-linked brain malformation phenotype with microcephaly and hypoplasia of the brainstem and cerebellum

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CASK is a multi-domain scaffolding protein that interacts with the transcription factor TBR1 and regulates expression of genes involved in cortical development such as *RELN*. Here we describe a previously unreported X-linked brain malformation syndrome caused by mutations of *CASK*. All five affected individuals with *CASK* mutations had congenital or postnatal microcephaly, disproportionate brainstem and cerebellar hypoplasia, and severe mental retardation.

Human microcephaly has previously been associated with defects in mitosis and DNA repair[1]. Recently, homozygous inactivation of the T-box family transcription factor TBR2, which is encoded by *EOMES* and putatively regulates neural identity and cortical neurogenesis[2], was associated with microcephaly and other brain malformations in four individuals from an inbred family[3]. These data indicate that disturbances in transcriptional regulation may also be responsible for microcephaly.

To further elucidate causes of human microcephaly, we analyzed an individual (individual 1) referred at 4 years because of congenital and marked postnatal microcephaly, severe mental retardation and sensorineural hearing loss. Her brain MRI showed reduced number and complexity of gyri, thin brainstem and severe cerebellar hypoplasia (**Supplementary Table 1** and **Supplementary Fig. 1a–c** online). Chromosome analysis showed a paracentric inversion of one X chromosome: 46,X,inv(X)(p11.4p22.3). This was not inherited from her mother, and her father could not be studied. We constructed a physical map using fluorescence *in situ* hybridization (FISH) for both breakpoint regions (**Supplementary Methods** online). The Xp22.33 breakpoint was narrowed to a ~20-kb gene-poor region (**Supplementary Table 2** online), and the Xp11.4 breakpoint interrupted the *CASK* gene (NM_003688) (**Fig. 1a,b** and **Supplementary Table 2**), and possibly the *GPR34* (NM_005300, NM_001033513 and NM_001033514) or *GPR82* (NM_080817) genes located in reverse orientation in *CASK* intron 5. *CASK* encodes a calcium/calmodulin-dependent serine protein kinase that belongs to the membrane-associated guanylate kinase (MAGUK) family. Members of this family target to neuronal synapses and regulate trafficking, targeting and signaling of ion channels. CASK has been proposed to be a 'pseudokinase' and functions as part of large signaling complexes in both pre- and postsynaptic sites[4]. However, CASK also translocates to the nucleus and interacts with the brain-specific T-box family member TBR1 (ref. 5).

Recently, a heterozygous ~3.2-Mb deletion resulting in loss of eight annotated genes, including *EFHC2*, *NDP* and *CASK* exons 1 and 2 but not *GPR34* or *GPR82*, was detected by X-chromosome array comparative genomic hybridization (aCGH) in a girl with multiple abnormalities that partly overlap with those of individual 1 (**Supplementary Table 1**)[6]. Available functional data make *CASK* an excellent candidate gene for this phenotype, as *Cask* mouse mutants have a small brain, abnormal cranial shape and cleft palate[7,8]. Further, CASK enhances transcriptional activity of TBR1, which regulates expression of the extracellular matrix protein Reelin (Reln), a key player in neuronal migration and lamination[9]. *Tbr1*$^{-/-}$ mice have severe microcephaly and defective cortical connectivity[10], and *Reln*$^{-/-}$ mice have severe cerebellar hypoplasia[9]. Both mutants have a forebrain malformation consisting of abnormal dispersion of neurons within and below the cortex, and inverted position of neurons within the cortex[9,10]. In humans, mutations in *RELN* are associated with the neuronal migration disorder lissencephaly as well as severe cerebellar and hippocampal hypoplasia[11]. These data suggest that CASK has a role in embryonic brain development.

The core phenotype for individual 1 consists of severe mental retardation, microcephaly and disproportionate pontine and cerebellar hypoplasia (MIC-PCH). We obtained genome-wide aCGH data (**Supplementary Methods**) on a clinical basis for two girls with this phenotype and found copy number losses in Xp11.4 in both (**Supplementary Table 1** and **Supplementary Fig. 1d–i**). In individual 2, we detected a ~740-kb heterozygous deletion encompassing *CASK*, *GPR34* and *GPR82*, which we confirmed by FISH (**Supplementary Methods** and **Supplementary Figs. 2a** and **3** online). Neither parent carried the deletion (data not shown; paternity confirmed). In individual 3, we found two separate deletions of *CASK*, including

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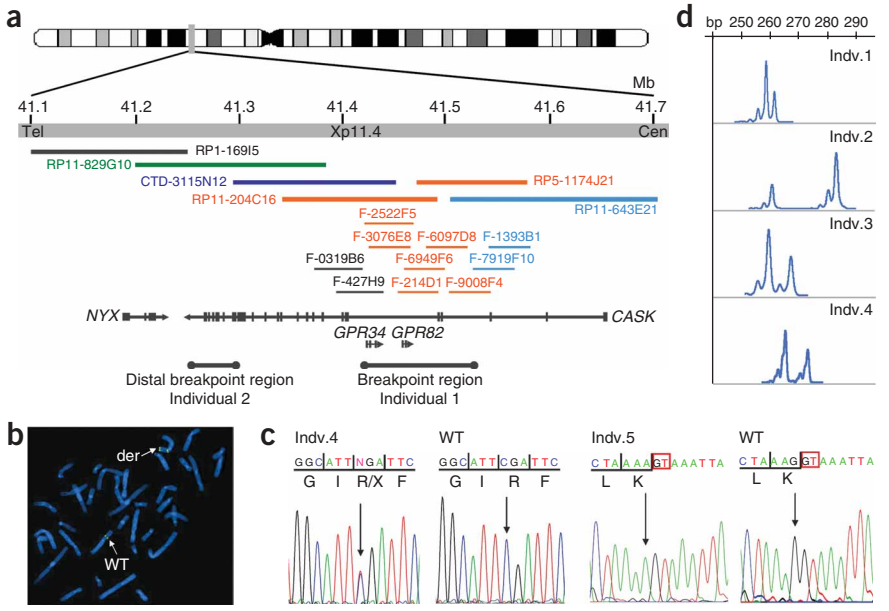

Figure 1 Mutations of *CASK* are associated with a previously unreported X-linked brain malformation phenotype. (**a**) Physical map of Xp11.4. BAC and fosmid (F–) clones used for mapping the Xp11.4 breakpoint of the individual with the Xp inversion (individual 1) and the distal deletion breakpoint of individual 2 are indicated by colored bars and names are given. Color code of BACs: black, mapped distal to both the deletion and inversion breakpoint; green, spanned the deletion breakpoint and mapped distal to the inversion breakpoint; dark blue, located within the deleted interval and distal to the inversion; red, spanned the inversion breakpoint; light blue, mapped proximal to the inversion. Color code of fosmids: black, mapped distal to the inversion breakpoint; red, spanned the breakpoint; light blue, proximal to the breakpoint. Exons of *CASK*, *GPR34*, *GPR82* and *NYX* are indicated by vertical bars, and the 5′→3′ orientation is given. Breakpoint regions are indicated. (**b**) FISH with fosmid clone F-6949F6 on metaphase spread from lymphocytes of individual 1 yielded split signals. Wild-type (WT) and derivative (der) X chromosomes are indicated by arrows. (**c**) Partial electropherograms depicting the heterozygous *CASK* mutation 1915C>T in individual 4 and the hemizygous 915G>A mutation in individual 5, as well as the respective wild-type sequence. (**d**) X chromosome inactivation data on genomic DNA from the four female subjects with a *CASK* mutation using the androgen receptor assay. Segments from the genotype plots after *Hpa*II restriction enzyme digestion are shown. The study was approved by all institutional review boards of the participating institutions, and written informed consent was obtained from all participants or their legal guardians.

~170 kb covering the 3′ region and ~150 kb encompassing the 5′ region that seem to be interrupted by a stretch of ~190 kb of normal copy number containing *GPR34* and *GPR82* (**Supplementary Fig. 2b** and data not shown). These data suggest a complex rearrangement such as an inversion-deletion. Notably, the distal deletion breakpoint of individual 2 and the most telomeric breakpoint in individual 3 are located in the same region (**Fig. 1a** and data not shown), suggesting that nonallelic recombination may be a common mutational mechanism. Quantitative PCR (**Supplementary Methods**) in individual 3 confirmed loss of one copy of *CASK* exons 2 and 24, and normal copy number of a probe ~92 kb centromeric to *CASK* (**Supplementary Table 3** online). We also identified several heterozygous SNPs in the middle of *CASK* (introns 4–12) as well as in intron 1 of the nearby *NYX* gene (data not shown). The parents of individual 3 have normal copy numbers in this region (**Supplementary Table 3**; paternity confirmed).

We next selected 46 individuals (33 males and 13 females, including individuals 2 and 3 discussed above) with MIC-PCH ascertained from our existing database and colleagues, and analyzed *CASK* for intragenic mutations (**Supplementary Methods**). We identified the heterozygous transition 1915C>T in exon 21 in individual 4, and the hemizygous transition 915G>A in exon 9 in individual 5, a boy who died at 2 weeks (**Fig. 1c**). Both had brain malformations resembling those of individuals 1–3, although these were more severe in the boy (**Fig. 2a**,**b**, **Supplementary Table 1**, and **Supplementary Fig. 1j–o**). The former mutation was not detected in the parents of individual 4 or in 150 control X chromosomes, and the latter was not found in the mother of individual 5 or in DNA samples from 515 healthy males (data not shown; all parental identities confirmed). The 1915C>T mutation must be pathogenic, as it results in a premature stop codon (R639X). However, the 915G>A transition is a synonymous mutation (K305) located in the last nucleotide of exon 9, where it could result in altered splicing[12]. We investigated the effect of this sequence change on splicing using three splice site prediction programs (**Supplementary Methods**) that all detected the wild-type donor site. Only one recognized the mutant sequence as a donor site with reduced splicing efficiency (**Supplementary Table 4** online). Because no RNA was available from individual 5, we carried out *in vitro* splicing analyses using 'minigene' constructs (**Supplementary Methods**) and identified skipping of exon 9 in about 20% of the mutant transcripts, suggesting a defect in splicing (**Supplementary Note** and **Supplementary Table 4** online).

Our finding of heterozygous loss-of-function mutations of *CASK* in four girls and a partly penetrant splice mutation in a severely affected boy suggests that the *CASK*-associated phenotype belongs to the group of X-linked disorders with reduced male viability or even *in utero* lethality[13]. However, 'mild' (hypomorphic) mutations, such as the synonymous mutation in *CASK* exon 9, may be compatible with livebirth in affected males. We investigated the X chromosome inactivation (XCI) pattern in genomic DNA extracted from lymphocytes (**Supplementary Methods**) in our four female subjects with a heterozygous *CASK* mutation and found random XCI (**Fig. 1d** and **Supplementary Note**).

Individuals with mutation of *CASK* show a previously undescribed and recognizable disease phenotype mainly characterized by severe or profound mental retardation and distinct structural brain anomalies (**Supplementary Note**, **Supplementary Table 1** and **Supplementary Fig. 4** online). We examined the brain of the deceased individual 5 in detail and identified a severely hypoplastic cerebellum, a small brainstem and abnormalities in cortical and cerebellar layering (**Fig. 2c–h** and **Supplementary Note**). We also analyzed the *Cask* knock-in mouse (*Cask-KI*), a hypomorphic *Cask* mutant[7] (**Supplementary Note** and **Supplementary Fig. 5** online). These mice showed disproportional cerebellar hypoplasia. Taken together, our data suggest that CASK is required for cerebellar (and forebrain) development in both human and mouse.

CASK has an important function during neuronal development. In embryonic neurons of the cerebral cortex, ~20% of CASK protein is present in the nucleus, where it regulates gene expression by interacting with the TATA-binding protein associated factor TAF9 (CINAP) and the transcription factor TBR1. These proteins form a

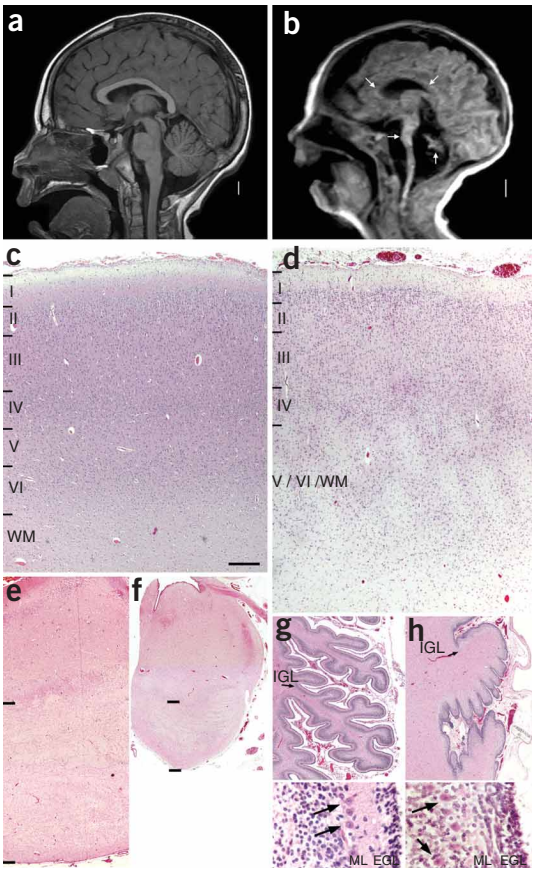

Figure 2 Brain imaging and pathologic features in a boy with the *CASK* 915G>A mutation (individual 5). (**a**,**b**) Compared to a normal control brain (**a**), magnetic resonance images in individual 5 (**b**) show a thin and unmyelinated corpus callosum (angled arrows) and severe hypoplasia of the brainstem (horizontal arrow) and cerebellum (vertical arrow). (**c**,**d**) The frontal cortex (**d**) is moderately disorganized and mildly thickened compared to an age-matched control (**c**). Cortical laminae I-IV appear normal, whereas layers V and VI merge together and show a vaguely nodular organization. These layers subtly merge into the white matter (WM), which differs from the well-defined border between layer VI and WM in the control cortex. (**e**,**f**) The pons is markedly reduced in size in the affected boy (**f**) compared to a control (**e**) because of loss of neurons in the basis pontis (borders defined by black lines). (**g**,**h**) The cerebellum (**h**) shows poorly formed, shallow and unbranched folia when compared to an age-matched control (**g**). In contrast to the normal external granular cell layer (EGL) in the age-matched control cerebellum (**g**, bottom panel), the EGL is abnormally thick in the cerebellum of individual 5 (**h**, bottom panel), and the internal granular cell layer (IGL) virtually absent (compare **g** and **h**). Purkinje cells are evenly spaced, forming a single layer in normal brain (arrows in **g**, bottom panel). In contrast, Purkinje cells in individual 5 are not seen in a single row (arrows in **h**, bottom panel). The bottom panels of **g** and **h** also show hypercellularity in the molecular layer (ML) and increased thickness of the EGL in individual 5 (**h**, bottom panel). Scale bar in **c** (lower right corner): 250 μm (**c**,**d**), 500 μm (**e**–**h**), 50 μm (bottom panels of **g** and **h**).

complex that induces transcription of genes containing TBR1 binding sequences, such as *RELN*[4,5]. The potential importance of the CASK-TBR1-RELN signaling cascade is supported by reports of similar malformations in *Tbr1* and *Reln* mouse mutants in brain regions where both are expressed[10,14]. Our data show that this resemblance applies to *CASK* as well, especially for brain size, where loss of CASK in humans and Cask or Tbr1 in mouse results in microcephaly, and for cerebellar cortex and brainstem, where loss of CASK in humans and Cask or Reln in mouse results in brainstem hypoplasia and defective inward migration of granule cells[9]. However, the cerebellar hypoplasia observed with loss of CASK in human or mouse cannot be mediated by TBR1 as it is not expressed in cerebellar cortex[2]. The CASK-interacting protein BCL11A (also known as Evi9 or CTIP1)[4], a zinc-finger transcription factor, is a good candidate to substitute for TBR1 in the cerebellum, as it is expressed in part of the cerebellum anlage during mouse development[15].

The cerebral cortical malformation found in individual 5 consists of abnormal dispersion of neurons only in the deeper layers V and VI (**Fig. 2d**), which are the primary expression domains of TBR1 in cortex[10,14]. This malformation might also result from disruption of CASK-TBR1 interactions, although it is less severe than that seen in *Tbr1* or *Reln* knockout mice.

A crucial role for CASK during synaptogenesis and a possible involvement of CASK in stereocilia and retinal function has recently been proposed (**Supplementary Note**). Identification and study of additional transcription factors that bind to CASK may help explain the pathogenesis of the *CASK*-associated phenotype, and identify candidate genes for related disorders.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

J.N. contributed to mutation analysis, X chromosome inactivation studies, *in vitro* splicing assay and manuscript writing. D.H. contributed to analysis of clinical data and manuscript writing and evaluated individual 2. I.W. contributed to FISH and mutation analysis and X chromosome inactivation studies. J.A.G. contributed to brain pathology of individual 5 and manuscript writing. V.V.C. contributed to analysis of *Cask-KI* mice. J.S. and S.L.C. interpreted the microarray, designed quantitative PCR assays and performed and interpreted the results in individual 3. R.U. contributed to design and production of BAC arrays. A.K. referred and evaluated individual 4. C.A.H. and A.F. contributed to obtaining and building up the *Cask-KI* mouse colony, genotyping of mice and preparing brains for morphological and histological analysis. L.R.C. referred and evaluated individual 5 and arranged for the brain to be obtained. G.U. interpreted brain scans of individual 4. U.F. referred and evaluated individual 1. E.K. performed and interpreted array CGH analysis for individuals 2 and 3 and FISH analysis for individual 2. W.B.D. contributed to patient ascertainment, clinical evaluation including interpretation of all brain scans, delineation of the phenotype, obtainment of the brain of individual 5, supervision of the mouse cerebellar studies and manuscript writing. K.K. designed the study, performed data analysis and contributed to manuscript writing.

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