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An N-terminal heterozygous missense CASK mutation is associated with microcephaly and bilateral retinal dystrophy plus optic nerve atrophy

Leslie E. W. LaConte¹, Vrushali Chavan¹, Stephanie DeLuca¹, Karol Rubin³, Jessica Malc¹, Susan Berry^{2,3}, C. Gail Summers^{2,4}, and Konark Mukherjee^{1,*}

¹Virginia Tech Carilion Research Institute, Roanoke, VA 24016, USA

²Department of Pediatrics University of Minnesota. 21 Church St SE, Minneapolis, MN 55455

³Department of Genetics, Cell Biology & Development University of Minnesota. 21 Church St SE, Minneapolis, MN 55455

⁴Department of Ophthalmology & Visual Neurosciences, University of Minnesota, 701 25th Ave. S. #300, Minneapolis, MN 55454

Abstract

Heterozygous loss-of-function mutations in the X-linked gene *CASK* are associated with mental retardation and microcephaly with pontine and cerebellar hypoplasia (MICPCH) and ophthalmological disorders including optic nerve atrophy (ONA) and optic nerve hypoplasia (ONH). Recently, we have demonstrated that *CASK*^(+/-) mice display ONH with 100% penetrance but exhibit no change in retinal lamination or structure. It is not clear if *CASK* loss-of-function predominantly affects retinal ganglion cells, or if other retinal cells like photoreceptors are also involved. Here, we report a heterozygous missense mutation in the N-terminal calcium/calmodulin-dependent kinase (CaMK) domain of the CASK protein in which a highly conserved leucine is mutated to the cyclic amino acid proline. *In silico* analysis suggests that the mutation may produce destabilizing structural changes. Experimentally, we observe pronounced misfolding and insolubility of the CASK^{L209P} protein. Interestingly, the remaining soluble mutant protein fails to interact with Mint1, which specifically binds to CASK's CaMK domain, suggesting a mechanism for the phenotypes observed with the CASK^{L209P} mutation. In addition to microcephaly, cerebellar hypoplasia and delayed development, the subject with the L209P mutation also presented with bilateral retinal dystrophy and ONA. Electroretinography indicated that rod photoreceptors are the most prominently affected cells. Our data suggest that the CASK interactions mediated by the CaMK domain may play a crucial role in retinal function, and thus, in addition to ONH, individuals with mutations in the *CASK* gene may exhibit other retinal disorders, depending on the nature of mutation.

*Corresponding Author, Konark Mukherjee, VTCRI, 2 Riverside Cir., Roanoke, VA 24014, **Fax:** 540-985-3373, **Telephone:** 540-526-2035, konark@vtc.vt.edu.

Conflict of interest

The authors have no conflicts of interest to report.

Keywords

CASK; MICPCH; optic nerve hypoplasia; optic nerve atrophy; retinal dystrophy

1 Introduction

CASK (calcium/calmodulin-activated serine kinase) is an X-linked intellectual disability (XLID) gene that plays a critical role in brain development. *CASK* is important for postnatal survival (Atasoy et al., 2007), therefore many of the loss-of-function (LOF) mutations are found in females who are heterozygous for a given mutation (Burglen et al., 2012). Boys have a single *CASK* gene, thus mutations in many cases are lethal. *CASK* missense mutations have, however, been documented in boys with variable XLID and autistic traits (Hackett et al., 2010); these mutations are likely to be partial LOF. More severe *CASK* mutations in boys have been associated with epileptic encephalopathies such as Ohtahara syndrome and West syndrome (Michaud et al., 2014; Saitsu et al., 2012). Girls with heterozygous LOF mutations typically exhibit postnatal microcephaly, severe global developmental delays, ponto-cerebellar hypoplasia, motor incoordination, hypotonia, seizures and ophthalmological phenotypes like optic nerve atrophy (ONA) and optic nerve hypoplasia (ONH) (Burglen et al., 2012; Moog et al., 2011). The brain phenotypes seen in humans can be faithfully replicated in female mice by deleting a single allele of the *CASK* gene, indicating that *CASK* has a highly conserved role in mammals (Liang et al., 2017; Srivastava et al., 2016).

The retinal and optic nerve pathology associated with *CASK* distinguishes it from other causes of postnatal microcephaly and pontocerebellar hypoplasia-related intellectual disabilities. The molecular mechanism by which *CASK* mutation produces microcephaly, hindbrain hypoplasia and ophthalmic phenotype is not yet known. Recently we have demonstrated that heterozygous deletion of *CASK* produces postnatal ONH and retinal ganglion cell (RGC) loss without affecting overall retinal lamination or pronounced retinal cell loss, even though *CASK* is ubiquitously expressed. ONH is a heterozygous group of disorders characterized by loss of RGCs and their axons. Electroretinogram (ERG) recordings from ONH subjects suggest, however, that in some cases of ONH, additional retinal dysfunction may be present (Chaplin, Borchert, Fink, Garcia-Filion, & McCulloch, 2009; Cibis & Fitzgerald, 1994). Indeed many instances of bilateral ONH (~65%) are also associated with other brain malformations (Ryabets-Lienhard, Stewart, Borchert, & Geffner, 2016). It is not clear, at present, if *CASK* plays a role in the development of non-RGC retinal cells (Liang et al., 2017).

CASK's gene product (also called CASK) is a multi-domain scaffolding molecule belonging to the membrane-associated guanylate kinase (MAGUK) group of proteins (Hata, Butz, & Sudhof, 1996). CASK has a unique CaMK domain at its N-terminus which is followed by two L27 domains, a PDZ motif, an SH3 domain and a guanylate kinase domain (most likely enzymatically inactive). CASK interacts with neurexin via its PDZ domain and phosphorylates neurexin with its CaMK domain in a divalent ion-sensitive manner (Mukherjee, Sharma, Jahn, Wahl, & Sudhof, 2010; Mukherjee et al., 2008; Pak et al., 2015).

Furthermore CASK links neurexin with other molecules such as Mint1 (Butz, Okamoto, & Sudhof, 1998), liprin- α (LaConte et al., 2016), and Caskin1 (Tabuchi, Biederer, Butz, & Sudhof, 2002), all of which interact with CASK via its CaMK domain. To identify the specific molecular defects in CASK that affect proper brain growth, we have begun to analyze CASK missense mutations that are associated with microcephaly and hindbrain hypoplasia. We recently identified two novel missense mutations in CASK that disrupt the CASK-neurexin interaction and are associated with microcephaly and hind brain hypoplasia but do not exhibit optic nerve anomaly (LaConte et al., 2018). In the current work, we describe a missense mutation (L209P) that disrupts protein interactions mediated by CASK's CaMK domain without directly affecting the CASK-neurexin interaction. Strikingly, the L209P mutation also has a strong tendency to destabilize CASK globally. The subject displays microcephaly and cerebellar hypoplasia, as well as bilateral retinal dystrophy. Genotype-phenotype correlation of the three missense mutations reported by us suggest that the N-terminal interactions of CASK are likely to play a unique role in retinal function.

2 Materials and Methods

2.1 Ethical statement

The Virginia Tech Institutional Review Board approved the collection and use of data from the subject. Informed consent was obtained from the family prior to participation.

2.2 Exome sequencing

Exome sequencing was done at GeneDx (Gaithersburg, MD). Briefly, the exonic and flanking splice junctions were sequenced using NextGen sequencing on an Illumina system. Alignment was done against GRCH37/UCSC hg19, and analysis was done for variants. Putative variants were confirmed using capillary sequencing.

2.3 Molecular dynamics simulations and analysis

Molecular dynamics (MD) simulations were performed using the program GROMACS 2018 (Abraham et al. 2015), with a crystal structure of CASK's CaMK domain as the starting structure (3C0H.pdb). The crystal structure was solved with 5'-AMP in the binding site (Mukherjee et al., 2008). To include 5'-AMP in the MD runs, ACPYPE was used to generate a GROMACS topology file for 5'-AMP (Sousa da Silva & Vranken, 2012). The UCSF Chimera software package (Pettersen et al., 2004) was used for molecular visualization, editing, and analysis. Chimera's Rotamers tool was used to replace the native leucine residue in the 3C0H structure at the position analogous to 209 with a proline to generate a model for CASK^{L209P}. The proline rotamer from the Dunbrack backbone-dependent rotamer library with the highest probability was chosen. The AMBER99SB-ILDN force field (Lindorff-Larsen et al., 2010) was used for all simulations. Both structures were solvated with a three-point water model (SPC) in an explicit rhombic dodecahedron water box (solute box distance of 1.0 nm) under periodic boundary conditions. Starting structures were energy minimized until convergence at $F_{\text{max}} < 1000$ kJ/mol/nm. A 100 ps position-restrained NVT equilibration simulation was run for water relaxation at 300K using a modified Berendsen (velocity rescaling) thermostat, followed by a 100 ps NPT equilibration simulation with

Berendsen pressure coupling. After equilibration, an unrestrained 100 ns NPT molecular dynamics simulation was run. Three trajectories initiated with different random seeds were run for each protein structure.

Trajectories were post-processed to correct for periodicity. Analysis of trajectories included calculation of root mean square deviation (RMSD) of the protein backbone from the starting structure at each trajectory time point using the GROMACS rms command and calculation of the radius of gyration at each trajectory time point using the GROMACS gyrate command. The root mean square fluctuation (RMSF) of all alpha carbons from each of the three trajectories was calculated using the rmsf command in GROMACS. B-factors for each residue were calculated from the RMSF values using the equation: $B\text{-factor} = (8\pi^2/3) \times (\text{RMSF})^2$. B-factors from all three trajectories were averaged. Cluster analysis of the three concatenated molecular dynamics trajectories (excluding the first 5 ns of each trajectory) for the wild-type and L209P CAMK domain structures was performed using the GROMACS cluster command with a 1.5 Å cutoff employing the gromos algorithm (Daura et al., 1999).

2.4 Plasmid and Point Mutagenesis

CASK was cloned into pZAC2.1 plasmid. Using Phusion® Polymerase (NEB), the point mutation L209P was generated in the human CASK gene by site-directed mutagenesis and sequenced in the Genomics Sequencing Center in the Biocomplexity Institute at Virginia Tech.

2.5 Cell Culture and Imaging

Human embryonic kidney (HEK-293) cells (ATCC) were plated on 50 µg/ml poly-L-lysine (Sigma Aldrich Inc.)-coated coverslips (Fisherbrand, Inc.) in 24-well plates (JetBiofil) and maintained in DMEM (Hyclone) containing 10% fetal bovine serum (Hyclone) supplemented with 5 mg/ml penicillin-streptomycin (Hyclone). Cells at 80% confluency were transfected with 0.5 µg of GFP-CASK-WT and GFP-CASK^{L209P} DNA per well using the calcium phosphate method. Twenty hours post-transfection, cells were washed twice with phosphate buffered saline (Sigma Inc.) and fixed for 15 minutes at room temperature using a 4% paraformaldehyde solution. Coverslips were mounted on microscope slides (Premiere) using Vectashield (Vector Laboratories Inc.) and visualized using confocal laser scanning microscopy (ZEISS Axio Examiner.Z1 LSM 710). The percentage of transfected cells with visible aggregates was counted using the cell counter plugin of the Image J program. For each condition, five high-power field images were analyzed for aggregation. Total cells and cells containing aggregates were visually identified and tallied. Percent of total cells containing aggregates was then calculated. The image analysis procedure was repeated 3 times for each condition and averaged.

2.6 Neurexin-mediated cell recruitment assay

Human embryonic kidney (HEK-293) cells (ATCC) were plated on 50 µg/ml poly-L-lysine (Sigma Aldrich Inc.)-coated coverslips (Fisherbrand, Inc.) in 24-well plates (JetBiofil) and maintained in DMEM (Hyclone) containing 10% fetal bovine serum (Hyclone) supplemented with 5 mg/ml penicillin-streptomycin (Hyclone). Cells at 80% confluency were co-transfected with 0.5 µg of FLAG-tagged neurexin1-β and GFP-CASK-WT or GFP-

CASK^{L209P} DNA per well using the calcium phosphate method. Twenty hours post-transfection, cells were washed twice with phosphate buffered saline (Sigma Inc.) and fixed for 15 minutes at room temperature using a 4% paraformaldehyde solution. Cells were permeabilized using PBS with 0.01% Triton-X 100 solution and blocked using 5% fetal bovine serum. Cells were immunostained using monoclonal anti-FLAG antibody (1:100) followed by Alexa-633(1:250) anti-mouse antibody. Mounting and imaging procedures are described above.

2.7 Pull-down assay using NxCT and GST beads

Human embryonic kidney (HEK-293) cells (ATCC) were plated in 6-well plates (JetBiofil) and maintained in DMEM (Hyclone) containing 10% fetal bovine serum (Hyclone) supplemented with 5 mg/ml penicillin-streptomycin (Hyclone). Cells at 80% confluency were transfected with 10 µg of EGFP-N1, GFP-CASK-WT or GFP-CASK^{L209P} DNA per well using the calcium phosphate method. Two days later, cells were harvested and solubilized in PBS containing 1% Triton X-100, 2mM EDTA, 2mM DTT and protease inhibitors (0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin, 0.1 mg/ml pepstatin, and 0.01 mg/ml PMSF; all purchased from Fisher Scientific). The lysates were incubated by rocking at 4°C for 30min and then centrifuged for 30 min. at 15,000 rpm at 4°C to collect the supernatants. The pre-cleared supernatants were incubated with 30ul of GST or NxCT-GST beads and incubated on a rocker for 2hr at 4°C. The beads were washed three times with PBS containing 1% Triton X 100 buffer. 30 µl of 2× SDS sample buffer was added to the beads and sample was boiled at 100°C for 10min. Protein samples were separated using SDS-PAGE and immunoblotted for CASK as described below.

2.8 Pull-down assay using GFP-Trap beads

HEK293 cells maintained in a 100mm dish were transfected using the calcium phosphate method with 10ug EGFP-N1, GFP-CASK-WT or GFP-CASK^{L209P} DNA per well. Media was changed 6 hrs post-transfection, and cells were incubated at 37°C, 5% CO₂ for 48 hr. All the following steps were performed at 4°C or on ice. Cells were harvested in PBS and solubilized in RIPA buffer containing PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 2mM EDTA, 1mM EGTA and protease inhibitors (0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin, 0.1 mg/ml pepstatin, and 0.01 mg/ml PMSF; all purchased from Fisher Scientific). Cell lysates were incubated on a rocker for 30min. Supernatants were collected by centrifuging the lysates at 15000rpm for 30min. Frozen rat brain was thawed and homogenized in RIPA buffer and incubated on rocker for 30min. Cell and brain lysates were mixed and incubated overnight in cold room on rocker. GFP-trapTM beads (75 µl) were added to the pre-cleared (incubated with plain agarose beads) cell/brain mixture for each condition and incubated for 1.5 hr at 4°C. The beads were washed three times with PBS containing 1% Triton-X 100. 75ul of 2× SDS sample buffer was added to the beads and boiled (100°C) for 10min. Protein samples were separated using SDS-PAGE and immunoblotted for Mint1, Velis and CASK as described below.

2.10 Immunoblots

Proteins were resolved using SDS-PAGE (sample volumes loaded were based on estimates of appropriate protein concentration), transferred to a nitrocellulose membrane (Fisher), and

immunoblotted for the proteins of interest. HRP-conjugated secondary antibody were used. The blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare Life Sciences) for HRP secondaries. All blots were imaged using a ChemiDoc™ MP System (BioRad).

2.11 Solubility assay

HEK293 cells transfected in 10 cm dish with proteins of interest were solubilized after 2 days in culture using 1 ml of RIPA buffer for 30 minutes. Supernatants were collected by centrifuging the lysates at 15000rpm for 30min and boiled with 200 µl 6× SDS PAGE sample buffer. Pellets were washed once with ice cold RIPA buffer and then boiled in 100 µl sample buffer. 20 µl of pellet and supernatant were used to perform immunoblotting.

3 Results

Here we describe a 9-year-old girl with microcephaly and mild cerebellar hypoplasia with severe intellectual and motor disability (Fig. 1A,B). She was born full term after an uneventful pregnancy. Parents noticed delay in achieving milestones. She rolled over at 6 months, sat at 12 months and walked at 5 years. She is a happy and social child with global developmental delay. She is ataxic, with motor incoordination in both upper and lower extremities which is more pronounced on left side. She has good core stabilization of the trunk with spinal exaggeration in extensor muscles, suggesting some truncal weakness. She exhibits atypical gait pattern and foot drop during walking and running. In addition, she internally rotates her left lower extremity likely due to decreased tone associated with left hip extensors. From a motor function perspective, she appears to be delayed in both gross and fine motor skills, with greater delays in fine motor abilities. Although she exhibits a clear desire to communicate, there is no verbal communication; only occasionally does she respond with appropriate syllable production. She responds to simple commands and demonstrates some self-stimulation behaviors.

In addition to the psychomotor and intellectual delays described above, the subject also has bilateral epicanthus and bilateral retinal dystrophy. The patient was first seen at the institution for an eye examination at 5 years of age. She had a history of myopia and an oculodigital reflex but no nyctalopia or photosensitivity. Paternal family history was positive for myopia and retinal detachment. Examination showed no paradoxical pupillary constriction in the dark or visual field constriction to confrontation and the optic nerves were pink with a scleral crescent, but the retinal arterioles were attenuated. However, the optic nerve looked normally developed at this stage (Fig. 1C). The girl also had ptosis and epicanthus. In view of the oculodigital reflex and arteriolar attenuation, an ERG was performed that showed severe loss of rod greater than cone photoreceptors, indicating a retinal dystrophy (Fig. 1D-E). She developed photosensitivity and difficulty adapting indoors after being outside. The optic nerves showed evidence of mild to moderate atrophy two years later, but the ERG remained relatively stable. At no time did she have nystagmus or strabismus. Examination remained relatively stable at age 9 years except for an increase in her myopia. She was unable to perform optotype acuity testing. Upon exome sequencing a single mutation in the *CASK* gene is observed (NM_003688.3(*CASK*):c.626T>C

(p.Leu209Pro); Variation ID: 432816). The variant was not observed in either parent, indicating that the mutation was *de novo*.

This mutation has been previously reported as a loss-of-function mutation, although the mechanism underlying the pathogenicity is not clear (Cristofoli, Devriendt, Davis, Van Esch, & Vermeesch, 2018). The substituted leucine in CASK's CaMK domain (Fig. 2A) at position 209 is highly conserved in all species surveyed (Fig. 2B), indicating that it may be a critical part of CASK's primary structure. The leucine is situated in the α F helix of the C-terminus lobe of the CaM-kinase domain (Fig. 2D) (Hanks & Hunter, 1995; Mukherjee et al., 2008). None of the CaM-kinases in the human kinome contain a cyclic amino acid at this site. A change in the side chain at this position is thus likely to alter intra-molecular interactions and produce secondary and tertiary structural defects. Molecular dynamics (MD) simulations were done to computationally model the movement of CASK at the atomic level over a period of time at room temperature in an aqueous environment; MD simulations can provide insight into the impact of a mutation on a protein's structure, if an atomic model of the wildtype structure is available (Karplus & Petsko, 1990). When MD simulations were done on the isolated CaM-kinase structure (a previously solved crystal structure; (Mukherjee et al., 2008), an analysis of the simulations indicates that the addition of a proline at this position does not destabilize the overall structure of the isolated domain, as indicated by the nearly identical average radii of gyration ($19.37398\text{\AA} \pm 0.20033$ and $19.5173\text{\AA} \pm 0.254$ for wildtype and CASK^{L209P}, respectively) and root mean square deviation of the backbone relative to the starting structure ($1.57455\text{\AA} \pm 0.31$ and $1.52034\text{\AA} \pm 0.29$ for wildtype and CASK^{L209P}, respectively) over the course of the 100 ns MD trajectories for CASK^{L209P} structure as compared to the wildtype structure (Fig. 2C). MD simulations, by their very nature, yield millions of individual protein conformations, but most of these conformations differ from each other very little. A cluster analysis allows investigators to identify and then examine the most common structure(s) populated during an MD simulation. When the two structures representing the most populated cluster are superimposed using the MatchMaker tool in Chimera, the root mean square deviation between the two structures is $1.252\text{\AA}$; for comparison, structures of identical proteins within the Protein Databank can exhibit RMSDs up to $1.2\text{\AA}$, due to variability arising from inherent protein flexibility as well as the resolution limits of the methods used to determine protein structure (Kufareva & Abagyan, 2012). From the MD trajectories, calculations of the root mean square fluctuation (RMSF) of the alpha-carbons for both wildtype and CASK^{L209P} were done to, in turn, calculate B-factors at each amino acid position within the CAMK domain. B-factors provide a measure of mobility at a given site, and a comparison between B-factor values by sequence position revealed very few dramatic differences throughout the domain due to the L209P mutation, with the exception of increased mobility in two specific regions (Fig. 2D,E), both located in the activation loop of the CAMK domain: residues 166–168 and 174–176. Both the α F helix, where the L209P mutation is located, and the activation loop, which is predicted by the MD simulations to be impacted by the L209P mutation, are considered critical structural components of the core kinase domain (McClendon, Kornev, Gilson, & Taylor, 2014), but these changes do not point, in isolation, to a global unfolding of CASK's CAMK domain due to the L209P residue. These

simulations are done on an isolated domain of CASK, however, making it impossible to rule out the possibility of disruption of native inter-domain contacts.

We have previously published a simple assay to test structurally unstable CASK mutations. This involves transient over-expression of a GFP-CASK fusion protein using a strong cytomegalovirus promoter (CMV) in human embryonic kidney (HEK 293) cells (LaConte et al., 2018; LaConte, Chavan, & Mukherjee, 2014). Typically GFP-CASK can be visualized using a confocal microscope and is evenly distributed throughout the cytoplasm but excluded from the nucleus (Figure 3A). Misfolding of CASK leads to formation of visible “cotton-wool”-like aggregates in the cytoplasm. Upon overexpression of CASK^{L209P}, aggregates are readily observable, indicating that the protein has a tendency to misfold and aggregate (Figure 3B). In cells where large aggregates are not observed (67%) (Figure 3C), we observe a small punctate distribution not previously seen with the other mutations that we have tested. CASK^{L209P} thus may also be producing much smaller aggregates, thereby resulting in an underestimate of the amount of protein aggregation caused by this mutation. We therefore tested the solubility of CASK^{L209P} by transfecting HEK293 cells with GFP, GFP-CASK and GFP-CASK^{L209P} and solubilizing after 2 days in culture using a solubilizing buffer. Both endogenous CASK and GFP-CASK exhibited a high degree of solubility with little protein left in pellet (Figure 3D). CASK^{L209P}, however, was less soluble, with large amounts remaining in pellet. Thus our data indicate that the L209P mutation results in a propensity for CASK to misfold in a disordered manner, reducing the amount of soluble and possibly functional protein present in the cell.

To determine if the small amount of solubilizable CASK^{L209P} retained any functionality, we examined the biochemical competence of CASK^{L209P}. In previous work, we demonstrated that CASK mutations associated with microcephaly disrupt the interaction between CASK and neuroligin. We therefore first tested the interaction between CASK^{L209P} and neuroligin in a cellular milieu using a previously described recruitment assay (LaConte et al., 2018; LaConte et al., 2016). The assay relies on the ability of membrane-bound neuroligin to recruit the cytosolic protein CASK. The recruitment can be easily observed using a confocal microscope in a large, flat cell (with a high cytosolic-to-nuclear ratio) such as HEK293 cells (Figure 4B). Although CASK^{L209P} aggregates in many cells, neuroligin is still able to recruit some CASK^{L209P}, indicating that soluble CASK^{L209P} retains its capacity to interact with neuroligin (Figure 4D). This finding was confirmed using a GST pull-down experiment (Figure 5A). The cytosolic tail of rat neuroligin-1 fused with GST was expressed, purified, and immobilized on glutathione beads. GST-neuroligin cytosolic tail (GST-Nx-CT) beads were used to precipitate CASK (both wild-type and CASK^{L209P}) that was expressed in HEK 293 cells. The results from the pull-down experiment confirm that the soluble population of CASK^{L209P} retains its ability to interact with neuroligin.

CASK is a scaffolding protein involved in an evolutionarily conserved ternary complex with Mint1 and Veli (Butz et al., 1998; Kaech, Whitfield, & Kim, 1998; Mukherjee, Slawson, Christmann, & Griffith, 2014). Veli interacts with CASK via its L27 domain whereas Mint1 interacts with CASK via its CaMK domain. To determine whether CASK^{L209P} is capable of forming this ternary complex, GFP-CASK was expressed in HEK293 cells, and the lysate from these cells was combined with rat brain lysate to allow the exogenous CASK to form

complexes with brain-derived proteins (Figure 5B). GFP-CASK was then immunoprecipitated with GFP antibodies, and immunoprecipitates were blotted for Veli and Mint1 (Figure 5D), revealing that CASK^{L209P} can still bind to Veli, but its interaction with Mint1 is disrupted. The L209P mutation in CASK is thus likely to disrupt its regulatory scaffolding function that links neurexin to molecules like Mint1 (LaConte et al., 2016).

4 Discussion

In this work, we describe a girl with a missense mutation in *CASK* that is associated with microcephaly, mild cerebellar hypoplasia, motor disability, intellectual disability and bilateral retinal dystrophy with optic atrophy. The mutated leucine is present in the C-terminal lobe of the CaMK domain of CASK within the α F helix (Mukherjee et al., 2008). Recently this specific mutation has been putatively shown to be pathogenic in a zebrafish model, although how the mutation affects the protein function remained unclear. Furthermore, the cause of visual impairment in the subject described in that report was not investigated (Cristofoli et al., 2018). We demonstrate that CASK^{L209P} mutation is capable of destabilizing the protein sufficiently to produce misfolding and insolubility in a large proportion of expressed protein when transiently overexpressed. The soluble fraction of CASK^{L209P}, however, retains enough structure to preserve interactions attributed to the domains subsequent to the CaMK domain, such as the L27 and PDZ domains. The evolutionarily conserved interaction of the CaMK domain with Mint1 is completely abolished, indicating that even in soluble CASK^{L209P}, the CaMK domain is not appropriately folded.

The analysis of the molecular dynamics simulations provides some insight into the structural effects of the L209P mutation. As seen in Figure 2D, the structure that represents the most populated cluster in the MD simulation of a CASK CAMK domain model with the L209P mutation suggests that there is no profound difference in CASK's tertiary structure induced by this substitution. The mutation is located in a helix that has been identified as a key component of the structural core of all kinases, the α F helix (Roskoski, 2012). The α F helix (Fig. 2D, blue) serves as the base for two structural motifs that create a hydrophobic core within eukaryotic protein kinases responsible for allosteric signal propagation: the R (regulatory) spine (Fig. 2D, red) that has been implicated in regulation of kinase activity and the C (catalytic) spine (Fig. 2D, yellow), which is involved in maintaining proper kinase structure for catalysis and relies upon the presence of ATP (Kornev, Taylor, & Ten Eyck, 2008). Notably, introduction of proline at position 209 in CASK does not alter the packing of the α F helix, and the positioning of the residues of the R spine is largely unimpacted by the mutation, with CASK's CAMK domain still exhibiting the same "DFG-in" orientation observed in the original crystal structure; the DFG motif (in CASK, GFG), located at the beginning of the activation segment, exerts some control over whether a kinase is in an active conformation with an extended activation segment (DFG-in) or an inactive conformation, with the activation segment collapsed onto the surface of the kinase (Taylor, Keshwani, Steichen, & Kornev, 2012).

All of the residues in the α F helix have been shown to be involved in a conserved spatial pattern, and Kornev et al. suggest that the residue at the position equivalent to 209 in CASK

plays a critical role in the anchoring of the α H helix (Fig. 2D, pink), which, in turn, is purported to help stabilize the hydrophobic core around the α F helix. Replacing the native leucine at this position with a proline is unlikely to exert a destabilizing pressure because both amino acids are hydrophobic; the native leucine forms stabilizing contacts with residues in the α H helix (Kornev et al., 2008) which may be weakened by the spatially smaller proline, but no distinct difference in mobility or orientation of the α H helix are noted in the CASK^{L209P} structure most populated during the MD simulations. The L209P mutation does induce increased mobility at two locations, as indicated by differences in B-factors calculated across the relevant MD trajectories (Fig. 2E). Both of these hyper-mobile regions (Fig. 2D, orange) are within the activation segment (Fig. 2D, green), the portion of the kinase structure described above considered to be responsible for shifting the kinase domain between an open and closed conformation (not always correlated with active and inactive kinase states) (Meharena et al., 2013). The implications of this increased mobility in the activation segment as relates to CASK's kinase activity are difficult to predict, given that CASK is inhibited by divalent ions, but the activation segment is also purported to be important for protein-protein interactions in some kinases (Hu, Liu, Ghirlando, Saltiel, & Hubbard, 2003). CASK is a multi-domain kinase (Fig. 2A), and although various domains of CASK have been crystallized in isolation, the overall tertiary structure of CASK is unknown, and we lack a thorough understanding of how the domains interact with each other and how these interactions are regulated. Based on our MD simulations, it is not possible to precisely predict the impact of the L209P mutation, but it is reasonable to speculate that the mobility induced in the activation segment of the CaMK domain by the L209P mutation disrupts either inter-domain interactions or CASK's interactions with one of its many binding partners. CASK^{L209P} could thus produce phenotypes via two different mechanisms: 1) predisposing the mutant protein to a gross misfolding due to disrupted inter-domain interactions, thus reducing the available functional protein, and/or 2) abolishing key interactions of the CaMK domain with other protein binding partners.

We have previously published two separate heterozygous *CASK* missense mutations that are associated with microcephaly in which the CASK-neurexin interaction is disrupted but N-terminal CaMK interactions are maintained. None of those subjects displayed obvious optic nerve or retinal defects. It may be possible that the CaMK domain-mediated interactions play a distinct role in the retina. It may also be possible that CASK^{L209P} produces a pathology due to misfolding or a gain of function effect. Presence of retinal degeneration lends credibility to such gain of function of CASK^{L209P}. A simpler explanation might be that the missense mutations that we have described fall on a spectrum of partial loss of function. Relatively preserved function produces microcephaly without any retinal or optic nerve defect, complete loss of function produces ONH, and mutations with functional loss in between the two produces a retinal defect with a delayed onset and thus appears as degenerative changes in childhood. We have shown that *CASK* haploinsufficiency in mice produces ONH with a reduction in the number of retinal ganglion cells but no change in retinal lamination nor a reduction in the thickness of the photoreceptor cell layer (Liang et al., 2017). Does *CASK* play a developmental role in the retina outside of the retinal ganglion cells? Based on the findings we present here, this idea needs to be further tested in the rodent model.

We have also made another striking observation regarding ocular phenotypes. Previously it has been reported that C-terminal hemizygous mutations of *CASK* specifically produced nystagmus. The phenotype has been attributed to the interaction of *CASK* via its C-terminus with a molecule called FRMD7 (Watkins et al., 2013). However, we have now described two independent heterozygous mutations (M519T in the middle of the protein (LaConte et al., 2018) and L209P at the N-terminus of the protein) that are associated with nystagmus. Importantly, two subjects have been reported for each of these mutations, and for both M519T and L209P, one subject exhibits nystagmus and the other does not. Thus even for the same *CASK* mutation, nystagmus seems to be an incompletely penetrant phenotype. Although ONH itself is often associated with congenital nystagmus (Lohmuller, Gangloff, Wenzel, & Lagreze, 2017), this relationship does not seem to hold for *CASK* mutations. Because mutations in *CASK* produce cerebellar hypoplasia, the nystagmus could be of a central vestibular nature.

Although the profound cognitive and motor impairment in subjects with *CASK* mutations makes vision assessment a challenge, our current findings support the idea that children with *CASK* mutations may have ERG abnormalities and that *CASK* plays a critical role in optic nerve and retinal function.

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Figure 1. Clinical findings for subject with CASK^{L209P} mutation.

A) Subject's clinical presentation. B) T2-weighted MRI brain scans from subject displaying small cerebellum (white arrow) and C) optic nerve (white arrow) at age 5 years. D) Wide field images of the retina of the right (left panel) and left (right panel) eyes show optic atrophy and arteriolar attenuation but no significant pigment migration at age 7 years. E) Results from full-field electroretinography examination. The scotopic maximal response, representing both cones and rods, is electronegative and is moderately reduced in right (top)

and left (bottom) eyes at 5 years. The dashed lines indicate the normal amplitude of trough and crest.

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Figure 2. Impact of L209P mutation on CASK structure.

A) Schematic of the domains of CASK, including locations of known interactions with other proteins, and the site of the L209P mutation. B) Conservation of the mutated site in various animal species. C) Box and whisker plots of the radius of gyration and root mean square deviation (RMSD) of the backbone of the CAMK domain of CASK or CASK^{L209P} over the course of three molecular dynamics (MD) trajectories, respectively. D) Structure of CASK's CAMK domain, modeled from a crystal structure (3C0H.pdb), with a proline substituted for leucine at position 209 (red) in the structurally important α F helix (blue). Other structurally critical regions of the core protein kinase catalytic domain are also shown: α H helix (pink),

R-spine (red space-filling residues), and C-spine (yellow space-filling residues). The activation segment is shown in green, with regions of increased mobility due to the L209P mutation as predicted by MD simulations shown in orange. E) Average B-factors ($\pm$ SEM) for the two regions (residues 166–168 and 174–176; shown in orange in panel D) within the activation segment (green, panel D) of CASK's CAMK domain that demonstrated notable differences between CASK and CASK^{L209P}.

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Figure 3. Properties of GFP-CASK^{L209P} expressed in cell culture.

A,B) Representative images of wildtype CASK and CASK^{L209P} GFP fusion protein expressed in HEK293 cells. Note the cotton-wool aggregates of CASK^{L209P} evident in some cells. Scale bar= 5µm. C) Quantitation of number of cells displaying large aggregations. Results represent mean and SEM from 6 different experiments. A total of ~1000 cells were examined in each sample. D) Blot for CASK in insoluble and soluble fractions of cell lysate. CASK antibody detects both expressed GFP-CASK and endogenous CASK present in HEK cells. Note that CASK^{L209P} is predominantly present in the insoluble fraction.

Figure 4. Neurexin-mediated recruitment assay in HEK293 cells.

Confocal images of HEK 293 cells transfected with A) GFP-CASK alone, B) GFP-CASK with FLAG-tagged neurexin1-β, C) GFP-CASK^{L209P} alone, and D) GFP-CASK^{L209P} with FLAG-tagged neurexin1-β. Scale bar= 5μm

Figure 5. Pull-down assays to evaluate interactions of CASK^{L209P} with other proteins.
 A) Schematic of GST pull-down from transfected HEK293 cells. B) Schematic of immunoprecipitation assay using GFP antibody. C) Immunoblot of CASK from HEK 293 cells. Endogenous CASK can be seen in GFP sample. D) Blots of indicated antigen from immunoprecipitation assay.