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

Juliane Najm1,2, Denise Horn1,2, Isabella Wimimplinger1, Jeffrey A Golden2, Victor V Chizhikov3, Jyotsna Sudi4, Susan L Christian4, Reinhard Ullmann5, Alma Kuechler6, Carola A Haas7, Armin Flubacher2, Lawrence R Charnas8, Gökhan Uyanik9, Ulrich Frank10, Eva Klopocki2, William B Dobyns4,11,12 & Kerstin Kutsche1

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. Recently, homozygous inactivation of the T-box family transcription factor TBR2, which is encoded by EOMES and putatively regulates neural identity and cortical neurogenesis, was associated with microcephaly and other brain malformations in four individuals from an inbred family. 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,XX,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_008017) 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. 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). 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. Further, CASK enhances transcriptional activity of TBR1, which regulates expression of the extracellular matrix protein Reelin (Reln), a key player in neuronal migration and laminar. Tbr1−/− mice have severe microcephaly and defective cortical connectivity, and Reln−/− mice have severe cerebellar hypoplasia. 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. In humans, mutations in RELN are associated with the neuronal migration disorder lissencephaly as well as severe cerebellar and hippocampal hypoplasia. 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...
Mutations of \textit{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, 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 \textit{CASK}, \textit{GPR34}, \textit{GPR82} and \textit{NYX} are indicated by vertical bars, and the 5’–3’ orientation is given. Breakpoint regions are indicated. (b) FISH with fosmid clone F-6949FB6 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 \textit{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 \textit{CASK} mutation using the androgen receptor assay. Segments from the genotype plots after \textit{HpaII} 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.

Supplementary Note and \textit{Supplementary Table 3} online. We also identified several heterozygous SNPs in the middle of \textit{CASK} (introns 4–12) as well as in intron 1 of the nearby \textit{NYX} gene (data not shown). The parents of individual 3 have normal copy numbers in this region (\textit{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 \textit{CASK} for intragenic mutations (\textit{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, \textit{Supplementary Table 1}, and \textit{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 (K305S) located in the last nucleotide of exon 9, where it could result in altered splicing. We investigated the effect of this sequence change on splicing using three splice site prediction programs (\textit{Supplementary Methods}) that all detected the wild-type donor site. Only one recognized the mutant sequence as a donor site with reduced splicing efficiency (\textit{Supplementary Table 4} online). Because no RNA was available from individual 5, we carried out \textit{in vitro} splicing analyses using ‘minigene’ constructs (\textit{Supplementary Methods}) and identified skipping of exon 9 in about 20% of the mutant transcripts, suggesting a defect in splicing (\textit{Supplementary Note} and \textit{Supplementary Table 4} online).

Our finding of heterozygous loss-of-function mutations of \textit{CASK} in four girls and a partly penetrant splice mutation in a severely affected boy suggests that the \textit{CASK}-associated phenotype belongs to the group of X-linked disorders with reduced male viability or even \textit{in utero} lethality. However, ‘mild’ (hypomorphic) mutations, such as the synonymous mutation in \textit{CASK} exon 9, may be compatible with livebirth in affected males. We investigated the \textit{X} chromosome inactivation (XCI) pattern in genomic DNA extracted from lymphocytes (\textit{Supplementary Methods}) in our four female subjects with a heterozygous \textit{CASK} mutation and found random XCI (Fig. 1d and \textit{Supplementary Note}). Individuals with mutation of \textit{CASK} show a previously undescribed and recognizable disease phenotype mainly characterized by severe or profound mental retardation and distinct structural brain anomalies (\textit{Supplementary Note}, \textit{Supplementary Table 1} and \textit{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 \textit{Supplementary Note}). We also analyzed the \textit{Cask} knock-in mouse (\textit{Cask-KI}), a hypomorphic \textit{Cask} mutant (\textit{Supplementary Note} and \textit{Supplementary Fig. 5} online). These mice showed disproportional cerebellar hypoplasia. Taken together, our data suggest that \textit{CASK} is required for cerebellar (and forebrain) development in both human and mouse.

\textit{CASK} has an important function during neuronal development. In embryonic neurons of the cerebral cortex, ~20% of \textit{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

$\sim 170$ kb covering the 3′ region and $\sim 150$ kb encompassing the 5′ region that seem to be interrupted by a stretch of $\sim 190$ kb of normal copy number containing \textit{GPR34} and \textit{GPR82} (\textit{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 (\textit{Supplementary Methods}) in individual 3 confirmed loss of one copy of \textit{CASK} exons 2 and 24, and normal copy number of a probe $\sim 92$ kb centromeric to \textit{CASK} (\textit{Supplementary Table 3} online). We also identified several heterozygous SNPs in the middle of \textit{CASK} (introns 4–12) as well as in intron 1 of the nearby \textit{NYX} gene (data not shown). The parents of individual 3 have normal copy numbers in this region (\textit{Supplementary Table 3}; paternity confirmed).

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complex that induces transcription of genes containing TBR1 binding sequences, such as RELN\textsuperscript{1,2}. 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\textsuperscript{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 Reln in mouse results in brainstem hypoplasia and defective inward migration of granule cells\textsuperscript{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\textsuperscript{2}. The CASK-interacting protein BCL11A (also known as Evi9 or CTIP1)\textsuperscript{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\textsuperscript{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\textsuperscript{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.