Functional studies of a missense mutation in the $CRADD$ gene in Mennonite patients with
autosomal recessive non-syndromic mental retardation
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I. Abstract

The Clinic for Special Children recently associated a novel variant (c.382G>C) in *CRADD* with autosomal recessive non-syndromic mental retardation in Mennonite patients. The *CRADD* gene, encodes a death-adaptor protein with a caspase recruitment domain (CARD) and a death domain (DD). CRADD interacts with caspase-2, a cysteine protease, and PIDD, a p53-induced protein with a DD, to form the PIDDosome. CRADD and PIDD interact within the PIDDosome to recruit and activate caspase-2, initiating apoptosis (Berube et al., 2005). Previous work has shown that a single amino acid residue change on an interface of CRADD DD and PIDD DD interaction inhibits formation of the PIDDosome (Jang et al., 2010). The c.382G>C variant is predicted to change the highly conserved Gly<sup>128</sup> to Arg<sup>128</sup> in the CRADD DD. If the Gly<sub>128</sub>Arg variant alters interaction between the DDs of CRADD and PIDD, then formation of the PIDDosome may be disrupted. Mouse (m) Cradd Gly<sub>128</sub>Arg in mIMCD3 cells and human (h) CRADD Gly<sub>128</sub>Arg in hARPE-19 cells formed clusters when co-overexpressed with mPidd or hPIDD, which were not observed in wild-type (wt) overexpression, suggesting a change in CRADD and PIDD interaction. Semi-quantitative western blotting demonstrated that overexpressed hCRADD Gly<sub>128</sub>Arg was 82.9 ± 2.3% (n=3) less abundant than overexpressed wt hCRADD, suggesting that hCRADD Gly<sub>128</sub>Arg may be unstable or that cells containing the Gly<sub>128</sub>Arg variant undergo apoptosis more readily. Mouse Cradd DD Gly<sub>128</sub>Arg did not co-immunoprecipitate mPidd DD, suggesting that the Gly<sub>128</sub>Arg variant may alter one of the interaction interfaces of CRADD DD to increase CRADD DD homo-oligomerization and decrease CRADD DD’s affinity for PIDD DD. Disruption of the PIDDosome and subsequent neurotrophin-mediated apoptosis in the developing central nervous system may underlie the cognitive impairment in *CRADD* c.382G>C patients.
II. Introduction

Clinical Phenotype

The Clinic for Special Children (CSC) diagnosed six related Mennonite patients with non-syndromic mental retardation (MR), characterized by mild cognitive impairment and delayed developmental milestones. With syndromic mental retardation, affected individuals display the MR phenotype in addition to other phenotypes outside of the nervous system. Patients with non-syndromic MR, inherited as an autosomal recessive disorder in 25% of cases (Philippe et al., 2009), do not show associated pathology outside of the MR phenotype. Mild cognitive impairment is classified based on an IQ range from 50-70 (Philippe et al., 2009) and precludes the patients’ ability to live independently (Philippe et al., 2009; Puffenberger et al., 2012). The affected Mennonites have mild speech and language impairment as part of the MR phenotype, but are able to communicate effectively (Puffenberger et al., 2012). Although MR affects 2-3% of the general population, the biological and genetic causes of MR remain relatively unknown, likely as a result of the phenotypic heterogeneity of the disorder (Philippe et al., 2009), typically rendering genetic mapping unsuccessful at the general population level. The Mennonite patients provide an opportunity to investigate one of the potential genetic causes underlying non-syndromic mental retardation because the affected individuals are within the same sibship and display nearly identical clinical phenotypes, which is beneficial to homozygosity mapping and candidate disease gene identification.

Candidate Disease Gene Mapping

Mennonites are of Swiss Anabaptist descent and immigrated to America during the 18th century, carrying a limited subset of alleles derived from their Western European ancestors and further divided into settlements of endogamous demes (Puffenberger, 2003; Strauss and
As a result of the founder effect and decreased gene flow due to endogamous population settlements, Mennonites experience an increased incidence of recessive alleles and therefore a higher incidence of inherited recessive disorders (McKusick et al., 1964). Settlement in endogamous demes allows for a greater sample size of affected individuals with inherited genetic disorders than in the general population, increasing the ease of mapping studies to identify candidate disease genes (McKusick et al., 1964). The CSC can use autozygosity mapping to identify homozygous haplotype blocks inherited through identity-by-descent in affected individuals relative to unaffected siblings, within which a single pathogenic variant associated with a recessively inherited disorder is likely to lie (Puffenberger et al., 2012) (Figure 1).

Autozygosity mapping was used to identify homozygous haplotype blocks shared among the six Mennonite patients with non-syndromic MR in comparison to unaffected family members. Analysis of the patients' genotypes using an Affymetrix 10K SNP microarray revealed a 3.6 Mb homozygous region, containing 46 genes, on chromosome 12 (12q22) unique to affected individuals relative to unaffected family members (Figure 2). Of the 46 genes within the homozygous haplotype, none were clearly linked to the non-syndromic MR phenotype, rendering Sanger sequencing insufficient for candidate disease gene identification.

DNA samples from five of the affected individuals were sent to The Broad Institute (Boston, Massachusetts) for exome sequencing (Puffenberger et al., 2012), which accelerates the identification of candidate disease genes by sequencing the coding region of an affected individual's genome (Gnirke et al., 2009) to determine variants common among affected individuals in comparison to the general population. Exome sequencing identified nine variants among affected individuals within the 3.6 Mb mapped interval, seven of which were identified
by dbSNP129 and the 1000 Genomes Project (Puffenberger \textit{et al.}, 2012) as known single nucleotide polymorphisms (SNPs) in the general population and have not been associated with disease. The two novel homozygous missense mutations were \textit{EEAI} (c.2882T>C) and \textit{CRADD} (c.382G>C) (Puffenberger \textit{et al.}, 2012). \textit{EEAI} c.2882T>C results in a codon change from ATA to ACA, predicted to substitute Thr$^{961}$ for Ile$^{961}$. Based upon multiple sequence alignments, Ile$^{961}$ is not highly conserved and Thr$^{961}$ is present in a variety of species, suggesting that the variant likely does not negatively affect \textit{EEAI} structure or function. The c.382G>C mutation in \textit{CRADD} changes the codon GGC to CGC, which is predicted to change Gly$^{128}$ to Arg$^{128}$ (Gly128Arg). The amino acid substitution is predicted to be “probably damaging” by the PolyPhen2 database, a score based on sequence homology of conserved functional domains, conservation of the affected residue among multiple sequence alignments (Figure 3), and amino acid structure between the wild-type and disease-associated residue (Adzhubei \textit{et al.}, 2010). The score is also based on a comparison of the affected residue against databases of known disease causing mutations (HumanDiv and HumanVar) and impacts on protein structure and function (Adzhubei \textit{et al.}, 2010). Therefore, evidence suggests that the highly conserved Gly$^{128}$ (Figure 3) likely plays a critical structural or functional role in wild-type \textit{CRADD}.

\textbf{CRADD and the PIDDosome}

The caspase-2 and RIPK1 domain-containing gene with a death domain (\textit{CRADD}), also known as \textit{RAIDD}, is a receptor interacting (RIP) adaptor protein containing a C-terminal death domain (DD) and an N-terminal caspase recruitment domain (CARD) (Duan and Dixit, 1997; Ahmad \textit{et al.}, 1997). \textit{CRADD} interacts with caspase-2 (a cysteine protease) (Duan and Dixit, 1997; Ahmad \textit{et al.}, 1997) and the DD of PIDD (a p53-induced DD protein) to form the PIDDosome. The DDs of \textit{CRADD} and PIDD interact following autoproteolytic cleavage of full-
length PIDD into a 37-kDa fragment, PIDD-CC, which contains the DD and is preferentially co-immunoprecipitated by CRADD (Tinel et al. 2007). Following interaction between CRADD DD and PIDD DD, caspase-2 is recruited to the CARD of CRADD to form the PIDDosome (Tinel et al., 2007; Park et al., 2007). Activated caspase-2 acts as an initiator caspase (Guo et al., 2002; Tinel and Tschopp, 2004; Tinel et al., 2007) upstream of the mitochondrial apoptotic pathway to induce Bid cleavage and subsequent cytochrome c release from the mitochondria (Stefanis et al., 1998; Guo et al., 2002; Baliga and Kumar, 2003). Cytochrome c release triggers the activation of downstream effector caspases, including caspase-3 and caspase-7 (Guo et al., 2002; Berube et al., 2005). Overexpression of either CRADD or PIDD induces apoptosis through the PIDDosome/caspase-2-mediated pathway (Duan and Dixit, 1997; Ahmad et al., 1997; Tinel and Tschopp, 2004). Caspase-2 processing does not occur when small interfering RNA (siRNA) inhibits endogenous CRADD (Tinel and Tschopp, 2004), suggesting that PIDDosome-mediated activation of caspase-2 depends on functional CRADD.

Park et al. (2007) characterized the crystal structure of the PIDDosome, consisting of interactions between seven CRADD DD and five PIDD DD, allowing for seven caspase-2 CARDs to associate with the seven CARDs of CRADD (Figure 4). Each DD has the ability to associate with six other DDs through interactions between amino acid residues within structurally similar helical regions: three CRADD DD:CRADD DD (R:R), three CRADD DD:PIDD DD (R:P), and two PIDD DD:PIDD DD (P:P) interactions (Figure 5A) (Park et al., 2007). Careful inspection by amino acid sequence alignment demonstrated that CRADD Gly128 aligns with PIDD Gly800 within a highly nonpolar region (between Leu127 and Pro129) associated with CRADD:CRADD, CRADD:PIDD, and PIDD:PIDD interactions (Figure 5A) (Park et al., 2007). The Gly128Arg variant in CRADD is predicted to replace the nonpolar side chain of
Gly$^{128}$ with the large, polar, positively charged side chain of Arg$^{128}$. The change from a nonpolar side chain to a polar, positively charged side chain as a result of the Gly128Arg variant may alter the neighboring nonpolar interactions of residues involved in DD interfaces. Interaction interfaces are highly conserved within each DD, suggesting that the residues involved are critical for oligomerization of CRADD DD and PIDD DD (Park et al., 2007). Therefore, based upon the work of Park et al. (2007), if CRADD Gly128Arg alters one of the DD residue interfaces necessary for CRADD:CRADD or CRADD:PIDD interactions, then PIDDosome formation and subsequent caspase-2 activation may be disrupted.

Given the nature and position of the Gly128Arg variant in the CRADD DD, the likelihood of disrupted interaction with PIDD DD is sufficiently strong. The focus of this study is on potential disruption of the PIDDosome as a result of the variant, however, subsequent work will focus on additional CRADD-associated pathways. CRADD DD interacts with RIPK1 (receptor TNFRSF-interaction serine threonine kinase 1) DD to associate CRADD with the tumor necrosis factor receptor 1 (TNFR1) pathway for apoptotic signaling (Duan and Dixit, 1997; Thakar et al., 2006; Guicciardi and Gores, 2009). The interaction between RIPK1 DD and CRADD DD may activate caspase-2 apoptosis via the TNFR1 signaling pathway (Shearwin-Whyatt et al., 2000; Berube et al., 2005; Thakar et al., 2006). The Gly128Arg variant may alter the DD interaction and disrupt TNFR1-mediated apoptosis. CRADD has also been identified as a component of the CARMA1 signalosome, involved in immunoregulatory functions through CARD interaction with BCL10 (Lin et al., 2010) to inhibit inflammatory cytokines. Although the DD is not involved (Lin et al., 2010), the Gly128Arg variant may indirectly affect CARD association with BCL10, disrupting the CARMA1 signalosome pathway.
Caspase-2 is activated through the PIDDosome in response to a variety of cellular apoptotic signals, including genotoxic stress leading to activation of effector caspase-3 (Tinel and Tschopp, 2004), cytoskeletal disruption leading to Apaf-1/caspase-9 activation (Ho et al., 2008), and DNA damage repair through p53-induced apoptosis (Baptiste-Okoh et al., 2008). In sympathetic neurons and PC-12 cells, CRADD/PIDDosome-mediated caspase-2 activation is required for apoptosis following neurotrophic factor withdrawal (Stefanis et al., 1996; Troy et al., 1997; Wang et al., 2006), suggesting a role for caspase-2-mediated apoptosis in the nervous system. Overexpression of CRADD has been shown to increase cell death through PIDDosome-mediated caspase-2 activation after neurotrophic factor withdrawal in sympathetic neurons (Jabado et al., 2004) and in non-neuronal cells (Guo et al., 2002). Disruptions in each of the three components of the PIDDosome have been shown to alter PIDDosome formation or subsequent PIDDosome/caspase-2 mediated apoptosis (Tinel and Tschopp, 2004; Berube et al., 2005; Baptiste-Okoh et al., 2008). Although alternate pathways of caspase-2 activation exist (Manzl et al., 2005; Thakar et al., 2006; Baptiste-Okoh et al., 2008; Ribe et al., 2012), CRADD and its' interactions within the PIDDosome play a critical role in at least one of the potential caspase-2 activating mechanisms that initiate apoptosis.

Based upon the extensive characterization of the crystal structure of the PIDDosome, the role of CRADD in capase-2 mediated apoptosis in neuronal cell death, and the highly conserved Gly\textsuperscript{128} in the region of CRADD DD known to interact with PIDD DD (Park et al., 2007), PIDDosome/caspase-2-mediated apoptosis is a likely mechanism to be associated with CRADD c.382G>C and the non-syndromic MR phenotype. Jang et al. (2010) introduced a point mutation in CRADD corresponding to an amino acid substitution at Arg\textsuperscript{147}, a residue involved in CRADD DD:CRADD DD and CRADD DD:PIDD DD interaction. The mutation substitutes Glu\textsuperscript{147} for
Arg^{147} (Arg147Glu) (Jang et al., 2010). Overexpression of CRADD DD Arg147Glu inhibited formation of the PIDDosome (Jang et al., 2010), suggesting that the alteration of a single amino acid residue involved in one of the interaction interfaces in the CRADD DD is sufficient to prevent oligomerization between CRADD DD and PIDD DD. If the Gly128Arg variant in Mennonite patients directly or indirectly alters one the interaction surface residues within the CRADD DD required for CRADD:CRADD or CRADD:PIDD interaction, then PIDDosome assembly and downstream caspase-2-mediated apoptosis may be disrupted.

**CRADD and caspase-2-mediated apoptosis in the developing central nervous system**

During embryonic development, mouse (m) *Cradd* is expressed in a time and tissue specific manner in the nervous system, notably within the forebrain (Motaln et al., 2005). Similarly, *caspase-2* is upregulated in embryonic mouse brain tissues during critical periods of neurogenesis and in post-mitotic neurons (Kumar et al., 1994). Collectively, the established role of PIDDosome/caspase-2-mediated apoptosis in neurotrophin-mediated apoptosis and upregulation of *Cradd/caspase-2* expression in the developing central nervous system, suggests that PIDDosome/caspase-2-mediated apoptosis may be involved in neuronal connectivity and patterning.

During central nervous system development, apoptosis is essential to the establishment of appropriate neuronal circuitry (Vanderhaeghen and Cheng, 2010), precise refinement of synaptic connectivity (Low et al., 2006), and the differentiation of immature neurons (Vanderhaeghen and Cheng, 2010). Caspase-2-mediated apoptosis has been associated with apoptosis in Aβ induced cell death in hippocampal neurons (Ribe et al., 2012), in post-mitotic mouse cortical neurons following mitochondrial oxidative stress to activate caspase-3 and capase-9 (Apaf-1 apoptosome) (Tiwari et al., 2011), and correlates temporally with caspase-3 activation following neurotrophic
factor withdrawal in sympathetic neurons (Stefanis et al., 1998). A downstream effector of caspase-2, caspase-3, is required for neuronal cell body death and effector caspase-6 is required for axon retraction during synaptic refinement (Nikolaev et al., 2009), illustrating the role of caspase-mediated apoptosis within the nervous system. Within the nervous system, parallel and compensatory pathways of caspase activation exist because in sympathetic neurons lacking caspase-2, caspase-9 is upregulated and activates apoptosis through the Apaf-1 apoptosome (Troy et al., 2001).

Activity dependent competition underlies the refinement of immature synaptic connections during development to eliminate 50% of neurons prior to adulthood (Vanderhaeghen and Cheng, 2010). Appropriate synaptic connections are strengthened through neurotrophic factor uptake (Cohen-Cory, 2002) and redundant or inappropriate connections are eliminated from neuronal circuitry (Kakizawa et al., 2000) through apoptosis (Troy et al., 2001) or neurite retraction (Rumpf et al., 2011). Changes in normal neuronal apoptosis and synaptic refinement have been implicated in neurodevelopmental and neurodegenerative disorders (Cohen, 1997; Micali et al., 2010), including MR (Engidawork et al., 2001; Philippe et al., 2009; Tessier and Broadie, 2009). Disruption of the PIDDosome and subsequent PIDDosome-mediated apoptosis through caspase-2 during activity dependent refinement may underlie the non-syndromic mental retardation observed in CRADD patients.

To determine the potential effects of CRADD Gly128Arg on subcellular localization, protein expression, and interaction with PIDD, I overexpressed fusion protein constructs in cell culture for use in immunofluorescence microscopy, western blotting, and co-immunoprecipitation. CRADD Gly128Arg formed clusters in the presence of PIDD in mammalian cell culture, displayed decreased abundance in cells, and disrupted interaction with
PIDD DD. Results suggest that CRADD Gly128Arg may be targeted for degradation due to instability and act to increase CRADD DD homo-oligomerization and decrease CRADD DD affinity for PIDD.

III. Methods

Animals and cell culture

This research was approved by the Franklin & Marshall College Institutional Animal Care and Use Committee. Wild-type C57BL6 mice were used for extraction of RNA from tissues. Mouse inner-medullary collecting duct (mIMCD3) cells (ATCC, Manassas, VA) and human adherent retinal pigmented epithelium (hARPE-19) cells (ATCC) were maintained at 37°C and 5% CO₂ in DMEM:F12 (1:1) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies).

Cloning

Total RNA was extracted from mouse tissue and from human adherent retinal pigmented epithelium (hARPE-19) cells (ATCC) using Trizol (Life Technologies). Reverse transcription of total RNA with SuperScript II reverse transcriptase (Life Technologies) was used to synthesize first strand complimentary DNA (cDNA) using oligo (dT) primers. Wild-type (wt) mouse (m) Cradd full-length (FL) coding sequence (NCBI Accession #: NM_009950.2) was amplified by the polymerase chain reaction (PCR) from first strand cDNA reverse transcribed from mouse brain, kidney, and liver tissue using forward (fwd) primer 5’-CACCATGGAAGCCAGAGACAAGC-3’ and reverse (rev) primer 5’-AGCCAGTGTGACTTGGGAAC-3’. The coding sequence for wt mCradd DD was PCR amplified from wt mCradd FL cloned into D-TOPO Gateway vector (see below) (Life Technologies) using fwd primer 5’-CACCCCTACAGGTGACTGGATGGC-3’ and rev primer
5'-AGCCAGTGTGACTTGGGAAC-3'. The coding sequence for wt mPidd FL was PCR amplified from pcDNA3.1/mPidd (a gift from Dr. S. Benchimol, York University, Toronto, Canada) using fwd primer 5'-CACCCTGATGGCTGCAGTGTTGG-3' and rev primer 5'-GGTGAAGGTAGAAGGATAGGGC-3'. Wild-type mPidd DD coding sequence was PCR amplified from pcDNA3.1/mPidd with fwd primer 5'-CACCACTGACTTCTCTGTATGCCC-3' and rev primer 5'-GTGAGGTAGAAGTGACTGGATGGC-3'. Wild-type human (h) CRADD FL coding sequence (NCBI Accession #: NM_003805.3) was PCR amplified from first strand cDNA reverse transcribed from hARPE-19 cells using fwd primer 5'-CACCATGGAGGCCAGACAAACAAAAG-3' and rev primer 5'-TCAGGATTCCAGCCCACATGAC-3'. The coding sequence for wt hCRADD DD was PCR amplified from first strand cDNA reverse transcribed from hARPE-19 cells using fwd primer 5'-CACCGCAGGTGACAGATGGG-3' and rev primer 5'-TCAGGATTCCACCACATGAC-3'. The coding DNA for wt hPIDD (NCBI Accession #: NM_145886.3, isoform 1) was amplified from first strand cDNA of hARPE-19 cells by PCR using fwd primer 5'-CACCAAGGACCCTCGTGGGCGAT-3' and rev primer 5'-ATCTGGGCAAGCTAAAAGTCTGTGG-3'. Wild-type hPIDD DD coding sequence was PCR amplified from first strand cDNA reverse transcribed from hARPE-19 cells using fwd primer 5'-CACCGAGGCTGCCCGACGAGG-3' and rev primer 5'-ATCTGGGCAAGCTAAAAGTCTGTGG-3'. PCR products were then subjected to agarose gel electrophoresis. The coding sequence constructs were excised from agarose gels and purified using the Qiaex II gel extraction kit, according to the manufacturer's protocol (Qiagen, Valencia, CA) and ligated into D-TOPO Gateway entry vectors (Life Technologies). The c.382G>C variant was introduced into D-TOPO Gateway entry vectors of mCradd FL, mCradd DD,
hCRADD FL, hCRADD DD using the Quickchange II site-directed mutagenesis kit, following the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Entry vector constructs were recombined into destination vectors with N-terminal fusion protein tags: pCAGIG/FLAG/RFC/A (FLAG), pCAGIG/V5/RFC/A (V5) (gifts of Q. Zhang and E.A. Pierce, University of Pennsylvania, Philadelphia, PA), or pcDNA3.1/n-V5 (V5) (Life Technologies) using LR Clonase II (Life Technologies). Sanger sequencing was used to confirm cloning and mutagenesis fidelity (Penn State Nucleic Acid Facility, State College, Pennsylvania).

Cell Culture and Transfection

Mouse IMCD3 cells and hARPE-19 cells were grown to 80-90% confluency on glass coverslips in 6-well plates for immunofluorescence or 10-cm dishes for protein collection and co-immunoprecipitation. Cells were transfected with FLAG, V5, or pcDNA3.1/n-V5 N-terminal fusion protein constructs (4 μg of DNA per well or 24 μg of DNA per 10-cm dish) using Lipofectamine 2000 (Life Technologies). Non-transfected cells were treated with Lipofectamine 2000, without the addition of plasmid DNA, and cultured identically to transfected cells.

Immunofluorescence

Following 40-48 h post-transfection and incubation at 37°C and 5% CO₂ in DMEM:F12 growth medium (Life Technologies) supplemented with FBS (Life Technologies), as above, cells on glass coverslips were washed with 1X phosphate buffered saline (PBS) and fixed for 10 min at room temperature (RT) with 4% paraformaldehyde in 1X PBS. Cells were permeabilized in 0.5% Triton-X 100 in 1X PBS for 10 min and subsequently incubated in blocking buffer (1% bovine serum albumin and 0.2% Triton-X 100 in 1X PBS) for 10 min at RT. Cells were then incubated for 1 h at RT with primary antibodies: anti-FLAG M2 monoclonal IgG₁ (1:1000; Sigma-Aldrich, St. Louis, MO) and/or anti-V5 monoclonal IgG₂a (1:400 or 1:500; Life
Technologies) diluted in blocking buffer for 1 h at RT. Cells were washed with 1X PBS three times for 10 min and were then incubated for 1 h at RT with secondary antibodies: AlexaFluor 488-conjugated goat anti-mouse IgG (1:400; Life Technologies) or AlexaFluor 594-conjugated goat anti-mouse IgG2a (1:400; Life Technologies) diluted in 0.3% Triton-X 100 in 1X PBS. Cell nuclei were stained with DAPI (4',6′-diamidino-2-phenylindole-1.5 μg/μl; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 1X PBS for 10 min. Coverslips were mounted with Fluoromount-G (Electron Microscopy Sciences, Ft. Washington, PA). Images were captured with a Leica DM RB fluorescence microscope using a Jenoptik MF-Cool camera (Jenoptik, Jena, Germany) with ProgRes Procapture software.

Co-immunoprecipitation and Western Blotting

Mouse IMCD3 cells transfected with N-terminal fusion protein constructs, as described above, were harvested on ice and lysed with Cell Lytic M (Sigma-Aldrich), supplemented with 1X Protease Inhibitor Cocktail (PIC) (Roche Applied Science, Indianapolis, IN) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). A 1:2 10-cm plate ratio of transfected cells was used for wt mCradd DD:mCradd DD Gly128Arg overexpression, in order to account for relatively equal protein levels. Because western blotting suggested that mCradd DD Gly128Arg expressed at lower levels than wt mCradd DD, lysis buffer volumes were adjusted to normalize total protein concentration across input cell lysates used for co-immunoprecipitation. Lysates were centrifuged at 14,000 rpm at 4°C for 15 min. The supernatant was collected, filtered with 0.45 μm filters (Millipore, Billerica, MA), and incubated at 37 °C for 1 h to activate complex formation (Tinel and Tschopp, 2004). A 1.0 ml aliquot of cleared lysate was added to 20 μl of equilibrated anti-FLAG M2 affinity red resin, following the manufacturer's instructions (Sigma-Aldrich), and rotated overnight at 4 °C. The resin beads were then washed three times.
with 1X Tris buffered saline (TBS), supplemented with 1X PIC (Roche Applied Science) and 1 mM PMSF (Sigma-Aldrich). FLAG fusion proteins were eluted from the resin with 100 μl of 3X FLAG peptide (150 ng/μl) (Sigma-Aldrich). Cell lysates co-overexpressing mCradd DD Gly128Arg and mPidd DD were eluted with 70 μl of 3X FLAG peptide to normalize protein concentrations across eluates. Eluates and/or crude lysate samples were diluted in a 3:1 ratio of NuPAGE 4X LDS Sample Buffer (Life Technologies), denatured through boiling, and loaded onto 4-12% polyacrylamide gels (Life Technologies). Biotinylated protein ladder (Cell Signaling, Danvers, MA) was used. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose (0.45 μm) (Bio-Rad, Hercules, CA). The membranes were blocked for 1 h at RT with blocking buffer (5% nonfat dry milk with 0.1% Tween-20 in 1X TBS). Membranes were incubated overnight at 4 °C in primary antibody (anti-FLAG-M2 mouse monoclonal IgG1-1:1000 or anti-V5 mouse monoclonal IgG2a-1:5000, and anti-β-actin mouse monoclonal-1:1,000,000) diluted in blocking buffer. Blots were washed 3 times for 5 min with 1X TBS supplemented with 0.1% Tween-20 (TBST) and then incubated for 1 h at RT in secondary antibody (HRP-conjugated goat anti-mouse IgG-1:1500) (Cell Signaling) and primary antibody (HRP-conjugated anti-biotin-1:2500) (Cell Signaling) diluted in blocking buffer. Membranes were washed with TBST and then incubated for 1 min in LumiGLO (Cell Signaling). Enhanced chemiluminescence was imaged using BioMax Light film (Kodak, Rochester, NY). Protein band densities were measured using ImageJ (http://rsbweb.nih.gov/ij) calibrated with an NIST calibrated step tablet (Kodak, Rochester, NY).
III. Results

**mCradd expression in mouse tissue and hCRADD expression in hARPE-19 cells**

To confirm that mCradd was expressed in mouse brain, as previously reported (Motaln et al., 2005), and to clone mCradd for use in fusion protein overexpression in mammalian cell culture, I used PCR to amplify the coding sequence for mCradd using cDNA reverse transcribed from total RNA of mouse brain, kidney, and liver tissue. Although expressed ubiquitously in the three mouse tissues used (Figure 6A), the PCR band from mouse brain tissue was used for D-TOPO Gateway entry vector cloning due to the association of CRADD c. 382G>C patients' phenotype with the nervous system. To clone human (h) CRADD (Figure 6B) and human (h) PIDD (Figure 6C), the coding sequences were PCR amplified from first strand cDNA reverse transcribed from total RNA of hARPE-19 cells for overexpression in mammalian cell culture. hARPE-19 cells are a well-established cell line for overexpression of nervous system related genes and provide an accessible model for future studies using neuronal cell lines.

**mCradd Gly128Arg localizes to the cytosol of mIMCD3 cells**

A single amino acid change in protein structure may be reflected in immunofluorescence as a change in protein localization within cells. In order to determine the subcellular localization pattern of FLAG-mCradd Gly128Arg in comparison to wild-type (wt) FLAG-mCradd distribution, I overexpressed wt FLAG-mCradd and FLAG-mCradd Gly128Arg in mIMCD3 cells using an antibody against the N-terminal FLAG tag of mCradd. Overexpressed wt FLAG-mCradd (Figure 7A) (n=2 transfected wells) and FLAG-mCradd Gly128Arg (Figure 7B) (n=2 transfected wells) both displayed a cytosolic distribution with minor nuclear localization in mIMCD3 cells. Non-transfected cells (Figure 7C) showed no anti-FLAG or anti-V5 immunoreactivity (n=2 transfected wells).
Overexpressed mPidd full-length (FL) and mPidd death domain (DD) localize to the cytosol of mIMCD3 cells

If the interaction between Cradd and Pidd DD is altered as a result of the Gly128Arg variant in the Cradd DD, as predicted, then interaction changes may be reflected in subcellular localization patterns as displayed in immunofluorescence. The first step in testing this hypothesis was to identify the cellular localization of overexpressed V5-mPidd in mIMCD3 cells using an antibody against the N-terminal V5 tag. Overexpressed V5-mPidd FL localized to the cytosol (Figure 7D) (n=2 transfected wells) and overexpressed V5-mPidd DD (Figure 7E) (n=2 transfected wells) localized to both the cytosol and the nucleus, consistent with previous literature (Berube et al., 2005; Tinel et al., 2007).

Autocatalytic cleavage of Pidd FL is necessary for Cradd and Pidd interaction (Tinel and Tschopp, 2004; Berube et al., 2005), so I wanted to determine whether overexpressed V5-mPidd FL undergoes autoprocessing in mIMCD3 cells. Cell lysates were collected from the well surface area surrounding the coverslips used in immunofluorescence, described above. Western blotting, using an anti-V5 antibody, displayed a fragment at ~110-kDa fragment, representative of V5-mPidd FL, and a cleaved fragment at ~57-kDa (Figure 7F) (n=3 transfected wells), likely representative of the previously reported ~51-kDa PIDD-C fragment (Tinel and Tschopp, 2004).

mCradd Gly128Arg forms clusters when co-overexpressed with mPidd FL and mPidd DD in mIMCD3 cells

To investigate whether the Gly128Arg variant disrupts interaction between mCradd Gly128Arg and mPidd, as shown through immunofluorescence, I used antibodies against the FLAG and V5 N-terminal tags to determine co-localization patterns in mIMCD3 cells. Wild-type and mutant (Gly128Arg) FLAG-mCradd were each co-overexpressed with V5-mPIDD FL
or V5-mPidd DD in mIMCD3 cells. When wt FLAG-mCradd was co-overexpressed with V5-mPidd FL (Figure 8A) (n=2 transfected wells) or V5-mPidd DD (Figure 8C) (n=2 transfected wells), FLAG-mCradd and V5-mPidd co-localized uniformly to the cytosol, as represented by the overlap of green (FLAG-mCradd) and red (V5-mPidd) immunofluorescent signals (yellow). FLAG-mCradd Gly128Arg formed clusters when co-overexpressed with V5-mPidd FL (Figure 8B) (n=2 transfected wells) or V5-mPidd DD (Figure 8D) (n=2 transfected wells), represented by bright green fluorescent circular structures (FLAG-mCradd Gly128Arg) against a red fluorescent (V5-mPidd) background.

**mCradd DD Gly128Arg forms clusters when overexpressed and when co-overexpressed with mPidd DD in mIMCD3 cells**

To further elucidate the impact of Gly128Arg, located within the Cradd DD, on mCradd subcellular localization and co-localization with mPidd, I overexpressed DD deletion constructs in mIMCD3 cells. Wild-type FLAG-mCradd DD overexpression displayed cytosolic localization (Figure 9A) (n=2 transfected wells). In 85% of cells (n=40 cells across 2 transfected wells) overexpressing FLAG-mCradd DD Gly128Arg, green fluorescent circular structures were dispersed throughout the cytosol (Figure 9B) (n=2 transfected wells), which were not observed in wt FLAG-mCradd DD overexpression (Figure 9A). V5-mPidd DD overexpression displayed localization to the cytosol and nucleus (Figure 9C) (n=2 transfected wells), as previously described (Figure 7E). In order to determine whether mCradd DD Gly128Arg alters interaction with mPidd DD, I co-overexpressed wt and mutant FLAG-mCradd DD with V5-mPidd DD in mIMCD3 cells. Overexpressed wt FLAG-mCradd DD co-localized with overexpressed V5-mPidd DD uniformly in the cytosol as demonstrated by the overlap of green (FLAG-mCradd DD) and red (V5-mPidd DD) fluorescent signals (yellow) (Figure 9D) (n=2 transfected wells).
Overexpressed FLAG-mCradd DD Gly128Arg formed clusters when co-overexpressed with V5-mPidd DD (Figure 9E) (n=2 transfected wells) as displayed by bright green circular structures (FLAG-mCradd Gly128Arg) against a red fluorescent (V5-mPidd DD) background. The clusters were absent in wt FLAG-mCradd DD co-overexpression with V5-mPidd DD (Figure 9D). Non-transfected cells (Figure 9F) showed no anti-FLAG or anti-V5 immunoreactivity (n=2 transfected wells).

**hCRADD Gly128Arg displays differential overexpression patterns in hARPE-19 cells**

Although overexpression of mCradd fusion proteins in mouse cell culture provides a sufficient model for the study of Cradd Gly128Arg, the variant is associated with a human developmental disorder, so it was necessary to repeat immunofluorescence microscopy with human CRADD fusion proteins in human cell culture to confirm results. To investigate the impact of hCRADD Gly128Arg on subcellular localization, I overexpressed wt V5-hCRADD and V5-hCRADD Gly128Arg in hARPE-19 cells. The pcDNA3.1/n-V5 N-terminal fusion protein tag was used for hCRADD in this study of immunofluorescence because it can be used to establish stable cell lines, which is a potential future goal of this study. Overexpressed wt V5-hCRADD localized to the cytosol of hARPE-19 cells (Figure 10A) (n=2 transfected wells), as reported in previous literature (Shearwin-Whyatt et al., 2000). The majority of cells (72.5%; n=40 cells across two transfected wells) overexpressing V5-hCRADD Gly128Arg displayed cytosolic distribution in hARPE-19 cells (Figure 10B). However, 27.5% of hARPE-19 cells (n=40 cells across two transfected wells) overexpressing V5-hCRADD Gly128Arg displayed hCRADD clusters, represented by red fluorescent circular structures dispersed throughout the cell (Figure 10C) that were not observed in wt V5-hCRADD overexpression (Figure 10A). To further determine the affect of the Gly128Arg variant within the CRADD DD on subcellular
localization, wt and mutant DD deletion constructs were overexpressed in hARPE-19 cells. Overexpressed wt V5-hCRADD DD (Figure 10D) (n=2 transfected wells) and V5-hCRADD DD Gly128Arg (Figure 10E) (n=2 transfected wells) each localized to the cytosol and the nucleus. Non-transfected cells (Figure 10F) displayed no anti-V5 and anti-FLAG immunoreactivity (n=2 transfected wells).

hCRADD Gly128Arg forms clusters when co-overexpressed with hPIDD DD

If hCRADD Gly128Arg disrupts interaction with hPIDD DD, as hypothesized, then the interaction may be displayed through changes in co-localization, as detected by immunofluorescence. In order to investigate the impact of hCRADD Gly128Arg on co-localization with hPIDD DD, wt and mutant V5-hCRADD were co-overexpressed with FLAG-hPIDD DD in hARPE-19 cells. Co-overexpression of wt V5-hCRADD (Figure 11B) and FLAG-hPIDD DD (Figure 11A) displayed co-localization in the cytosol of hARPE-19 cells represented by the overlap of green (FLAG-hPIDD DD) and red (V5-hCRADD) fluorescent signals (yellow) (Figure 11C) (n=2 transfected wells). When V5-hCRADD Gly128Arg (Figure 11E) was co-overexpressed with FLAG-hPIDD DD (Figure 11D), V5-hCRADD formed clusters, as represented by red fluorescent circular structures (V5-hCRADD Gly128Arg) dispersed in the cytosol against a green (FLAG-hPIDD DD) fluorescent background (Figure 11E and 11F) (n=2 transfected wells). Non-transfected cells were from the same transfection as in Figure 10.

Overexpressed hCRADD Gly128Arg & hCRADD DD Gly128Arg display reduced abundance in hARPE-19 cells

Qualitative analysis of immunofluorescence microscopy of V5-hCRADD Gly128Arg overexpression suggested that fewer hARPE-19 cells overexpressed the mutant protein, relative to wt V5-hCRADD overexpression. To quantify V5-hCRADD Gly128Arg overexpression in
hARPE-19 cells in the immunofluorescence study described above, I calculated the percentage of hARPE-19 cells that overexpressed wt or mutant V5-hCRADD, relative to the total number of nuclei in a randomly selected 10X field of view using a fluorescence microscope. I used the overexpression rate of each V5-hCRADD fusion protein (n=2 transfected wells), to determine whether hARPE-19 cells equally overexpressed wt V5-hCRADD and V5-hCRADD Gly128Arg. An independent samples t-test was used to determine whether there was a statistically significant difference between the number of hARPE-19 cells overexpressing V5-hCRADD Gly128Arg relative to wt V5-hCRADD and between the number of hARPE-19 cells overexpressing V5-hCRADD DD Gly128Arg relative to wt V5-hCRADD DD (Table 1). When overexpressed in hARPE-19 cells, 5.19 ± 2.93 % of cells overexpressed wt V5-hCRADD (n=20 images of two transfected wells) and 1.29% ± 0.686% of hARPE-19 cells overexpressed V5-hCRADD Gly128Arg (n=20 images of two transfected wells). Overexpression of V5-hCRADD Gly128Arg was reduced by 75.2% relative to wt V5-hCRADD overexpression (p<0.05) (Table 1). When co-overexpressed with hPIDD DD, 7.81 ± 7% of hARPE-19 cells overexpressed wt V5-hCRADD (n=20 images of two transfected wells) and 1.08 ± 1.5% of cells overexpressed V5-hCRADD Gly128Arg (n=20 images of two transfected wells). Overexpression of V5-hCRADD Gly128Arg was reduced by 85% in hARPE-19 cells, relative to wt V5-hCRADD overexpression (p<0.001) (Table 1). Wild-type V5-hCRADD DD was overexpressed in 4.31± 2.1% of hARPE-19 cells (n=20 images of two transfected wells) and V5-hCRADD DD Gly128Arg was overexpressed in 0.43 ± 0.08% of hARPE-19 cells (n=20 images of two transfected wells). Overexpression of V5-hCRADD Gly128Arg was reduced by 89.9% in hARPE-19 cells, relative to overexpression wt V5-hCRADD DD (p<0.001) (Table 1).
Collectively, the quantitative analysis of immunofluorescence suggested that across three different overexpression studies, expression of the Gly128Arg variant was reduced in hARPE-19 cells. If hCRADD Gly128Arg is expressed by fewer cells, then its abundance in hARPE-19 cells may be reduced relative to wt hCRADD. Based upon those results, I used western blotting of the cell lysates collected from the well surface area surrounding the coverslips used in immunofluorescence, described above, to determine relative protein abundance of overexpressed hCRADD Gly128Arg in comparison to wt hCRADD overexpression. I used an anti-V5 antibody to label V5-hCRADD fusion proteins and anti-β-actin antibody to label endogenous β-actin in hARPE-19 cells, which was used as a control for comparative analysis purposes. When overexpressed in hARPE-19 cells, V5-hCRADD Gly128Arg abundance was reduced by an average of 82.9 ± 2.3% relative to wt V5-CRADD abundance (n=3 transfections) (Figure 12A). Overexpressed V5-hCRADD DD Gly128Arg was 98.9% (n=1 transfection) less abundant than overexpressed wt V5-CRADD DD (Figure 12B).

**mCradd DD Gly128Arg does not interact with mPidd DD**

Based upon correlative evidence from immunofluorescence that suggested a change in co-localization between mCradd Gly128Arg and mPidd, as well as between hCRADD Gly128Arg and hPIDD DD, if mCradd Gly128Arg alters interaction with mPidd, then the ability of mCradd DD Gly128Arg to co-immunoprecipitate mPidd DD may increase or decrease in comparison to wt mCradd DD. Because others have shown that overexpression of the DDs of PIDD and CRADD is sufficient to induce interaction between CRADD and PIDD, (Tinel et al., 2007; Jang et al., 2010), I used the co-overexpression of DD deletion constructs in mIMCD3 cells to probe the impact of the Gly128Arg variant on mCradd DD and mPidd DD interaction through co-immunoprecipitation. I co-overexpressed wt FLAG-mCradd DD with V5-mPidd DD
and FLAG-mCradd DD Gly128Arg with V5-mPidd DD in mIMCD3 cells. Complex activation of mCradd and mPidd was induced in cell lysates following incubation at 37 °C, as described by Tinel and Tschopp (2004). Overexpressed wt FLAG-mCradd DD, FLAG-mCradd Gly128Arg, and V5-mPidd DD were each present in cell lysates prior to co-immunoprecipitation (Figure 13), serving as a control for fusion protein expression. Wild-type FLAG-mCradd DD, immunoprecipitated by anti-FLAG M2-conjugated agarose beads, co-immunoprecipitated V5-mPidd DD (Figure 13). Overexpressed FLAG-mCradd DD Gly128Arg did not co-immunoprecipitate V5-mPidd DD, as demonstrated through western blotting (Figure 13). Non-transfected cells displayed no anti-FLAG or anti-V5 immunoreactivity (Figure 13).

IV. Discussion

Through RT-PCR, I demonstrated that mCradd was expressed in the total mRNA isolated from mouse brain, kidney, and liver tissue (Figure 6A), suggesting that mCradd expression is not limited to one tissue type. This is consistent with previous work that detected mCradd expression in a variety of midgestation mouse embryonic tissues, including the heart, kidney, stomach, midgut, spinal cord, forebrain, optic vesicle, lens fibre cells, and ependymal cells of the fourth ventricle (Motaln et al., 2005). Collectively, these results are surprising, given the non-syndromic MR phenotype of patients with CRADD c.382G>C. This suggests that post-mitotic cells, such as neurons (Troy and Salvesen, 2002), may be more susceptible to the effects of the mutant CRADD protein. Kumar et al. (1994) detected caspase-2 expression in post-mitotic neurons, despite the reduced expression of caspase-2 in the majority of adult mouse tissues, suggesting that post-mitotic cells display an increased sensitivity to caspase-mediated apoptosis. Because caspase pathways can be redundant (Stefanis et al., 1998; Troy et al., 2001; Troy and Salvesen, 2002; Manzl et al., 2005; Ho et al., 2008; Tiwari et al., 2011), it is possible
that disruptions in the CRADD/PIDDosome/caspase-2-dependent apoptosis pathway may be supplemented by the redundancy of caspase pathways in other tissues. The redundant and compensatory nature of caspase-mediated apoptotic pathways correlates with the non-syndromic MR phenotype associated with CRADD c.382G>C patients. No abnormalities have been identified in other tissues of CRADD c.382 G>C patients, despite evidence of ubiquitous CRADD expression across tissue types (Duan and Dixit, 1997; Ahmad et al., 1997; Motaln et al., 2005). This suggests that the central nervous system may be increasingly sensitive to CRADD expression or CRADD-associated apoptosis causing a nervous-system specific mild phenotype. The ubiquitous expression of mCradd therefore warrants further study to determine the expression and behavior of mCradd in various tissues. To gain further insight into the non-syndromic MR phenotype in CRADD c.382G>C patients, future investigation through CRADD expression in primary neuronal cell culture is required to determine the impact and role of CRADD Gly128Arg in neuronal cells.

Immunofluorescence microscopy did not display an appreciable difference in the localization of overexpressed FLAG-mCradd Gly128Arg in mIMCD3 cells (Figure 7B), V5-hCRADD Gly128Arg (Figure 10B) in hARPE-19 cells, or V5-hCRADD DD Gly128Arg in hARPE-19 cells, relative to wild-type overexpression. Although, 27.5% of hARPE-19 cells overexpressing V5-hCRADD Gly128Arg displayed cluster formation (Figure 10C) and 85% of cells overexpressing FLAG-mCradd DD Gly128Arg (Figure 9B) formed similar clusters of mutant protein, not observed in wild-type overexpression. The cause of localization changes in the two overexpressed proteins requires further investigation. However, collectively, immunofluorescence results suggest that the Gly128Arg variant does not appear to significantly alter subcellular localization when overexpressed alone.
Because the Gly128Arg variant is located within the DD of CRADD, it was predicted that the variant might alter interaction with PIDD DD, which may be displayed through changes in immunofluorescence. Immunofluorescence microscopy displayed a collective difference in FLAG-mCradd Gly128Arg localization when co-overexpressed with V5-mPidd in mIMCD3 cells and in V5-hCRADD Gly128Arg localization when co-overexpressed with FLAG-hPIDD DD in hARPE-19 cells. Both FLAG-mCradd Gly128Arg (Figure 8B & 8D) and V5-hCRADD Gly128Arg (Figure 11F) formed clusters in the presence of overexpressed V5-mPidd or V5-hPIDD DD that were not observed in wild-type co-overexpression. This qualitative evidence suggests that Gly128Arg may change the interaction between CRADD and PIDD to increase CRADD homo-oligomerization portrayed in immunofluorescence as CRADD clusters.

A potential model to explain the formation of mCradd Gly128Arg and hCRADD Gly128Arg clusters in immunofluorescence, is based on the work of Shearwin-Whyatt et al. (2000). Shearwin-Whyatt et al. (2000) demonstrated through co-immunoprecipitation that the CARD and DD of wt CRADD interact in a compact formation that prevents higher order oligomerization between CARDs (Figure 14A). The compact unit may prevent oligomerization between the CARDs of CRADD proteins themselves or with other proteins that contain a CARD, such as caspase-2. Shearwin-Whyatt et al. (2000) suggests that under normal conditions, the folding of CRADD via interaction between the CARD and the DD may undergo a conformational change in response to death signals through its’ DD that unfolds the compact unit. This conformational change allows for CARD:CARD oligomerization, such as that between CRADD and caspase-2 during caspase-2 mediated apoptosis (Figure 14A). If the Gly128Arg variant increases homo-oligomerization between DDs, then the DD of CRADD Gly128Arg may not preferentially bind to its’ CARD under normal conditions. Under normal
conditions with no DD signaling present, CRADD Gly128Arg may take on a linear conformation (Figure 14B) rather than the compact unit observed by Shearwin-Whyatt et al. (2000) in wild-type CRADD. As a result, inappropriate oligomerization of CARD:CARD would not be prevented, as CRADD CARD is exposed and able to bind to another CARD, including that of other CRADD proteins (Figure 14B). This effect may be exacerbated in the presence of overexpressed PIDD because although PIDD may not interact with CRADD, there may be enough PIDD in the vicinity to induce the death signals that under normal circumstances, would trigger conformational change in the compact unit, but may instead trigger homo-oligomerization in the Gly128Arg variant model. Based upon the crystal structure analysis and sequence alignments conducted by Park et al. (2007), the exposed CRADD DD in this model with an increased affinity for self may be capable of interacting with a maximum of six CRADD DDs. Therefore, recruitment of multiple CRADD Gly128Arg to a single CRADD Gly128Arg, as a result of homo-oligomerization between CARD:CARD and DD:DD, may result in the formation of extensive higher order oligomerized CRADD structures (Figure 14B) that manifest themselves in cell culture as CRADD clusters. This hypothetical model can be tested in the future using both co-immunoprecipitation to determine whether the DDs of CRADD Gly128Arg co-immunoprecipitate each other and whether the CARDs of CRADD Gly128Arg co-immunoprecipitate each other, which would be suggestive of homo-oligomerization. It would also be necessary to determine, through co-immunoprecipitation, whether the DD of CRADD Gly128Arg interacts with the CARD of CRADD Gly128Arg, which would not be expected to occur based on the proposed Gly128Arg variant model.

Qualitatively, immunofluorescence microscopy suggests that fewer cells overexpressed the Gly128Arg variant. Due to the redundant quality of immunofluorescence results between
mCradd and hCRADD overexpression, I quantified V5-hCRADD Gly128Arg overexpression in mIMCD3 cells, extrapolating, based on immunofluorescence, that the results may also be reflective of overexpressed FLAG-mCradd Gly128Arg in mIMCD3 cells. A significantly reduced number of hARPE-19 cells overexpressed V5-hCRADD Gly128Arg (a 75.2% reduction) and V5-hCRADD DD Gly128Arg (an 89.9% reduction) relative to wt V5-hCRADD and wt V5-hCRADD DD. The number of hARPE-19 cells co-overexpressing V5-hCRADD Gly128Arg with FLAG-hPIDD DD was also significantly reduced (an 85% reduction), in comparison to the number of hARPE-19 cells co-overexpressing wt V5-hCRADD and FLAG-hPIDD DD. Consistent with immunofluorescence, western blotting revealed an average 82.9 ± 2.3% reduction in V5-hCRADD Gly128Arg abundance and a 98.9% reduction in V5-hCRADD DD Gly128Arg abundance in hARPE-19 cell lysates, relative to wild-type protein abundance (Figure 12). Collectively, immunofluorescence and western blotting suggests that the Gly128Arg variant may have a two-fold impact. CRADD Gly128Arg may be unstable and possibly targeted for degradation in the cell. Consequently, the mutant protein may be sequestered in cell lysosomes or proteasomes, resulting in the cluster formation observed in immunofluorescence. In future immunofluorescence microscopy, lysosome and proteasome markers will be used to determine if the Gly128Arg variant clusters are localized to intracellular organelles. Fractionation using differential centrifugation can also be used to determine whether CRADD Gly128Arg is present in cytosolic cellular fractions (Wang et al., 2006; Tinel et al., 2007). To determine whether the instability lies at the transcript level, RT-PCR will be used to quantify the overexpression of hCRADD c.382G>C in hARPE-19 cells and mCradd c.382G>C in mIMCD3 cells. Potential CRADD Gly128Arg protein instability may also prevent interaction with PIDD, as displayed in immunofluorescence, because if the mutant protein were sequestered
in organelles, then it would not be able to interact with PIDD in the cytosol. An alternate hypothesis for reduced CRADD Gly128Arg overexpression and abundance is that CRADD Gly128Arg may undergo apoptosis more readily than wild-type CRADD. Overexpressed CRADD has been shown to induce apoptosis in non-neuronal cells (Duan and Dixit, 1997) and in sympathetic neurons (Jabado et al., 2004). Based upon this previous work, in order to fully characterize the impact of the Gly128Arg variant, its’ affect on cellular apoptosis must be elucidated. Apoptosis can be analyzed using flow cytometry or antibodies against the effector caspases activated by caspase-2, such as caspase 3. However, other mechanisms of caspase-2 activation exist (Manzl et al., 2009), so it is possible that apoptosis may occur through a pathway that does not rely on functional CRADD.

To further characterize the potential change in interaction between CRADD and PIDD observed in immunofluorescence, co-immunoprecipitation was used to determine whether overexpressed FLAG-mCradd DD Gly128Arg co-immunoprecipitated V5-mPidd DD. If the interaction between Cradd DD Gly128Arg and Pidd DD is disrupted, then FLAG-mCradd DD Gly128Arg should not co-immunoprecipitate V5-mPidd DD. Based upon the decreased mutant protein abundance observed in previous western blotting, mMCD3 total lysate concentrations were adjusted to increase the relative level of FLAG-mCradd DD Gly128Arg overexpression to match that of wt FLAG-mCradd DD overexpression. Overexpressed wt FLAG-mCradd DD co-immunoprecipitated V5-mPidd DD following induced complex activation (Tinel and Tschopp, 2004), but overexpressed FLAG-mCradd DD Gly128Arg did not co-immunoprecipitate (Figure 13) V5-mPidd DD. These results suggest that FLAG-mCradd DD Gly128Arg disrupts interaction with V5-mPidd DD, and warrants future replication using co-overexpressed hCRADD DD Gly128Arg and hPIDD DD in hARPE-19 cells.
One interpretation of the immunofluorescence results displaying CRADD Gly128Arg cluster formation in the presence of PIDD and the disrupted interaction between mCradd DD Gly128Arg and mPidd DD, as demonstrated by co-immunoprecipitation, is that the Gly128Arg variant increases CRADD DD homo-oligomerization and decreases affinity for PIDD DD. Park et al. (2007) determined that there are eight interfaces required for CRADD DD:PIDD DD interaction and those eight interfaces are divided into three types of interactions: CRADD DD:CRADD DD, CRADD DD:PIDD DD, and PIDD DD:PIDD DD. It is possible that the Gly128Arg variant alters one of the nearby interaction surfaces of CRADD DD (Figure 5A) that strengthens one of the CRADD:CRADD interactions or changes a CRADD:PIDD interface to increase CRADD DD affinity for self (Figure 15). The Gly128Arg variant replaces the nonpolar side chain of Gly\textsuperscript{128} with the large, positively charged polar side chain of Arg\textsuperscript{128}. The change in side chain structure likely allows Arg\textsuperscript{128} to interact with surrounding nonpolar residues differently than Gly\textsuperscript{128}, which may alter CRADD DD interaction interfaces. Jang et al. (2010) demonstrated that a point mutation altering a highly conserved amino acid in CRADD DD required for CRADD DD:CRADD DD and CRADD DD:PIDD DD interaction inhibited interaction with PIDD and subsequent formation of the PIDDosome. Based upon the results from Jang et al. (2010), if the Gly128Arg variant alters interaction between the side chains of neighboring nonpolar residues known to participate in CRADD DD:CRADD DD and CRADD DD:PIDD DD interaction interfaces (Park et al., 2007), then it is likely that the variant may prevent formation of the PIDDosome (Figure 15A) as a result of increased CRADD DD homo-oligomerization and decreased CRADD DD affinity for PIDD DD (Figure 15B). Complex association assays using gel filtration chromatography can be used to determine the molecular weight of complexes formed in cell lysates, from which you can deduce the individual
components of the complex. This would allow for the determination of CRADD DD:CRADD DD complex formation based upon the molecular weights of eluted fractions. Complex shifts from CRADD DD:PIDD DD to CRADD DD:CRADD DD can be visualized using native PAGE. This experiment can also be expanded to include caspase-2 to determine whether caspase-2 is recruited to the CARD of CRADD Gly128Arg, despite the impaired interaction between CRADD DD:PIDD DD. These functional studies would provide evidence to determine whether homo-oligomerization occurs when hCRADD DD Gly128Arg is overexpressed in cell culture and to determine whether CRADD DD:PIDD DD complex formation is reduced at the expense of CRADD DD:CRADD DD interaction.

Functional studies of overexpressed CRADD Gly128Arg and PIDD in mammalian cell culture provide evidence suggesting that formation of the PIDDosome may be disrupted as a result of an altered interaction between CRADD DD Gly128Arg and PIDD DD. Disrupted PIDDosome formation may therefore alter PIDDosome/caspase-2 mediated apoptosis in CRADD c.382G>C patients. Early in development, the nervous system is characterized by synaptic overgrowth to ensure that all appropriate neural targets required for mature circuitry are present (Tessier and Broadie, 2009). In order to establish and maintain synaptic connections during nervous system development, neurons must have access to neurotrophic factor (Vanderhaeghen and Cheng, 2009) and those that don’t are eliminated through apoptosis (Troy et al., 2001) or undergo synaptic pruning through neurite retraction (Rumpf et al., 2010). Both neuronal apoptosis and axon pruning have been associated with caspase-mediated mechanisms (Rumpf et al., 2010; Nikolaev et al., 2009; Yan et al., 2010), including PIDDosome/caspase-2-mediated apoptosis (Kumar et al., 1994; Stefanis et al., 1998; Wang et al., 2006; Tiwari et al., 2011; Ribe et al., 2012). Disruption of apoptosis and synaptic pruning within the nervous system has been
linked to a variety of neurodevelopmental and neurodegenerative disorders, such as mental retardation (Philippe et al., 2009), Fragile X syndrome (Tessier and Broadie, 2009), Down syndrome (Engidawork et al., 2001), and Alzheimer’s disease (Troy and Salvesen, 2002). Based upon the established role of caspase-mediated mechanisms in the refinement of neuronal circuitry during nervous system development, it is likely that the non-syndromic MR observed in CRADD c.382G>C patients may be due to a disruption of apoptosis through PIDDosome/caspase-2-mediated pathways associated involved in activity dependent refinement.

The scope of the pathways associated with CRADD and its’ binding partners is extensive and are only beginning to be thoroughly investigated. The phenotype associated with CRADD c.382G>C patients provides a sufficient basis for beginning to elucidate the role of CRADD and its’ associated pathways in the context of human development. Behavioral analyses and neuroanatomical studies using Cradd^−/− mice (a gift from Dr. Tak Mak, University of Toronto) (Berube et al., 2005), which have yet to be characterized based on behavior, will allow for further connections to be drawn between molecular mechanisms and development in the mouse model in the future. Evidence from the functional studies of the CRADD Gly128Arg variant, suggesting an altered interaction with PIDD and subsequent PIDDosome assembly, provide a foundation for future investigation into the impact of the Gly128Arg variant at the cellular and molecular level. Knowledge of the pathways and mechanisms disrupted by CRADD Gly128Arg can then be related to the non-syndromic MR phenotype displayed by CRADD c.382G>C patients, likely associated with disruptions of activity dependent synaptic refinement during the development of the central nervous system.
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Figure 1. Model of a pedigree depicting inheritance of a recessive disorder. Within endogamous demes, homozygous haplotypes are assumed to be inherited through identity-by-descent (Puffenberger, 2003). As a result of recombination through successive generations the inherited haplotype block containing the mutant allele (the disease interval) is narrowed among affected family members. This allows for the use of autozygosity mapping to identify candidate disease gene intervals in affected individuals relative to unaffected family members (Lander and Botstein, 1987; Strauss and Puffenberger, 2009). This figure was modified from autozygosity.org.
Figure 2. Homozygosity mapping of six Mennonite patients with non-syndromic mental retardation. Affymetrix Genechip 10K Single Nucleotide Polymorphism microarrays were used to identify homozygous SNP blocks surrounding the disease gene locus, assuming identity-by-descent. The yellow peaks represent homozygous SNPs shared between affected individuals in comparison to their unaffected family members. The purple peaks represent LOD scores, a statistical estimate of the likelihood that two loci are linked and inherited together. Mapping revealed a 3.6 Mb homozygous block on chromosome 12 (12q22) in patients with non-syndromic mental retardation (Puffenberger et al., 2012) containing 46 genes. The mapped interval is the most likely region to contain the disease gene associated with mental retardation, as it is a region consistent with linkage among affected family members.
<table>
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<th>QUERY</th>
<th>Callithrix jacchus</th>
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**Figure 3.** Sequence alignment of the primary amino acid sequence of CRADD Gly128Arg (Query) with 17 of 75 mammalian and non-mammalian species using the PolyPhen 2 database (Adzhubei et al., 2010). The boxed G represents the highly conserved Gly128 in CRADD predicted to change to an Arg128 in the mutant protein (indicated by the bold arrow). The mutation is predicted to be “probably damaging” with a score of 1 on a scale of 0 (benign) to 1 (probably damaging) based on multiple sequence alignments of the affected residue, sequence homology of conserved domains, amino acid structure, and comparison to databases of known disease causing mutations (Adzhubei et al., 2010). CRADD Gly128 is a highly conserved residue, suggesting that Gly128 is likely critical for CRADD protein structure or function.
Figure 4. Model based on the crystal structure of the PIDDosome as determined by Park et al. (2007). Seven CRADD death domains interact with five PIDD death domains to form the core oligomeric complex of the PIDDosome. Following interaction between CRADD DD and PIDD DD, seven caspase-2 are recruited to the CARD of each of the seven CRADD to form the complete PIDDosome and initiate apoptosis.
A. Type Ia, Ib, R:P  RR RR PP P P P R RR RR PP
Type Ia, Ib, R:P  RR RR PP P P P R RR RR PP
Type Ia, Ib, P:P  PP P P P P P P P P P P P
Type IIa, IIb, R:P  R P R R R P P R P R R P P
Type IIa, IIb, R:P  RR RR P P P P P P P P
Type IIIa, IIIb, R:P  P P P P P P P P P P P P
Type IIIa, IIIb, R:P  R R R R R R R R R R R R
Type IIIa, IIIb, P:P  P P P P P P P P P P P P

H1 110 H2 120 H3 130 H4 140 H5 150 H6 160

CRADD HILNNSPSDRQINQLQRGPWEEMVLLGSLGQCDYRCKANHPHNQVSQQVEAFLIRWRQRFGKATFAQSLHGLRAVEVDPSSLHHMLE
PIDD AETGFLTFQSN LLSVGRLGLOWPVALHGLVSYTVQRIRHEFRDDLDEQIRHMLPSWAERQAGQPAGVGLLVQALEQSDRQ0VAEEVRAVLELG

Wild-type CRADD
PIDD

CRADD Gly128Arg
PIDD

B. CRADD CARD domain
PIDD interaction
Death domain

CRADD 1 94 116 198

Glycine Side Chain
Arginine Side Chain

Park et al., 2007
Figure 5. (A) Sequence alignment of CRADD death domain (DD) (residues 94-199) and PIDD DD (residues 778-883) (Park et al., 2007). Eight possible interfaces can form between interaction between DD residues: Type I: CRADD DD:CRADD DD (R:R), CRADD DD:PIDD DD (R:P), PIDD DD:PIDD DD (P:P); Type II: R:P, R:R; Type III: R:R, R:P, P:P. Residues on CRADD DD and PIDD DD known to participate in interaction interfaces are highlighted in yellow (Park et al., 2007). Gly^{128} was not identified by Park et al. (2007) to be a residue involved in one of the eight interaction interfaces, but is located in a region of CRADD (between Leu^{127} and Pro^{129}) responsible for nonpolar Type III R:P, R:R, and P:P interactions. Gly^{128} is predicted to be replaced by Arg^{128} in CRADD in patients with non-syndromic mental retardation. The wild-type Gly^{128} side chain is nonpolar and replaced by the large, polar, and positively charged side chain of Arg^{128}, which may alter the nonpolar interactions of neighboring residue interfaces. (B) The Gly128Arg variant in CRADD is located within the DD region of CRADD (amino acids 116-198), specifically in the region responsible for CRADD and PIDD interaction.
Figure 6. PCR of coding sequences amplified from first strand complimentary DNA (cDNA) reverse transcribed from total RNA of (A) wild-type mouse tissue or (B and C) hARPE-19 cells using gene specific primers, as described in methods. PCR bands at (A and B) ~658 bp (B) ~354 bp (C) ~554 bp were excised from agarose gels and purified for D-Topo Gateway entry vector cloning. In (A), only coding DNA amplified from brain tissue was used in Gateway entry vector cloning. Sanger sequencing confirmed the identity of PCR products: (A) wt mouse Cradd FL (~658 bp). (B) wt human (h) CRADD FL (~658 bp) and wt hCRADD death-domain (DD) (~354 bp). (C) wt FL hPIDD (~2780 bp) and wt hPIDD (DD) (~554 bp).
Figure 7. Immunofluorescence of overexpressed N-terminal tagged fusion mouse protein constructs in mouse IMCD3 cells using antibodies against N-terminal FLAG or V5 tags. (A) Wild-type (wt) FLAG-mCradd displays a cytosolic and minor nuclear distribution. (B) FLAG-mCradd Gly128Arg displays a cytosolic and minor nuclear distribution consistent with wt overexpression. (C) Non-transfected mIMCD3 cells, used as a control, showing no immunoreactivity (labeled with primary antibodies: anti-FLAG M2 and anti-V5; secondary antibodies: AlexaFluor 488-conjugated goat anti-mouse IgG<sub>1</sub>, AlexaFluor 594-conjugated goat anti-mouse IgG<sub>2a</sub>). (D) Overexpressed wt V5-mPidd FL localizes to the cytosol. (E) Overexpressed V5-mPidd DD displays a cytosolic and nuclear distribution. (F) Western blot of mIMCD3 cell lysates overexpressing V5-mPidd FL collected from the surface area surrounding the coverslips used for immunofluorescence. A band was detected at ~110-kDa, likely representative of V5-mPidd FL (Berube et al., 2005) and a band at ~57-kDa, likely representative of the cleaved PIDD-C (~51-kDa) fragment described by Tinell et al. (2007). Primary antibodies A-E: anti-FLAG-M2 mouse monoclonal IgG<sub>1</sub> (1:1000) or anti-V5 mouse monoclonal IgG<sub>2a</sub> (1:400). Secondary antibodies in A-E: AlexaFluor 488-conjugated goat anti-mouse IgG<sub>1</sub> (1:400) (green fluorescent signal), AlexaFluor 594-conjugated goat anti-mouse IgG<sub>2a</sub> (1:400) (red fluorescent signal). Nuclei were labeled with DAPI (1.5 μg/μl) (blue fluorescent signal). Each image is representative of two independent transfections. Scale bar in A= 10 μm for A-I. Primary antibodies in F: Anti-V5 mouse monoclonal IgG<sub>2a</sub> (1:5000) & HRP-conjugated anti-biotin (1:2500). Secondary antibody: HRP-conjugated goat-anti-mouse IgG (1:1500).
Figure 8. Immunofluorescence of co-overexpressed wild-type (wt) FLAG-mCradd or FLAG-mCradd Gly128Arg and V5-mPidd full-length (FL) or V5-mPidd death domain (DD) in mIMCD3 cells. Cells in A-D are a continuation of the transfection from Figure 7. (A) Wild-type FLAG-mCradd co-overexpressed with V5-mPidd FL displays co-localization in the cytosol. (B) FLAG-mCradd Gly128Arg forms Cradd clusters when co-overexpressed with V5-mPidd FL. (C) Wild-type FLAG-mCradd co-localized with V5-mPidd DD in the cytosol and the nucleus. (D) FLAG-mCradd Gly128Arg forms Cradd clusters when co-overexpressed with V5-mPidd DD. See Figure 7C for non-transfected control cells, demonstrating no immunoreactivity. Primary antibodies: anti-FLAG-M2 mouse monoclonal IgG1 (1:1000) and anti-V5 mouse monoclonal IgG2a (1:400). Secondary antibodies: AlexaFluor 488-conjugated goat anti-mouse IgG2a (1:400) (green fluorescent signal) and AlexaFluor 594-conjugated goat anti-mouse IgG2a (1:400) (red fluorescent signal). Nuclei labeled with DAPI (1.5 μg/μl) (blue fluorescent signal). Each image is representative of two independent transfections. Scale bar in A= 10 μm for A-D.
Figure 9. Immunofluorescence of overexpressed N-terminal tagged fusion protein death domain (DD) constructs in mIMCDS cells using antibodies against N-terminal FLAG and V5 tags. (A) Wild-type (wt) FLAG-mCradd DD displays a cytosolic and minor nuclear distribution. (B) FLAG-mCradd DD Gly128Arg forms Cradd clusters in the cytosol in 85% of cells overexpressing FLAG-mCradd DD Gly128Arg (n=40 cells). (C) V5-mPidd DD wt displays a cytosolic and nuclear distribution. (D) wt FLAG-mCradd DD co-overexpressed with V5-mPidd DD displays co-localization in the cytosol. (E) FLAG-mCradd DD Gly128Arg forms Cradd clusters when co-overexpressed with V5-mPidd DD wt. (F) Non-transfected cells used as a control (labeled with anti-FLAG and anti-V5 primary antibodies and secondary antibodies: AlexaFluor 488-conjugated goat anti-mouse IgG<sub>1</sub> and AlexaFluor 594-conjugated goat anti-mouse IgG<sub>2a</sub>), demonstrating no immunoreactivity. Primary antibodies: anti-FLAG M2 mouse monoclonal IgG<sub>1</sub> (1:1000) or anti-V5 mouse monoclonal IgG<sub>2a</sub> (1:500). Secondary antibodies: AlexaFluor 488-conjugated goat anti-mouse IgG<sub>1</sub> (1:400) (green fluorescent signal) or AlexaFluor 594-conjugated goat anti-mouse IgG<sub>2a</sub> (red fluorescent signal). Nuclei were labeled with DAPI (1.5 µg/µl) (blue fluorescent signal). Each image is representative of two independent transfections. Scale bar in A= 10 µm for A-F.
Figure 10. Overexpression of N-terminal V5-fusion protein constructs in hARPE-19 cells. (A) Wild-type (wt) pcDNA3.1/nV5 (V5)-hCRADD full-length (FL) displays cytosolic and minor nuclear distribution. (B) V5-hCRADD FL Gly128Arg displays cytosolic and minor nuclear distribution. (C) V5-hCRADD FL Gly128Arg forms CRADD clusters in 27.5% of cells overexpressing CRADD Gly128Arg (n=40 cells). (D) wt V5-hCRADD DD localizes to the cytosol and the nucleus. (E) V5-hCRADD DD Gly128Arg localizes to the cytosol and the nucleus. (F) non-transfected cells, used as a control and labeled with anti-FLAG and anti-V5 primary antibodies and secondary antibodies to demonstrate no immunoreactivity. Each image is representative of two independent transfections. Primary antibody: anti-V5 mouse monoclonal IgG<sub>2a</sub> (1:500). Secondary antibody: AlexaFluor 594-conjugated goat anti-mouse IgG<sub>2a</sub> (1:400) (red fluorescent signal). Nuclei labeled with DAPI (1.5 μg/μl) (blue fluorescent signal). Scale bar in A = 10 μm for A-F.
Figure 11. Co-overexpression of wild-type (wt) V5-hCRADD or V5-hCRADD Gly128Arg with FLAG-hPIDD death domain (DD) in hARPE-19 cells. (C) and (F) are merged images from (A-B) and (D-E). (A) FLAG-hPIDD DD localizes to the cytosol when co-overexpressed with wt V5-hCRADD. (B) wt V5-hCRADD localizes to the cytosol when co-overexpressed with FLAG-hPIDD DD. (C) wt V5-hCRADD co-overexpression with FLAG-hPIDD DD displays co-localization in the cytosol. (D) FLAG-hPIDD localizes to the cytosol when co-overexpressed with V5-hCRADD Gly128Arg. (E) V5-hCRADD Gly128Arg forms clusters when co-overexpressed with FLAG-hPIDD DD. (F) V5-hCRADD Gly128Arg forms clusters (red) when co-overexpressed with FLAG-hPIDD DD (green). Non-transfected cells not shown—see Figure 10F from the same transfection. Each merged image is representative of two independent transfections. Primary antibodies: anti-FLAG M2 mouse monoclonal IgG$_1$ (1:1000) and anti-V5 mouse monoclonal IgG$_{2a}$ (1:500). Secondary antibodies: AlexaFlour 488-conjugated goat anti-mouse IgG$_1$ (1:400) (green fluorescent signal), AlexaFluor 594-conjugated goat anti-mouse IgG$_{2a}$ (1:400) (red fluorescent signal). Nuclei were labeled with DAPI (1.5 µg/µl) (blue fluorescent signal). Scale bar in A = 10 µm for A-F.
Table 1. Average percent of hARPE-19 cells overexpressing wild-type V5-hCRADD or V5-hCRADD Gly128Arg.

<table>
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<th>Overexpressed Fusion Protein Constructs</th>
<th>% Overexpression</th>
<th>Standard Deviation</th>
<th>% Change</th>
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<tr>
<td>wt V5-hCRADD</td>
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<tr>
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<tr>
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<tr>
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Figure 12. Western blotting of overexpressed V5-hCRADD fusion protein constructs in hARPE-19 cells. (A) Wild-type pcDNA3.1/n-V5 (V5)-hCRADD full-length (FL) and V5-hCRADD FL Gly128Arg overexpressed as indicated. Overexpressed hCRADD FL Gly128Arg was 82.9 ± 2.3% (n=3 transfections) less abundant relative to wild-type. (B) Wild-type V5-hCRADD death domain (DD) and V5-hCRADD DD Gly128Arg overexpressed as indicated. CRADD DD Gly128Arg was 98.9% (n=1 transfection) less abundant relative to wild-type hCRADD DD. β-actin was used as a loading control. Primary antibodies: anti-V5 mouse monoclonal IgG2a (1:1500), anti-β actin (1:1,000,000), & HRP-conjugated anti-biotin (1:2500). Secondary antibody: HRP-conjugated goat anti-mouse IgG (1:1500).
Figure 13. Co-immunoprecipitation and western blotting of overexpressed wild-type FLAG-mCradd death domain (DD) or FLAG-mCradd DD Gly128Arg with V5-mPidd DD in mIMCD3 cell lysates using FLAG M2-conjugated agarose beads. Wild-type FLAG-mCradd DD co-immunoprecipitates V5-mPidd DD. FLAG-mCradd Gly128Arg does not co-immunoprecipitate V5-mPidd DD.

Primary antibodies (as indicated): anti-FLAG mouse monoclonal IgG1 (1:1000) or anti-V5 mouse monoclonal IgG2A (1:5000), and HRP-conjugated anti-biotin (1:2500). Secondary antibody: HRP-conjugated goat anti-mouse IgG (1:1500).
Figure 14. Model of caspase recruitment domain (CARD) and death domain (DD) (CARD:DD) CRADD homo-oligomerization. (A) Model of wild-type (wt) interaction between CRADD CARD:DD within wt CRADD. Under normal conditions, CRADD is in a folded conformation that prevents inappropriate oligomerization of CRADD CARD with other CARD domains (Shearwin-Whyatt et al., 2000), such as the CARD of caspase-2. DD signaling is predicted to induce a conformational change in CRADD, opening the folded unit, and allowing CRADD CARD and caspase-2 CARD to interact (Shearwin-Whyatt et al., 2000). (B) Potential conformation of CRADD Gly128Arg under normal conditions, exposing CRADD DD and CARD surfaces and allowing for oligomerization with other CRADD CARD domains and homotypic binding between CRADD DD. Death signaling as a result of overexpressed PIDD may exacerbate homo-oligomerization because under normal conditions, death signals through the DD induce conformational change. Therefore, even though PIDD may not interact with CRADD Gly128Arg, its’ proximity may be enough to send death signals that would promote homo-oligomerization of CRADD.
Figure 15. (A) Model of wild-type PIDDosome structure based upon the crystal structure of the PIDDosome, as determined by Park et al. (2007). Seven CRADD death domains (DD) interact with five PIDD DD to facilitate interaction between the caspase recruitment domain (CARD) of caspase-2 and the CARD of CRADD. (B) One potential impact of CRADD Gly128Arg on formation of the PIDDosome. The Gly128Arg variant may increase CRADD DD homo-oligomeric binding and decrease CRADD DD affinity for PIDD. As a result, the impaired interaction between CRADD DD and PIDD DD may disrupt PIDDosome formation and subsequent PIDDosome/caspase-2-mediated apoptosis.