Ubiquitylation of Neuronal Pentraxin with Chromo Domain by the E3 Ubiquitin Ligase Mayven/KLHL2 and Effects on Aggresome Formation and Neuronal Cytotoxicity

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UBIQUITYLATION OF NEURONAL PENTRAxin WITH CHROMO DOMAIN BY THE E3 UBIQUITIN LIGASE MAYVEN/KLHL2 AND EFFECTS ON AGGRESOME FORMATION AND NEURONAL CYTOTOXICITY

By
LeinWeih Andrew Tseng

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Neuronal pentraxin with chromo domain (NPCD) belongs to a family of neuronally-expressed pentraxin proteins thought to be involved in synaptic refinement and plasticity. One isoform of Npcd, neuronal pentraxin receptor (NPR), is a type-II transmembrane protein responsible for the clustering of related neuronal pentraxins 1 and 2. However, recently identified cytosolic NPCD isoforms with no known function were discovered through their interaction with the intracellular domain of a receptor protein tyrosine phosphatase PTPRO. PTPRO is a signaling molecule known to be involved in the development of the nervous system. Additionally, upregulated expression of neuronal pentraxins has been implicated in neuronal cytotoxicity and associated with neurodegenerative diseases. Here, we demonstrate that a novel cytosolic NPCD isoform interacts with the BTB-Kelch protein Mayven/KLHL2. This interaction was identified through a yeast two-hybrid screen using the C-terminal pentraxin domain region of NPCD and confirmed through mammalian cell colocalization and co-immunoprecipitation studies. Domain truncation analysis suggests that the kelch domains of Mayven/KLHL2 are responsible for this interaction with NPCD. We also
show that Mayven/KLHL2 is capable of interacting with Cullin 3, an integral protein in the Cullin-RING ubiquitin ligase complex. An in-vivo ubiquitylation assay demonstrates that overexpression of Mayven/KLHL2 increases NPCD ubiquitylation, and suggests a novel E3 ubiquitin ligase function of Mayven/KLHL2 with NPCD as its substrate. Furthermore, we observed an increased propensity of overexpressed NPCD to form aggresomes with coexpression of Mayven/KLHL2. As the formation of aggresomes is often associated with protein aggregation and deposition diseases, including a multitude of neurodegenerative diseases, we tested NPCD overexpression and the effects of Mayven/KLHL2 coexpression on neuronal cytotoxicity and apoptosis. Overexpressed NPCD in hippocampal neuron cultures resulted in increased cytotoxicity and apoptosis, further exacerbated by Mayven/KLHL2 coexpression. Our findings report an interaction between NPCD and Mayven/KLHL2, demonstrate a novel role of Mayven/KLHL2 as an E3 ubiquitin ligase, and explore a possible intersection between the ubiquitin-proteasome degradation pathway, neuronal pentraxins, and neurodegenerative disease.
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Chapter I: Introduction

Receptor Protein Tyrosine Phosphatases and PTPRO

Protein phosphorylation is an essential biochemical process that regulates diverse and numerous cellular functions. Phosphorylation has been shown to affect enzymatic activity as well as protein conformation and binding in a wide range of substrates. The addition and removal of phosphate groups from amino acids (usually serine, threonine, and tyrosine in eukaryotic proteins) is mediated by kinases and phosphatases, respectively. A subset of the protein phosphatases, the receptor protein tyrosine phosphatases (RPTPs), are a family of type-I transmembrane proteins with either one or two intracellular tyrosine phosphatase domains and extracellular domains reminiscent of cell-adhesion molecules (Bixby, 2001; Stoker, 2005). As their name suggests, RPTPs are thought to receive signals via their extracellular domains like receptors, and transduce those signals into intracellular signaling cascades by way of their tyrosine phosphatase activity.

RPTPs are further grouped in several sub-families based on the similarities of their non-catalytic extracellular domains. For example, type IIa RPTPs have extracellular domain structures that are similar to immunoglobulin superfamily cell adhesion proteins, and type III RPTPs have an extracellular domain consisting of fibronectin type III repeats (Figure 1.1). These two sub-families, type IIa and type III, and others (including type IIb and type IV) have been implicated as having an important role in both invertebrate and vertebrate neural development (Bixby, 2001). Genetic experiments in Drosophila have
shown that type IIa and type III RPTP knockouts display disrupted CNS and motor axon pathfinding as well as other axonal growth and guidance defects (Alonso et al., 2004; Stoker, 2005). Several type II RPTP knockouts in mice (including PTPRD/LAR and PTPRS/PTPsigma) have shown defects in motor and proprioceptive function, as well as aberrant neuronal development (Wallace et al., 1999; Yeo et al., 1997). It is thought that the loss of RPTP expression in areas of the brain mediating these functions (including the dorsal root ganglion, spinal motor neurons, cerebellum, forebrain, and hippocampus) leads to the disrupted function and/or aberrant growth and guidance of these neuronal populations, resulting in these phenotypes (Uetani et al., 2000; Wallace, et al., 1999; Yeo, et al., 1997). While less is known about the role of type III RPTPs in neural development, both in-vitro and in-vivo studies have suggested a role of PTPRO, a type III RPTP, in the developing chick and mouse nervous systems. PTPRO is a neuronally expressed and developmentally regulated RPTP that has been shown to act as a repulsive guidance cue and is critical in chick motor axon development (Bodden & Bixby, 1996; Stepanek, Stoker, Stoeckli, & Bixby, 2005; Stepanek, Sun, Wang, Wang, & Bixby, 2001). PTPRO has also been demonstrated to regulate Eph receptor activity in retinal axons and is involved in sensory nervous system development and function (Gonzalez-Brito & Bixby, 2009; Shintani et al., 2006). While these findings strongly suggest an important role for PTPRO in neuronal development, the pathways through which PTPRO mediates axonal growth and guidance effects are still mostly undiscovered. In the hopes of identifying a downstream signaling partner for PTPRO, a yeast two-hybrid screen was performed using the intracellular phosphatase domain of PTPRO (Chen & Bixby, 2005a).
A protein belonging to the neuronal pentraxin family was identified: neuronal pentraxin with chromo domain (NPCD).

**Neuronal Pentraxins and NPCD**

The pentraxins are an evolutionarily conserved family of proteins first identified by their ability to multimerize into a 5 member (pentameric) radial structure (Osmand et al., 1977). Pentraxins are divided up into two main groups: the classical “short” pentraxins consisting of serum amyloid P (SAP) component and C-reactive protein (CRP), or the more recently discovered “long” pentraxins including pentraxin 3 (PTX3), apexin, neuronal pentraxin 1 (NP1), neuronal pentraxin 2 (NP2), and neuronal pentraxin receptor/neuronal pentraxin with chromo domain (NPR/NPCD). Classical “short” pentraxins are best known for their role as pattern recognition receptors in the innate immune system. Both serum amyloid P component (SAP) and C-reactive protein (CRP) are secreted proteins produced mainly in the liver that recognize and bind to pathogens in order to aid in complement deposition and phagocytosis (Agrawal, Singh, Bottazzi, Garlanda, & Mantovani, 2009; Deban, Bottazzi, Garlanda, de la Torre, & Mantovani, 2009; Yuste, Botto, Bottoms, & Brown, 2007). Additionally, CRP and the long pentraxin PTX3 have been shown to mediate the recognition and phagocytosis of dying (apoptotic) cells (Baruah et al., 2006; Gershov, Kim, Brot, & Elkon, 2000). Apexin is a long pentraxin discovered in the guinea pig sperm acrosome (Reid & Blobel, 1994). The acrosome is an organelle derived from the Golgi apparatus that forms in the anterior region of the spermatozoa head, and functions in dissolving the outer membrane of the ovum during fertilization. While the reason for the presence of apexin in the acrosome is
still unknown, apexin is shown to covalently oligomerize and is easily purified in stable protein aggregates (Noland, Friday, Maulit, & Gerton, 1994). Both the short and long pentraxins share a highly conserved C-terminal pentraxin domain, with the “long” pentraxins containing an extended and a more variable N-terminal region. While PTX3 is involved in the innate immune system and apexin has no known function, a subset of the long pentraxins expressed in the brain, known as the neuronal pentraxins (NP1, NP2, and NPR/NPCD), are reported to have a role in synaptic development and maintenance.

NP1 and NP2 are secreted proteins that have been shown to cluster post-synaptic AMPA-type glutamate receptors and promote synaptogenesis (O'Brien et al., 1999; Sia et al., 2007; D. Xu et al., 2003). Knockdown of NP1 in cultured hippocampal neurons (cocultured with glial cells transfected with glutamate receptors) resulted in reduced glial cell glutamate receptor recruitment to the artificial synapses formed (Sia, et al., 2007). Overexpression of NP2 and dominant-negative NP2 in cultured spinal neurons showed increased and decreased levels of AMPA-type glutamate receptor clustering, respectively (O'Brien, et al., 1999). These results demonstrate NP1 and NP2 function in the clustering of post-synaptic AMPA-type glutamate receptors. NP1/NP2 knockout mice show aberrant electrophysiological activity in retinal ganglion cells and defective patterning of retinal axonal projections to the lateral geniculate nucleus (Bjartmar et al., 2006). This result suggests that NP1 and NP2 are involved in regulating retinal activity during development, possibly through their effect on glutamate receptor activity. Regulation of glutamate receptor activity may in turn affect synapse refinement through activity-dependent degradation and turnover of synaptic sites. It is believed that these two secreted proteins, NP1 and NP2, are tethered to the cell surface through
heteromultimerization with the membrane-bound NPR protein (Kirkpatrick, Matzuk, Dodds, & Perin, 2000).

NPR is a type-II transmembrane protein first identified through purification on an affinity column with the snake venom neurotoxin taipoxin (Dodds, Omeis, Cushman, Helms, & Perin, 1997). Sharing approximately 50% amino acid identity with NP1 and NP2, NPR has an extremely short N-terminal intracellular domain (~6 amino acids), a transmembrane domain, and an extracellular region containing a pentraxin domain (Dodds, et al., 1997). As NPR is believed to tether NP1 and NP2 to the cell membrane surface, a role in promoting AMPA-type glutamate receptor clustering and synaptogenesis has also been reported. In an in-vitro artificial synapse formation system, knockdown of NPR in hippocampal neurons is capable of reducing glutamate receptor clustering at the artificial synapse formed with co-cultured glial cells transfected with GluR4 (Sia, et al., 2007). However, recent findings suggest that the cleavage and release of NPR by the tumor necrosis factor-α converting enzyme (TACE) may be essential to endocytosis and internalization of post-synaptic glutamate receptors, giving NPR a dual role in both the formation and negative regulation of synapses (Cho et al., 2008).

While most of the literature continues to refer to the NPR protein as the single transcript from the neuronal pentraxin receptor (Nptxr) gene, our lab has reported the existence of several alternatively-spliced cDNA transcripts combining sequence from the Nptxr gene and the adjacent gene on the chromosome, chromobox homolog 6 (Cbx6) (Chen & Bixby, 2005a). Consequently, these two genes were combined as one and renamed neuronal pentraxin with chromo domain (Npcd). Two NPCD isoforms represent the two originally described protein products, NPR and CBX6 (Figure 1.2, Isoforms I and
II). Four novel NPCD isoforms lack the transmembrane domain and represent cytosolic isoforms of NPCD with no known function (Figure 1.2, Isoforms III, IV, V, and VI). Three of these cytosolic NPCD isoforms combine portions of both CBX6 and NPR (Figure 1.2, Isoforms IV, V, and VI) (Chen & Bixby, 2005a).

Functions for the previously identified NPCD isoforms have been described. As mentioned before, the membrane-bound and mostly extracellular NPR isoform of Npcd has a role in AMPA-type glutamate receptor clustering with effects on synapse formation and maintenance (Bjartmar, et al., 2006; Cho, et al., 2008; Kirkpatrick, et al., 2000; Sia, et al., 2007). The CBX6 isoform of Npcd lacks both the pentraxin domain and the transmembrane domain, but contains a chromatin organizer modifier (chromo) domain. Proteins that contain a chromo domain belong to the polycomb or chromobox family of proteins. Members of this family are best known for their role in regulating expression of homeotic genes, and recent findings suggest an expanded role in epigenetic transcriptional regulation and repression, mediated through their chromo domain interactions with methylated histones (Bernstein et al., 2006; Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001; Ruddock-D'Cruz et al., 2008; Vincenz & Kerppola, 2008).

CBX6 is reported to be localized in the nucleus (Bernstein, et al., 2006; Vincenz & Kerppola, 2008), however the function of CBX6 in transcriptional regulation during development is unknown.

Of the four remaining novel NPCD isoforms, three contain both the chromo and pentraxin domains (Figure 1.2, Isoforms IV, V, and VI). While these cytosolic NPCD isoforms have no known function, they were discovered by our lab through their interaction with the intracellular phosphatase domain of the type III RPTP PTPRO (Chen
& Bixby, 2005a). Subsequently, they were shown to be important in nerve growth factor (NGF)-induced process outgrowth in pheochromocytoma (PC12) cells (Chen & Bixby, 2005b). The PC12 cell line is derived from a rat adrenal pheochromocytoma and responds to NGF by differentiating into neuronal-like cells with processes (Greene & Tischler, 1976). After siRNA knockdown of NPCD (targeting sequence found in the pentraxin domain), PC12 cell process outgrowth in response to NGF treatment was abolished (Chen & Bixby, 2005b). These knockdown results in PC12 cells, taken together with the interaction of NPCD with the developmentally-important RPTP PTPRO, suggest a role for cytosolic NPCD isoforms in neurite outgrowth and neuronal development. How the newly identified cytosolic NPCD isoforms function, and with what proteins they interact with, is currently unknown and unlikely to mirror the differentially localized NPR or CBX6 Npcd isoforms.

In addition to reported functions in synaptic development and maintenance, as well as a putative function in neurite outgrowth, neuronal pentraxins are implicated in neuronal cell death and neurodegeneration. Overexpression of NP1 in cerebellar granule cells results in apoptotic neuronal cell death (DeGregorio-Rocasolano, Gasull, & Trullas, 2001). Furthermore, NP1 expression is increased in cultured cortical neurons after exposure to the Alzheimer’s disease-associated protein amyloid-beta (Aβ). Increased NP1 expression resulted in a loss of synapses, reduction in neurite outgrowth, and apoptotic neuronal death (Abad, Enguita, DeGregorio-Rocasolano, Ferrer, & Trullas, 2006). Prevention of the Aβ-mediated increase in NP1 expression (through shRNA knockdown of NP1) abrogated these neurotoxic effects and overexpression of NP1 alone mimicked them (Abad, et al., 2006).
Protein levels of NP1 were found to be increased in the brains of patients with Alzheimer’s disease (AD) as well as in AD mouse models; in these situations, NP1 protein was found to be associated with tau protein deposits and amyloid plaques in or around dystrophic neurites (Abad, et al., 2006). These results together suggest NP1 may be responsible for mediating Aß-mediated neurodegeneration in AD.

NP2 is the most highly upregulated gene in the substantia nigra of patients with Parkinson’s disease (PD) (Moran, et al., 2008). Additionally, NP2 protein was found to accumulate with alpha-synuclein in Lewy bodies, an abnormal PD-associated protein aggregate found inside of nerve cells. The role of NP2 in PD is not well understood. Based on the only reported function of NP2 (AMPA-type glutamate receptor clustering), the authors hypothesis a role for excitotoxicity in PD; that is, increased NP2 may lead to increased glutamate receptor clustering and activity, resulting in excitotoxic neuronal cell death (Moran, et al., 2008).

The role of NPCD in a neurodegenerative context is less well understood. While increased levels of the NPR isoform of NPCD are found in the cerebrospinal fluid (CSF) of AD patients, the reasons behind this increase are unknown (Yin, Lee, Cho, & Suk, 2009). One possibility is that neuronal populations expressing NPR are undergoing neurodegeneration and releasing their cellular contents into the CSF in death. However, another explanation may be related to the cleavage and release of NPR from the cellular membrane by TACE (Cho, et al., 2008). TACE is shown to cleave the full length ~62 kDa NPR protein in one of two extracellular sites: cleavage at site A (L36) results in a ~60 kDa NPR fragment lacking the N-terminal intracellular and transmembrane domains and cleavage at site B (D176) results in a ~50 kDa NPR fragment; both fragments contain the
pentraxin domain (Cho, et al., 2008). While a portion of the cleaved product was shown to stay associated with the cell membrane, NPR was also detected in early endosomes with internalized glutamate receptors. Additionally, some cleaved NPR was detected in the culture medium (Cho, et al., 2008) allowing for the possibility of cleaved NPR to be released into the CSF. The authors speculate that cleavage of NPR plays an important role in endocytosis of glutamate receptors and as an extension, synaptic plasticity. Although the role of dysfunctional glutamate receptor activity is implicated in AD (H. G. Lee et al., 2002), how this leads to increased NPR in the CSF is unclear.

The effects on glutamate receptor clustering and internalization are likely limited to the NPR isoform of Npcd, as differences in localization suggest cytosolic NPCD isoforms act in a distinct manner. However, no clear role of the cytosolic NPCD isoforms is described in a neurodegenerative context.

**Functions and Mechanisms of Ubiquitylation**

Ubiquitylation is a conserved biological process involved in the post-translational modification of a large number of eukaryotic cellular proteins (Chan & Hill, 2001; Ciechanover, 2005; Hershko & Ciechanover, 1986, 1992; Hicke, 2001; Marfany & Denuc, 2008; Pickart, 2001; Schwartz & Ciechanover, 1999). Ubiquitylation entails the covalent attachment of the 76-amino acid ubiquitin protein to a lysine residue on the protein substrate. Best known for targeting proteins for proteasomal degradation, several ubiquitin moieties are attached to a protein in the form of a polyubiquitin chain that is then directly recognized by the proteasome or by an adapter to the proteasome. The generally accepted model of polyubiquitin chain attachment involves the sequential
addition of ubiquitin proteins to the distal end of the growing ubiquitin chain. In the case of polyubiquitin chain addition, the terminal \( G^{76} \) of the unbound ubiquitin protein is covalently attached to one of the seven lysine residues on the distal ubiquitin protein of the chain. The most common ubiquitin lysine residue used in polyubiquitin chain addition is \( K^{48} \); four attachments of this type efficiently target the substrate protein for proteasome degradation (Thrower, Hoffman, Rechsteiner, & Pickart, 2000). Other types of polyubiquitin chain linkage such as \( K^{63} \) or monoubiquitylation can result in consequences other than direct proteasome degradation. \( K^{63} \) linkage has been shown to affect trafficking of the GAP1 permease into multi-vesicular bodies (Lauwers, Jacob, & Andre, 2009) and implicated in cellular functions such as DNA repair (Hofmann & Pickart, 1999), NF-\( \kappa \)B activation (Deng et al., 2000), and receptor endocytosis (Galan & Haguenauer-Tsapis, 1997). Monoubiquitylation of either one or several lysine residues on the protein substrate is also a potent signaling mechanism, leading to internalization and lysosomal degradation of both the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Haglund et al., 2003). Monoubiquitylation is also implicated in cellular functions such as cell cycle progression through histone monoubiquitylation (Robzyk, Recht, & Osley, 2000) and endocytosis leading to lysosomal degradation of other transmembrane proteins, including growth hormone receptor (Strous, van Kerkhof, Govers, Ciechanover, & Schwartz, 1996), the epithelial Na\(^+\) channel (Staub et al., 1997), and the colony-stimulating factor-1 receptor (P. S. Lee et al., 1999). While ubiquitin is found only in eukaryotes, a ubiquitin-like process is used in some prokaryotes to attach the prokaryotic ubiquitin-like protein (PUP) to a lysine residue on the protein substrate (pupylation). Pupylation has been shown to
lead to protein degradation, similar to that seen with ubiquitin (Pearce, Mintseris, Ferreyra, Gygi, & Darwin, 2008). However, polypuppylation (the addition of multiple PUP proteins in a chain, similar to polyubiquitylation) has not been reported, and whether puppylation regulates pathways other than proteasome degradation is unknown.

Since the discovery of ubiquitin, a larger family of ubiquitin-like proteins (Ubls) has been identified, utilizing similar chemistry, enzymes, and mechanisms to target proteins for degradation, effect protein localization, or regulate protein activation. Best studied of these Ubls includes the “small ubiquitin-like modifier” (SUMO) in sumoylation and “neural precursor cell expressed developmentally down-regulated protein 8” (NEDD8) in neddylation. Sumoylation was first identified in the cytosolic-to-nuclear pore trafficking of RanGAP1 (Mahajan, Delphin, Guan, Gerace, & Melchior, 1997), and has subsequently been shown to have a role in transcriptional regulation, protein stability, progression through the cell cycle, and apoptosis (Wilkinson & Henley, 2010). In neddylation, the first identified substrates were Cdc53 in *Saccharomyces cerevisiae* (Lammer et al., 1998) and cullin 4A in humans (Osaka et al., 1998). Both proteins are members of the cullin protein family, and neddylation was subsequently shown for all known yeast and mammalian cullin proteins, including cullin 3 (Rabut & Peter, 2008). Cullin proteins are involved in the addition of ubiquitin protein to substrates: the post-translational attachment of NEDD8 to cullin proteins is thought to stimulate their activity, promoting ubiquitylation (Merlet, Burger, Gomes, & Pintard, 2009).

The mechanism of ubiquitylation is a well-characterized multi-step process that involves three ubiquitin enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-
conjugating enzyme, and the E3 ubiquitin ligase (Hershko & Ciechanover, 1986, 1992; Pickart, 2001; Scheffner, Nuber, & Huibregtse, 1995). The ubiquitin protein is first adenylated at the C-terminal end by the E1 ubiquitin-activating enzyme in an ATP-dependent reaction. The high-energy thioester bond formed is immediately transferred to the cysteine at the active site of the E1 ubiquitin-activating enzyme. The activated ubiquitin protein is then transferred to the active site cysteine of the E2 ubiquitin-conjugating enzyme. From here, ubiquitin is transferred from the E2 ubiquitin-conjugating enzyme to the protein substrate through the action of the E3 ubiquitin ligase. E3 ubiquitin ligases belong to one of two major classes: the “homologous to E6-AP C-terminus” (HECT) type, or the “really interesting new gene” (RING) type (Pickart, 2001; You & Pickart, 2001). In the case of HECT type E3 ubiquitin ligases, the E2 ubiquitin-conjugating enzyme directly transfers the activated ubiquitin protein to the active site cysteine in the HECT domain of the E3 ubiquitin ligase. From here, the E3 ubiquitin ligase binds to and directly transfers the ubiquitin moiety to the protein substrate (You & Pickart, 2001). In contrast, with RING type E3 ubiquitin ligases, the ubiquitin protein is directly transferred from the E2 ubiquitin-conjugating enzyme to the protein substrate.

RING type E3 ubiquitin ligases are often multi-protein complexes that function as substrate adapters by bringing the E2 ubiquitin-conjugating enzyme and the protein substrate into close proximity. While there are only a handful of E1 ubiquitin-activating enzymes and approximately ~40-50 E2 ubiquitin-conjugating enzymes, there are over 600 proteins predicted to have an E3 ubiquitin ligase function in the human genome (W. Li et al., 2008). These numbers suggest that E3 ubiquitin ligases are capable of selectively targeting a large number of proteins for ubiquitylation, and regulating a wide
range of cellular processes. The largest subset of RING type E3 ubiquitin ligases are the cullin-RING ligases (CRLs). The discovery of the first CRL was simultaneously reported by several groups and the associated RING finger protein was named “RING-box protein 1” (RBX1), “regulator of cullins 1” (ROC1), and “high level expression reduces Ty3 transposition protein 1” (HRT1) by the different teams (Kamura et al., 1999; Ohta, Michel, Schottelius, & Xiong, 1999; Seol et al., 1999; Tan et al., 1999). For clarity, we will refer to this protein as simply ROC1. All four groups’ initial findings demonstrated binding of ROC1 to cullin 1 (CUL1). Furthermore, these groups described the activation of the E2 ubiquitin-conjugating enzyme CDC34 by ROC1 in association with the SCF complex (a complex consisting of SKP1, CUL1, and a variable F-box protein). Together, ROC1 and the SCF complex were described as the first CRL. It was later shown that ROC1 was capable of interacting with all cullin proteins (Ohta, et al., 1999).

In the first identified CRL, CUL1 provides the structural backbone, bridging ROC1 and the attached E2 ubiquitin-conjugating enzyme (the “working” end, functioning in the attachment of ubiquitin to the substrate) to the “substrate identification end” consisting of SKP1 and the F-box protein. SKP1 binds to a variety of F-box proteins, and the numerous F-box proteins are responsible for identifying and binding to the substrates for ubiquitylation (Figure 1.3A) (Bai et al., 1996; Kipreos & Pagano, 2000). In the case of cullin 2 (CUL2), SKP1 function is replaced by the elongin C / elongin B protein complex and the F-box protein function is replaced by the numerous members of the SOCS-box protein family (Figure 1.3B) (Stebbins, Kaelin, & Pavletich, 1999). With cullin 3 (CUL3), the substrate adapter is a single protein with domains
mimicking the SKP1/elongin C and F-box/SOCS-box functions; these single substrate adapter proteins all share an N-terminal "broad complex, tramtrack and bric a brac" (BTB) domain, thought to function in the interaction with CUL3, and contain a C-terminal substrate recognition domain (Figure 1.3C) (Perez-Torrado, Yamada, & Defossez, 2006). While some of these BTB domain-containing proteins contain “meprin and TRAF homology” (MATH), ankryin, or zinc-finger domains as their C-terminal substrate recognition domain, a large number utilize the kelch domain (Perez-Torrado, et al., 2006). Proteins with both an N-terminal BTB domain and C-terminal kelch repeats belong to the BTB-Kelch family.

**BTB-Kelch Family Proteins and Mayven/KLHL2**

All BTB-Kelch family members contain the N-terminal BTB domain and several C-terminal kelch repeats. More recently described, the majority contain a “BTB And C-terminal Kelch” (BACK) domain.

The BTB domain, first identified in Drosophila zinc-finger transcription factors and involved in the recruitment of transcriptional protein complexes, is found in many proteins as a protein-interaction motif (Zollman, Godt, Prive, Couderc, & Laski, 1994). The varied and diverse functions of BTB domain-containing proteins range from involvement in transcriptional regulation, to ubiquitin-mediated degradation, to regulation of cell-cycle and actin dynamics (Perez-Torrado, et al., 2006; Stogios, Downs, Jauhal, Nandra, & Prive, 2005). This family’s involvement in many cellular functions is unsurprising in light of the identification of several hundred BTB domain-containing
proteins. Interestingly, both SKP1 and elongin C contain domains that share structural homology to the BTB domain (Stogios, et al., 2005; Zheng et al., 2002).

The kelch domain was first identified as regulating actin dynamics of the ring canal during oogenesis in *Drosophila* (Kelso, Hudson, & Cooley, 2002; Xue & Cooley, 1993). Since then, close to 100 kelch domain-containing proteins have been identified in a wide range of organisms, from a handful in yeast to over 71 in the human genome (Prag & Adams, 2003). Tandem kelch repeats are most often found as a single group of six (although the number ranges from 4-7) and these repeats form a structural domain known as a beta-propeller (Ito, Phillips, Yadav, & Knowles, 1994; X. Li, Zhang, Hannink, & Beamer, 2004; Prag & Adams, 2003). Kelch beta-propeller structural motifs share only modest sequence identity, but this diversity is thought to mediate a wide range of unique protein-protein interactions (Prag & Adams, 2003).

Found between the N-terminal BTB domain and the C-terminal kelch repeats, the BACK domain is a conserved motif found in the majority of BTB-Kelch proteins (Stogios & Prive, 2004). Since the BTB domain is thought to mimic the CUL3 binding activity of the SKP1 protein and the kelch repeats are thought to function like F-box proteins as the substrate adapter interface, the intervening BACK domain has no analogous function when compared to the SCF complex. However, the structural motifs predicted in the BACK domain suggest it mimics both the C-terminal end of SKP1 and the N-terminal region of F-box proteins, and may function by allowing for some structural flexibility in the BTB-Kelch protein. Stogios and Prive speculate that the structural flexibility afforded by the BACK domain is important in the positioning of the kelch domain-bound proteins in the CRL complex, however they also suggest that the
BACK domain may interact with the BTB domain for some unknown function (Stogios & Prive, 2004).

As mentioned above, BTB-Kelch family members have been recently described as having an important role in the process of ubiquitylation. At least thirteen BTB-Kelch proteins have been identified as having E3 ubiquitin ligase function with an identified substrate (Furukawa, He, Borchers, & Xiong, 2003; Petroski & Deshaies, 2005; Pintard, Willems, & Peter, 2004; Willems, Schwab, & Tyers, 2004; L. Xu et al., 2003). To name a few, KLHL8 targets rapsyn, a protein involved in nicotinic acetylcholine receptor clustering at the neuromuscular junction (Nam et al., 2009), KLHL12 targets both the D4 dopamine receptor and dishevelled (DSH) in the Wnt signaling pathway (Angers et al., 2006; Rondou, Haegeman, Vanhoenacker, & Van Craenenbroeck, 2008; Rondou et al., 2010), Gigaxonin/KLHL16 targets MAP1B light chain (Allen et al., 2005), KEAP1/KLHL19 targets NRF2, a protein involved in the cytoprotective response to environmental and endogenous stresses (Furukawa & Xiong, 2005), and KLHL20 targets DAPK, a protein involved in interferon-induced cell death (Y. R. Lee et al., 2010). Several other BTB-Kelch family members demonstrate CUL3 binding but lack an identified substrate, suggesting that these proteins, along with other family members yet to be tested, may also be involved in E3 ubiquitin ligase activity.

Early in this project, a protein-protein interaction screen identified the BTB-Kelch protein known as Mayven or kelch-like 2 (KLHL2) as interacting with NPCD. Mayven/KLHL2 was first identified as an actin-binding protein highly-expressed in the brain (Soltysik-Espanola et al., 1999). Northern blot analysis shows Mayven/KLHL2 mRNA present in the majority of human adult and fetal tissues tested, as well as a
number of cell lines including HEK293T cells (Soltysik-Espanola, et al., 1999; Zagranichnaya, Wu, Danos, & Villereal, 2005). In the brain, Mayven/KLHL2 mRNA is found most highly expressed in the hippocampus, as well as the amygdala, caudate nucleus, and corpus callosum (Soltysik-Espanola, et al., 1999). Mayven/KLHL2, like its BTB-Kelch family members, contains three domains: the N-terminal BTB domain, the BACK domain, and 6 C-terminal kelch repeats (Figure 1.4). The BTB domain of Mayven/KLHL2 has a reported function in mediating increased expression of c-Jun protein and increased transcriptional activity of activating protein 1 (AP-1) in MCF-7 and T47D breast cancer cells (Bu 2005). The kelch repeats of Mayven/KLHL2 have been reported to interact with actin and mediate oligodendrocyte process outgrowth (Jiang et al., 2005; Soltysik-Espanola, et al., 1999; Williams et al., 2005). However, no reported function of Mayven/KLHL2 in the context of CRLs or ubiquitylation has been described.

**Proteasome Degradation, Aggresomes, and Neurodegenerative Disease**

Proteolysis of polyubiquitylated proteins is often carried out by the 26S proteasome, whereby ubiquitin-conjugated proteins destined for degradation are recognized by the regulatory 19S particle of the 26S proteasome (Bochtler, Ditzel, Groll, Hartmann, & Huber, 1999). In an ATP-dependent manner, proteins undergo deubiquitylation (the removal of the polyubiquitin chain) and are partially unfolded; the removal of the bulky polyubiquitin chain and unfolding are both required for the protein to enter the limited internal space of the proteasome where proteolysis occurs (C. W. Liu et al., 2006). While the extent of unfolding necessary for translocation into the proteasome is unclear, disulfide bonds have been shown to be sufficient to inhibit
degradation (Wenzel & Baumeister, 1995). Once inside the barrel-shaped proteasome structure, internal catalytic sites are able to cleave peptide bonds to form short peptide strands that are released from the proteasome and further degraded into individual amino acids in a subsequent step (Voges, Zwickl, & Baumeister, 1999). In some cases, ubiquitylation and targeting to the proteasome is a necessary step in the processing of proteins into their active form. In the case of two budding yeast transcription factors, Spt23p and Mga2p (Zhang, Skalsky, & Garfinkel, 1999), it is believed a loop region of the protein enters the proteasome and is proteolytically processed, with the majority of the protein remaining outside (Rape & Jentsch, 2002, 2004). A homolog to these two yeast proteins, the transcription factor NF-κB, is also shown to be activated through this process; however, ubiquitylation of NF-κB is not required for its targeting to the proteasome (Rape & Jentsch, 2002). In certain cases, misfolded or damaged proteins become highly-oxidized and are targeted to the proteasome for degradation in both ubiquitin-independent and dependent processes (Iwai, 2003; Shringarpure, Grune, Mehlhase, & Davies, 2003).

Protein oxidation is the attachment of reactive oxygen species to amino acids of proteins. Reactive oxygen species are involved in many beneficial cellular processes, including cellular metabolism, the immune response, and redox signaling; however protein oxidation of proteins can sometimes lead to cross-linking and increased hydrophobicity, leading to aggregation and protein accumulation (Rada & Leto, 2008; Shringarpure, et al., 2003; Thannickal & Fanburg, 2000). An increase in protein oxidation has been reported as a result of increased levels of aberrant protein production (Dukan et al., 2000). Additionally, increased levels of oxidized proteins and
accumulation are associated with aging (Davies, 2001). Components of the cellular stress response, including the chaperone/heat shock protein HSP70, are recruited to these oxidatively damaged or misfolded proteins and target them for ubiquitylation and proteasomal degradation (Park et al., 2007). However, as mentioned previously, high levels of oxidation in these misfolded or damaged proteins can lead to cross-linking, resistance to degradation, and aggregation. The accumulation of these aggregates is proposed to be aging-related and implicated in neurodegenerative disease pathophysiology (Davies, 2001; Lehman, 2009; McNaught, Jackson, JnoBaptiste, Kapustin, & Olanow, 2006). In these situations, dysfunction of the ubiquitin-proteasome system, either through decreased activity (caused by pharmacological inhibition, genetic aberrations, or cellular stress) or increased production of misfolded or damaged proteins overloading the system, is thought to be responsible.

In either case, dysfunction of the ubiquitin-proteasome pathway can lead to the aggregation of proteins. Aggregated proteins may form degradation-resistant structures, and the cytosolic accumulation of these degradation-resistant protein aggregate structures can lead to the formation of insoluble inclusion bodies or deposits (Rubinsztein, 2006). One well-studied example of aggregation occurs with activation of the endoplasmic reticulum associated degradation pathway (ERAD), responsible for protein folding and quality control (Romisch, 2005). Misfolded integral membrane or secreted proteins are recognized as being irrevocably damaged or misfolded and transported out of the endoplasmic reticulum (ER) into the cytosol for proteasomal degradation (Kopito & Sitia, 2000). The rapid exposure of these often hydrophobic proteins to the cytosolic environment often leads to aggregation if the degradation pathway is overwhelmed or
impaired; these circumstances often occur in situations of cellular stress or in the case of mutated proteins (Markossian & Kurganov, 2004). Similarly, production of cytosolic proteins is tightly associated with quality control mechanisms; nascent chain binding proteins such as HSP70 and prefoldin work to prevent protein misfolding and aggregation (Hartl & Hayer-Hartl, 2002). Like the ERAD system, cytosolic proteins that are misfolded or otherwise aberrant are targeted for ubiquitylation and proteasome degradation. Work on the cystic fibrosis transmembrane conductance regulator (CFTR) first demonstrated what happens when this system is overwhelmed: cells with impaired or overwhelmed degradation machinery form structures known as aggresomes (Johnston, Ward, & Kopito, 1998).

Aggresomes are cytosolic protein inclusion bodies that are detergent-insoluble and contain high levels of polyubiquitylated proteins (Johnston, et al., 1998; Kopito & Sitia, 2000; Markossian & Kurganov, 2004). Aggresomes typically have a perinuclear localization and are found near the microtubule organizing center (Johnston, et al., 1998). Surrounded by a cage of the intermediate filament vimentin, the aggresome structure is formed by active transport of smaller protein aggregates along microtubule fibers using the dynein/dynactin motor and HDAC6 (Johnston, et al., 1998; Kawaguchi et al., 2003). Components of the ubiquitin-proteasome degradation pathway such as the proteasome and ubiquitin enzymes, as well as members of the protein folding machinery such as chaperones and heat shock proteins (including HSP40, HSP70, and HSP90) are found to be present in aggresomes in high levels (Markossian & Kurganov, 2004).

Aggresomes are most often seen in cultured cells, often with overexpression of wild-type or mutant disease-associated proteins. Some examples include alpha-synuclein
protein, associated with Parkinson’s disease (PD), huntingtin protein from Huntington’s
disease (HD), presenilin-1 in Alzheimer’s disease (AD), and prion protein in prion
disease (Beaudoin, Goggin, Bissonnette, Grenier, & Roucou, 2008). Aggresomes have
been connected with protein deposition diseases as early as their discovery in cystic
fibrosis, where the expression of CFTR with disease-associated mutations in cultured
cells results in inefficient protein folding, aggregation, and aggresome formation
(Johnston, et al., 1998). The expression of several of the above-mentioned diseases-
associated proteins in cultured neurons reveals the same phenotype in cultured cell
expression experiments. (Taylor, Hardy, & Fischbeck, 2002). As a result, the
relationship of aggresomes to the symptoms and progression of neurodegenerative
diseases is currently under considerable investigation.

These protein inclusions, either in the form of aggresomes in cultured cells or
plaques and deposits seen in diseased brains, were initially thought to be the cause of cell
death and neurotoxicity. Of the several tri-nucleotide repeat disorders, Huntington’s
disease (HD) is one of the most well-known (Walker, 2007). In HD, mutations in the
huntingtin protein (HTT) result in expanded glutamine repeats, resulting in protein that is
prone to aggregation. An increased length of the glutamine repeats, shown to increase
protein aggregation, was associated with earlier onset and higher penetrance of HD
symptoms (Andrew et al., 1993; Rubinsztein & Carmichael, 2003; Snell et al., 1993).
Another tri-nucleotide repeat disorder, spinal and bulbar muscular atrophy (SBMA)
involves expanded glutamine repeats in the androgen receptor (AR). Pharmacological
induction of the heat shock proteins, thought to prevent the aggregation of, or solubilize
misfolded mutant AR protein aggregates, alleviates cellular toxicity in SBMA disease
Overexpression of HSP70 has been shown to protect against neurodegeneration seen in another tri-nucleotide repeat disease, Spinocerebellar ataxia type 1 (SCA1), and is thought to act in the same manner on mutant ataxin-1 (Atx1) aggregates (Cummings et al., 2001). Therefore, preliminary evidence in the literature suggested that the products of protein aggregation, such as plaques or protein inclusions, were cytotoxic. As a result, the initial focus of developing treatments for protein deposition diseases was in the removal of protein plaques and deposits. However, recent studies suggest aggresome formation and protein aggregation may be more complex, and that aggresome formation may be a cellular cytoprotective measure (Olzmann, Li, & Chin, 2008). For example, the presence of alpha-synuclein and synphilin-1 containing aggresomes in cultured cells correlated with increased cell survival (Tanaka et al., 2004). Disrupting aggresome formation of mutant AR in cultured cells resulted in increased cytotoxicity (Taylor et al., 2003). These results suggest aggresomes may sequester cytotoxic protein aggregates and quarantine their deleterious effects. Whether aggresomes are truly analogous to protein deposits and plaques seen in neurodegenerative diseases is unknown.

One of the most promising AD treatment clinical trials involving the AN1792 vaccine recently reported disappointing findings: this vaccine, designed to induce the removal of Aβ plaques, was shown in both animal models and humans to be effective in the removal of plaques. However, the removal of plaques was unable to lessen the severity of symptoms and had no correlation with survival (Holmes et al., 2008). These findings have stimulated the investigation into the process of protein aggregation, as the
emerging evidence suggests that the presence of aggresomes (and as an extension, protein plaques and deposits) are not the underlying cause of neuronal degeneration.

As impairment of the proteasome may cause enhanced protein aggregation, investigation into whether proteasomal impairment is associated with aging revealed no link, at least in mouse aging models (Cook et al., 2009). Therefore, other contributors to cellular stress and proteasome dysregulation are currently being investigated.

While impairment and dysfunction of the proteasome machinery most likely contributes to protein aggregation, recent studies in the dysfunction of the ubiquitylation pathway have yielded potential neurodegenerative disease mechanisms. A direct role of ubiquitin pathway dysfunction is seen in PD: mutations in the parkin protein, an E3 ubiquitin ligase, lead to altered degradation of several substrates (including alpha-synuclein) resulting in aggregation and accumulation (Dawson & Dawson, 2010; Moore, 2006). The mouse neurological mutant, *lister*, was shown to have a mutation in LISTERIN, a RING type E3 ubiquitin ligase, and presented neurodegenerative symptoms such as dystrophic neurites, tau accumulation, and gliosis (Chu et al., 2009). Mutations in the E3 ubiquitin ligase malin (NHLRC1) are implicated in Lafora disease (LD), a fatal neurological disorder (Gentry, Worby, & Dixon, 2005). RNF182 is an E3 ubiquitin ligase upregulated in AD (Q. Y. Liu, Lei, Sikorska, & Liu, 2008), and a mutation in the E3 ubiquitin ligase Mahogunin results in a prion-like spongiform neurodegeneration (Kim, Olzmann, Barsh, Chin, & Li, 2007). Mutations in two BTB-Kelch family E3 ubiquitin ligases, Gigaxonin/KLHL16 and KLHL7, lead to giant axonal neuropathy and retinitis pigmentosa, respectively; both are neurological disorders (Allen, et al., 2005; Friedman et al., 2009). These results suggest that the ubiquitin pathway may be an
important target in the development of treatments for neurodegenerative diseases. Further strengthening this argument, proteins found in disease-associated protein deposits and plaques, including huntingtin protein (HD), prion protein (prion disease), and presenilin-1 (AD), are normal targets for ubiquitylation and proteasomal degradation (Kovacs, Lentini, Ingano, & Kovacs, 2006; Sieradzan et al., 1999; Yedidia, Horonchik, Tzaban, Yanai, & Taraboulos, 2001).

In our study, we identified and characterized a novel interaction between the neuronal pentraxin NPCD and Mayven/KLHL2. During the course of investigating the significance of this interaction, we discovered an E3 ubiquitin ligase function for Mayven/KLHL2. Additionally, we observed aggresome formation of overexpressed NPCD. These results, taken together with the emerging role of both neuronal pentraxins and ubiquitylation in neurodegenerative diseases, encouraged the study of NPCD and Mayven/KLHL2 in the context of neuronal cytotoxicity.
Chapter I Figures

Figure 1.1 - Receptor Protein Tyrosine Phosphatases (RPTPs). Type I – Type IV receptor protein tyrosine phosphatase families. FN = fibronectin type III domain, Ig = Immunoglobulin domain, MAM = meprin/A2/mu domain, D1/D2 = tyrosine phosphatase catalytic domain. Figure is adapted from Alonso et al., 2004.
Figure 1.2 - Npcd Gene Structure and Alternatively-Spliced NPCD Isoforms. Npcd consists of 10 exons spread over mouse chromosome 15. Exons 1 through 5 encode for the gene formerly known as Cbx6. Exons 6 through 10 encode for the gene formerly known as Nptxr. The transmembrane domain (TM) is located in exon 6, and the chromo domain (CD) and pentraxin domain (PTX) are found in exons 2/3 and 8/9/10 respectively. Due to the identification and consolidation of Nptxr and Chx6 into Npcd, the protein products of the original two genes are also known as NPCD Isoform I (NPR) and NPCD Isoform II (CBX6). NPCD Isoform III is similar to Isoform 1, but lacks the TM. NPCD Isoform IV, V, and VI combine regions containing both the CD and PTX domains. Figure is adapted from Chen & Bixby, 2005a.
Figure 1.3 – Cullin-RING Ubiquitin Ligases. (A) SCF complex with SKP1, Cullin1, and an F-box protein. Cullin 1 (CUL1) binds to the SKP1 and F-box proteins that form the substrate adapter complex. CUL1 also binds to ROC1 (labeled as ROC in figure) which binds to the E2 ubiquitin-conjugating enzyme. Neddlylation (NEDD8) of CUL1 promotes ubiquitylation activity. (B) Cullin 2 and Cullin 5 (CUL2.5) bind to elongin B/elongin C which bind to SOCS-box proteins to form the substrate adapter complex. (C) Cullin 3 (CUL3) is shown to bind to a single BTB-Kelch protein which is responsible for substrate adapter function. Figure is adapted from Barry & Fruh, 2006.
Figure 1.4 – Mayven/KLHL2 Protein Domain Structure. Mayven/KLHL2 consists of three known domains. The BTB (Broad Complex, Tramtrack, Bric a brac) domain, the BACK (BTB and C-terminal Kelch) domain, and 6 tandem kelch repeats.
Chapter II: Materials and Methods

Cell Culture

Human embryonic kidney cells (HEK293T) and COS-7 cells were acquired from the American Tissue Culture Collection. Both types of cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1x GlutaMAX (Invitrogen). Both HEK293T and COS-7 cells were passaged every three days at a 1:10 ratio using 0.05% Trypsin-EDTA (Invitrogen) and grown at 37°C at 5% CO2. Cells were routinely grown on tissue-cultured treated plastic in T75 flasks (BD Biosciences). However, for confocal microscopy experiments, HEK293T cells were grown on ethanol and UV sterilized glass coverslips. Briefly, glass coverslips were dipped in 95% ethanol and allowed to dry under UV light. Coverslips were washed three times with sterile water and once with pre-warmed cell culture media. In transfection experiments, cells were seeded into smaller tissue-culture treated plastic plates and dishes as required.

Primary hippocampal neurons were prepared as previously described (Moore et al., 2009) and grown in modified Enriched Neurobasal (ENB) media (Blackmore et al., 2010). Tissue-culture treated 12- or 24-well plates (BD Biosciences) were coated with PDL (10ug/ml in Hanks Buffered Salt Solution (HBSS) (Invitrogen), overnight at 4°C) and laminin (10ug/ml in HBSS (Invitrogen), four hours at RT). Neuronal media was equilibrated to 37°C and 5% CO2 for 4 hours before use. Hippocampal neurons were
allowed to grow for 8h before fixation (TUNEL assay) or 12h before staining (Live/Dead assay).

**Plasmids and Cloning**

The bait plasmid used in the NPCD yeast two-hybrid screen was pGBDU-C1 (James, Halladay, & Craig, 1996), a generous gift from Dr. Sandra Lemmon, Department of Molecular and Cellular Pharmacology, University of Miami. PCR amplification of NPCD sequence was performed using Taq polymerase (Invitrogen) and PCR product was ligated into pGEM-T Easy. PCR products in pGEM-T Easy were then cut out using appropriate restriction enzymes and ligated in frame into the MCS of the cut pGBDU-C1 vector. Plasmids were then sequenced to confirm sequence fidelity. Sequence from NPCD Exons 1-5 and 7-10 were used as bait proteins in the yeast two-hybrid screen.

The pCMV-Myc and pCMV-HA expression vectors were purchased from Clontech. Full length cDNA sequences for NPCD (Isoform IV, 1.1kb variant), Mayven/KLHL2, and KEAP1 were PCR-amplified using Phusion high-fidelity polymerase (New England BioLabs), cut with restriction enzymes, and directly ligated in frame into the MCS of both pCMV-Myc and pCMV-HA plasmids. Full length cDNA sequences of Mayven/KLHL2 and KEAP1, used as template for PCR-amplification, were obtained from the Open Biosystems MGC full-insert expression library.

Mayven/KLHL2 truncation mutants were constructed by PCR amplification from full length Mayven/KLHL2 cDNA. The following truncation mutant plasmids of Mayven/KLHL2 were constructed by Phusion PCR amplification, digestion with restriction enzymes, and ligation into the pCMV-Myc expression vector before vector
sequencing to verify proper insertion and sequence fidelity: Myc-BTB (BTB domain only), Myc-BTB-BACK (BTB and BACK domains), Myc-BACK-Kelch (BACK domain and kelch repeats), and Myc-Kelch (kelch repeats only).

HA-Cullin1, -Cullin2, and -Cullin3 were generous gifts from Dr. Mark Hannink (Department of Biochemistry, University of Missouri) and were constructed in the pCI-HA expression vector (Clontech).

Expression plasmids for fluorescently tagged NPCD domains (PTX-GFP and CD-GFP) for NPCD domain analysis were constructed using Taq PCR amplification, ligation into pGEM-T Easy, digestion with restriction enzymes, and ligation into the pEGFP-N2 expression vector (Clontech). Plasmids were sequenced to confirm sequence fidelity.

Expression plasmids for mCherry/mVenus-NPCD and mCherry/mVenus-Mayven/KLHL2 were constructed through Phusion PCR amplification, digestion with restriction enzymes, and direct ligation into a modified pSport6 expression vector (Invitrogen). The pSport6 vector was modified by the insertion of fluorescent-tag sequence (mVenus, accession: DQ092360; mCherry, accession: AY678264) into the MCS, followed by the in-frame insertion of full length NPCD (Isoform IV, 1.1kb variant) or full length Mayven/KLHL2 cDNA sequence. The Myc-Cherry expression plasmid was constructed by, and obtained from Dr. Murray Blackmore. The mVenus and mCherry control plasmids were the modified pSport6 expression vectors with mVenus or mCherry sequence but without a cDNA insert.

The HA-ubiquitin plasmid was purchased from Addgene (Addgene plasmid 17608), obtained from Ted Dawson (Lim et al., 2005).
Antibodies

Ubiquitin antibody (ab19247, Abcam), vimentin antibody (ab8545, Abcam), and HSP70 antibody (sc-65521, Santa Cruz) were purchased and used according to the manufacturer’s recommended protocols. E7 monoclonal beta-tubulin antibody (Developmental Studies Hybridoma Bank) was used for tubulin staining in COS-7 cells for NPCD domain expression experiments. Hoechst dye was used for nuclear staining. Myc-epitope (M4439, Sigma), HA-epitope (H9658, Sigma), and GAPDH (G8795, Sigma) antibodies were purchased and used according to manufacturer’s recommended protocols. Cullin3 antibody (ab1871, Abcam) was purchased used according to manufacturer’s recommended protocol. Alexa Fluor conjugated secondary antibodies were used with appropriate species targeting primary antibody: Alexa Fluor 488 (green) and Alexa Fluor 546 (red) were used in immunocytochemistry experiments. For western blot analysis, IRDye 700/800 secondary antibodies (Rockland) were used in the Odyssey Infrared Imaging System (LI-COR Biosciences).

Yeast Two-Hybrid Screen

An optimized reporter yeast strain pJ69-4A (MAT\(\alpha\) trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-AD\(E2\) met2::GAL7-lacZ) was used (James, et al., 1996) and was a kind gift from Dr. Sandra Lemmon (Department of Molecular and Cellular Pharmacology, University of Miami). The yeast two-hybrid screen was performed using the Mate & Plate Mouse Embryo 17-day cDNA library in yeast strain Y187 (MAT\(\alpha\) ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ met-gal80Δ MEL1 URA3::GAL1\(UAS\)-GAL1\(TATA\)-lacZ) from Clontech. To test for self-
activation, the NPCD chromo domain and pentraxin region bait plasmids were separately transformed into pJ69-4A and checked for growth on SD-Ura-Ade plates. The library plasmid used in the Mate & Plate Mouse Embryo 17-day cDNA library in yeast strain Y187 was pGADT7-Rec. Mating and selection were performed as recommended by the Mate & Plate cDNA library protocol. For the library screen, pJ69-4A transformed with NPCD bait plasmid was mated with the pre-transformed yeast two-hybrid cDNA library in Y187 (Clontech) and transformed diploids were first selected for on SD-Leu-Ura media before being streaked onto SD-Leu-Ura-Ade plates for selection of candidate clones expressing NPCD-interacting proteins. Candidate clones underwent a second round of positive selection by beta-galactosidase activity and negative selection using 5-FOA, which tests for self-activation of prey candidates by loss of the bait URA3 plasmid. Plasmid DNA was isolated from the positive clones and subjected to restriction digest analysis. Unique patterns of DNA digestion indicated clones to be grouped into candidate families. Two representative members from each candidate family were sequenced and gene names were identified through BLAST search of the NCBI nucleotide sequence database. Purified cDNA plasmids were co-transformed with the NPCD bait into pJ69-4A and rechecked for growth on SD-Leu-Ura-Ade media as confirmation.

**HEK293T and COS-7 Cell Transfection**

HEK293T and COS-7 cells were grown in supplemented DMEM as previously described. One day before transfection, cells were seeded and allowed to grow overnight to reach ~90% confluency by the next day. All cultured cell transfections were
performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommended protocol. Briefly, plasmid DNA and Lipofectamine 2000 were independently diluted in Opti-MEM (Invitrogen) and allowed to incubate for 5 minutes at RT. Diluted DNA and Lipofectamine were mixed and allowed to incubate at RT for 20 minutes. DNA/Lipofectamine mixture was then added directly to culture media. Media was not changed before fixation or cell lysis. Cells were regularly fixed or lysed at 10h, 20h, 24h, or 48h post-transfection.

**HEK293T Cell Lysis and Immunoprecipitation**

HEK293T cells were transfected in 6-well plates as described and allowed to grow for 10h, 20h, 24h or 48h (depending on the assay) before protein lysates were collected. Cells were rinsed with 1x room temperature PBS before cell lysis. Cells were lysed in 500ul of ice cold CelLytic M Cell Lysis Reagent (C2978, Sigma) with 1x protease inhibitor cocktail (Complete, EDTA-free, Roche) for 15 minutes on ice with gentle shaking before cells were scraped and collected into pre-chilled 1.5ml microcentrifuge tubes. Lysates were vortexed briefly to homogenize any remaining cells before pelleting of the insoluble fraction with centrifugation at 16,000xg at 4° C for 15 minutes. Cleared protein lysates were transferred to a fresh pre-chilled tube and 400ul was transferred to the immunoprecipitation spin column. The remaining protein lysate was stored at -80° C as the input fraction. 200ul of pre-chilled 1x IP buffer (anti-c-Myc Immunoprecipitation Kit, Sigma) with 1x protease inhibitor cocktail was added to the 400ul of protein lysate and 30ul of anti-c-Myc Affinity Gel (Sigma). Immunoprecipitation was carried out overnight at 4° C with gentle end-over-end shaking.
Washing of the anti-c-Myc Affinity Gel was carried out with pre-chilled 1x co-IP buffer with 1x protease inhibitor cocktail in a modified flow-through method. Spin columns were placed in ice and 500ul of wash buffer was added and allowed to drain through by gravity flow. 6ml of wash buffer for each column was used, resulting in 12 applications. The last wash step was carried out with 0.1x pre-chilled IP buffer with 1x protease inhibitor cocktail and the affinity gel was spun dry into a wash buffer collection tube. The spin column was then plugged and 100ul of pre-heated 2x Laemmli sample buffer with 5% beta-mercaptoethanol (BME) was applied to the anti-c-Myc affinity gel and allowed to incubate at 95°C for 10 minutes. Eluted proteins were spun down into a fresh 1.5ml microcentrifuge tube and placed on ice before directly loading onto the gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or stored at -80°C for future use.

In all co-immunoprecipitation experiments, cells were allowed to grow for 48h before cell lysis. Each well of cells in a 6-well plate was transfected with 4ug of plasmid DNA with equal amounts of both expression plasmids used. In the NPCD ubiquitylation assay and the in-vivo ubiquitylation assay with Mayven/KLHL2 and NPCD, cells were allowed to grow for 24h post-transfection before cell lysis. Each well of cells in a 6-well plate was transfected with 4ug of plasmid DNA with equal amounts of both expression plasmids used. Cells were harvested using a SDS lysis buffer (2% SDS, 1% NaCl, and 0.2% Tris-HCl, pH 8) at RT and incubated at 95°C for 20 minutes before being vortexed thoroughly. Cell lysate was diluted with the same lysis buffer without SDS to a final SDS concentration of 0.5% before use in the standard immunoprecipitation procedure. In the in-vitro ubiquitylation assay, a total of 5ug of plasmid DNA was used for transfection of each well of cells in a 6-well plate: 1ug of plasmid expressing HA-Ubiquitin, 2ug of
plasmid expressing a “substrate,” and 2μg of plasmid expressing an “effector.” At 24h post-transfection, cells were viewed using fluorescence microscopy to confirm expression of mVenus-tagged “effector” or “control effector” before protein lysates were harvested in a similar manner as described with the NPCD ubiquitylation assay. Lysis of cells for the NPCD protein expression level assay was modified to separate soluble from insoluble protein fractions. At 10h and 20h post-transfection, cells were lysed with CelLytic M Lysis Reagent as previously described. Soluble protein (supernatant) was removed after centrifugation at 16,000xg to pellet the insoluble fraction. The insoluble pellet fraction was subjected to three rounds of sonication (25s, 30% Duty Cycle, 60 watt output) and ultracentrifugation (100,000 rpm, Beckman Airfuge) in CelLytic M Lysis Reagent. The resulting pellet was dissolved in 2x Laemmli sample buffer with 5% BME at 95°C for 30 minutes with occasional vortexing. Samples were stored on ice before loading or stored at -80°C prior to use.

**Western Blot Analysis**

Protein lysate from each experiment was run on an 8% or 10% polyacrylamide gel (appropriately chosen for proper resolution of the proteins run) and resolved using SDS-PAGE. The protein ladder used was the Precision Plus Protein Standards All Blue (Bio-RAD). After SDS-PAGE, proteins were transferred in chilled transfer buffer (2.2% sodium bicarbonate in water) onto nitrocellulose membranes (Bio-RAD). Membranes were washed 1x with PBS and then blocked with near-infrared blocking buffer (Rockland) overnight at 4°C or 1h at RT before primary antibody addition. After primary antibody incubation (antibody diluted in blocking buffer and incubated overnight
at 4°C or 1h at RT), blots were washed 5x for 5min with PBS. Secondary antibody incubation (1:10,000 Rockland IRDye700/800 in PBS) occurred for 1h at RT, protected from light. Blots were washed 5x with PBS and visualized using the Odyssey Infrared Imaging System (LI-COR).

**Immunocytochemistry and Fluorescence Microscopy**

HEK293T and COS-7 cells were fixed by diluting fixative stocks solution (16% paraformaldehyde / 16% sucrose in PBS) into cell culture media to a concentration of 4% paraformaldehyde / 4% sucrose. Cells were fixed for 20 minutes at RT protected from light and washed 5 times with PBS. With cells expressing only fluorescent proteins, cells were incubated for a minimum of 1h with 1:5000 dilution of Hoechst nuclear stain in PBS and washed with PBS before visualization using fluorescence microscopy.

In NPCD and Mayven/KLHL2 colocalization experiments, 24h post-transfection, cells were trypsinized and replated at a lower density onto glass coverslips. Cells were allowed to adhere for 12h before fixing as normal. After Hoechst nuclear staining, coverslips were rinsed quickly with ddH2O before being mounted using Prolong Gold antifade reagent (Invitrogen) onto glass microscope slides and allowed to dry overnight at RT protected from light, before visualization using wide-field fluorescence and confocal microscopy. In aggresomal marker colocalization experiments, cells were incubated with blocking solution (2% fish gelatin and 0.03% Triton X-100 in PBS) overnight at 4°C before incubation with primary antibody in blocking solution. Primary antibody incubation occurred overnight at 4°C and cells were washed 5x with PBS before secondary antibody addition in blocking solution for 1h at RT, protected from light.
Cells were washed 5x in PBS and quickly rinsed in ddH₂O before mounting using Prolong gold antifade reagent. Microscope slides were allowed to dry overnight at RT, protected from light, before confocal microscopy. COS-7 cells were immunostained in a similar method to HEK293T cells, however cells were directly fixed, stained, and visualized on tissue-culture treated plastic and viewed using an inverted Nikon wide-field fluorescence microscope. Observation of aggresome formation was performed on fixed cells at 10h and 20h post-transfection. Cells were transfected at the same time, and fixed at the two time points. Cells were stored at 4°C in PBS with sodium azide as a preservative, protected from light. Aggresomes in time course experiments were identified based on morphology, brightness, and size. NPCD-containing aggresomes can range from small punctate dots near the nucleus (~1-2um in diameter) to large masses (~10-15um in diameter) occupying the cytoplasm. NPCD-containing aggresomes were observed to be cytosolic (non-nuclear) dense masses that appeared extremely bright compared to all other cellular fluorescence. A cell was counted as having an aggresome if it had either a small punctate aggresome or a large aggresome. Transfection efficiency was consistently at or near 100%; therefore the percentage of aggresomes was calculated by the number of cells with aggresomes divided by the total number of cells. The cell viability assay was performed on neuronal cultures 12h post-transfection. Disassociated neurons were transfected using the Amaxa Rat Neuron Nucleofector Kit (Lonza) with program O-03 according to the recommended protocol. Calcein AM (live) and ethidium homodimer-1 (dead) staining was performed using the Live/Dead Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen), following the manufacturer’s recommended protocol. Cells were plated in individual 35mm plates so that staining and quantification
could be done on each transfection condition sequentially. TUNEL staining was performed on neuronal cultures fixed at 8h post-transfection using the APO-BrdU TUNEL Assay Kit (Invitrogen). Disassociated neurons were transfected using the Amaxa Rat Neuron Nucleofector Kit (Lonza) with program O-03 according to the recommended protocol. Cells were fixed using 4% paraformaldehyde / 4% sucrose on ice for 20 minutes and permeabilized with 70% ethanol overnight at -20° C. The staining procedure was followed according to the manufacturer’s recommended protocol.

**Densitometry and Statistical Analysis**

Densitometry was performed using the Odyssey Infrared Imaging System and the Odyssey software. Integrated intensity was measured for discrete bands and normalized to the integrated intensity of other bands as appropriate. In the in-vivo ubiquitylation assay, monoubiquitylated Myc-NPCD as detected by HA antibody was normalized to total levels of non-ubiquitylated Myc-NPCD as detected by Myc antibody. In the assay measuring NPCD protein level expression (soluble / insoluble), integrated intensity of the insoluble HA-NPCD band was normalized to soluble NPCD in the same sample. The statistical analyses used on the in-vivo ubiquitylation assay (Figure 3.11) and on Mayven/KLHL2 aggresome formation (Figure 3.14) were unpaired Student’s t-tests. The statistical analyses used on the NPCD protein expression levels (soluble / insoluble) assay (Figure 3.12), on the NPCD aggresome formation time course experiment (Figure 3.13), on the neuronal viability live/dead staining (Figure 3.15), and on the TUNEL staining assay (Figure 3.16) were one-way ANOVA using Tukey post-hoc tests to determine significance between conditions.
Chapter III: Results

Yeast Two-Hybrid Screen Identifies NPCD Interacting Proteins

A yeast two-hybrid screen was performed to identify protein-protein interactions with NPCD. We chose two regions of NPCD as our bait proteins: exons 1-5 containing the chromo domain and exons 7-10 containing the pentraxin domain (Figure 3.1).

The NPCD baits in pJ69-4A were mated to Y187 containing an embryonic day 17 mouse cDNA library (Clontech) and diploids were selected on SD–Leu-Ura-Ade plates to identify proteins that interact with the NPCD bait. This is the same library used in the PTPRO yeast two-hybrid screen that identified the cytosolic NPCD isoforms (Chen & Bixby, 2005). Although we did not identify any proteins as interacting with the NPCD chromo domain region bait, we identified two promising candidates as interacting with the NPCD pentraxin region bait: PINCH1/LIMS1 and Mayven/KLHL2 (Table 3.1). Confirmation of the positive candidate clones was accomplished by isolation of prey plasmids followed by cotransformation with the NPCD pentraxin bait plasmid into the yeast reporter strain pJ69-4A and rechecked for growth on SD–Leu-Ura-Ade plates. Only Mayven/KLHL12 and PINCH1/LIMS1 were successfully confirmed in this manner.

A large majority (41 out of 42) of the confirmed candidates contained sequence from the gene kelch-like 2 (*Klhl2*), also known as Mayven (Table 3.1). Candidate families were chosen by their unique restriction enzyme digest patterns, and multiple Mayven/KLHL2 candidate families resulted from distinct N-terminal truncations of Mayven/KLHL2. These truncations ranged from full length cDNA sequence to clones
missing as much as half the N-terminal BTB domain. The single PINCH1/LIMS1 cDNA was full length. N-terminal truncation of cDNA sequences was not unexpected, as the generation of this cDNA library utilized oligo-dT primers and incomplete cDNA synthesis resulted in N-terminal truncations. These results identified two potential protein-protein interactions of NPCD and suggested that the full length BTB domain of Mayven/KLHL2 was not required for NPCD interaction.

Although work was initially performed on both Mayven/KLHL2 and PINCH1/LIMS1, preliminary results in co-immunoprecipitation and colocalization studies suggested that investigation of Mayven/KLHL2 would prove to be more fruitful. Since co-immunoprecipitation of Mayven/KLHL2 and NPCD was more robust, and colocalization between Mayven/KLHL2 and NPCD was stronger and more consistent, we chose to focus our efforts on confirmation and characterization of the interaction between these two proteins.

NPCD Selectively Co-immunoprecipitates with Mayven/KLHL2

Confirmation of yeast two-hybrid results is often done through mammalian cell co-immunoprecipitation experiments. To confirm the interaction of Mayven/KLHL2 with NPCD outside of the yeast two-hybrid system, HEK293T cells were transfected with plasmids expressing HA-NPCD and one of three Myc-tagged proteins: Myc-Cherry (control), Myc-KEAP1 (control, another BTB-Kelch family member), or Myc-Mayven/KLHL2. At 48h post-transfection, protein lysate was collected and either directly loaded or first immunoprecipitated using Myc antibody before SDS-PAGE (Figure 3.2). Our results showed that HA-NPCD only co-immunoprecipitated with Myc-
Mayven/KLHL2, and not the Myc-Cherry or Myc-KEAP1 negative controls. This result demonstrates that the interaction between Mayven/KLHL2 and NPCD also occurs in mammalian cells. Our finding that NPCD does not co-immunoprecipitate with the BTB-Kelch protein KEAP1 suggests that NPCD selectively interacts with Mayven/KLHL2 and not other BTB-Kelch family proteins.

**Expressed NPCD and Mayven/KLHL2 Colocalize in Mammalian Cells**

To validate our results from the yeast two-hybrid screen and co-immunoprecipitation experiments, colocalization studies were conducted by expressing fluorescently-tagged mCherry-NPCD (Figure 3.3A, Red) and fluorescently-tagged mVenus-Mayven/KLHL2 (Figure 3.3B, Green) in HEK293T cells. After 48h in culture, cells were fixed, stained with Hoechst nuclear dye, and viewed using both wide-field fluorescence and confocal microscopy. Wide-field fluorescence microscopy was used to observe nuclear staining, as the confocal microscope used was not equipped with a UV excitation laser to view the Hoechst nuclear dye. Confocal microscopy showed strong colocalization of Mayven/KLHL2 and NPCD in a distinct structure (Figure 3.3C; arrow), observed using wide-field fluorescence microscopy to be generally perinuclear. These results suggest that these two proteins strongly interact in distinct perinuclear structures when expressed in HEK293T cells.

**Mayven/KLHL2 Kelch Domains Are Required for NPCD Interaction**

To identify the domain of Mayven/KLHL2 responsible for its interaction with NPCD, four Myc-tagged Mayven/KLHL2 domain truncation mutants were generated
(Figure 3.4, top schematics). Each of the Myc-tagged Mayven/KLHL2 domain truncation mutants, along with full length Myc-Mayven/KLHL2 and the Myc-Cherry negative control, was individually coexpressed with HA-NPCD in HEK293T cells. At 48h post-transfection, protein lysates were generated and either directly loaded or first immunoprecipitated with Myc antibody before SDS-PAGE (Figure 3.4). Our results showed that HA-NPCD only co-immunoprecipitated with kelch domain-containing Mayven/KLHL2 domain truncation mutants (Figure 3.4; lanes 3, 4) as well as full length Mayven/KLHL2 (Figure 3.4; lane 5). These findings demonstrate that the kelch repeats of Mayven/KLHL2 are necessary for interaction with NPCD, and suggest that the Mayven/KLHL2 kelch repeats form a protein interaction domain that binds to NPCD. We observed that the inclusion of the BACK domain in the Mayven/KLHL2 domain truncation mutant (Figure 3.4; lane 3) improved co-immunoprecipitation with NPCD, compared to the kelch repeats alone (Figure 3.4; lane 4). This finding suggests that the BACK domain may enhance or stabilize the interaction of NPCD to the kelch repeats of Mayven/KLHL2. This finding is consistent with a proposed role of the BACK domain in providing structural flexibility and assisting in the favorable positioning of kelch domain-bound proteins (Stogios & Prive, 2004).

**Expressed NPCD Forms Aggresomes**

During the course of NPCD and Mayven/KLHL2 colocalization studies (Figure 3.3), both proteins were found to strongly colocalize in dense, perinuclear structures. A literature search of this phenotype suggested an aggresomal identity for these structures. To test this hypothesis, fluorescently-tagged NPCD was expressed in HEK293T cells and
the cells were immunostained with antibodies recognizing three commonly reported aggresome markers: vimentin, HSP70, and ubiquitin (Figure 3.5).

Vimentin (Figure 3.5A, red), an intermediate filament fiber protein reported to form a cage around aggresomes, was seen to wrap in a cage-like structure around the mass of NPCD (Figure 3.5B; green). Confocal microscopy showed vimentin staining as a ring surrounding the mass of NPCD in an optical slice through the center of the structure (Figure 4.1C; arrow). An optical slice closer to the top of the structure (Figure 4.1C, inset) showed a cage-like presentation of vimentin fibers. Another marker commonly associated with aggresomes is the chaperone protein HSP70. Confocal microscopy of HSP70 antibody immunostaining (Figure 3.5D; red) and NPCD-containing structures (Figure 3.5E; green) showed strong colocalization between these two proteins (Figure 3.5F; arrows). Immunostaining and confocal microscopy revealed strong colocalization (Figure 3.5I) between ubiquitin (Figure 3.5G; red) and NPCD (Figure 3.5H; green).

The cage-like condensation of vimentin fibers around the NPCD-containing structure suggests that this structure is an aggresome. The co-localization with HSP70 also suggests an aggresomal identity. Our finding that the NPCD structure is highly immunoreactive to the ubiquitin antibody suggests that either NPCD is highly ubiquitylated or the structure that NPCD is localized to contains a high level of ubiquitin protein or ubiquitylated proteins (or both). These results together strongly suggest that overexpressed NPCD forms aggresomes. Additionally, we found that NPCD expression in the absence of overexpressed Mayven/KLHL2 results in the same, strong localization to distinct perinuclear structures (Figure 3.5B, E, H; arrows). However, as
Mayven/KLHL2 is reported to be expressed in HEK293T cells, aggresome localization may still be related to Mayven/KLHL2 expression (Zagranichnaya et al., 2005).

The punctate nature of non-aggresome localized mVenus-NPCD expression (Figure 3.5B, E, H) may reflect small protein aggregates in the cytosol. Vimentin fibers do not appear to colocalize with these cytosolic puncta (Figure 3.5C). Of the three markers, HSP70 staining is the most punctate, and shares partial colocalization with cytosolic NPCD puncta (Figure 3.5F). This finding is consistent with the role of HSP70 in protein folding and aggregation, where HSP70 interacts with newly synthesized proteins to prevent misfolding, and is recruited to sites of protein aggregation (Hartl & Hayer-Hartl, 2002; Park et al., 2007).

**Role of the NPCD Pentraxin Domain Region in Aggresome Formation**

To identify which region of NPCD is involved in aggresome formation, we expressed two fluorescently-tagged NPCD constructs in COS-7 cells. The first construct, CD-GFP, consists of NPCD exons 1-5 and contains the chromo domain (Figure 3.6B; green). The second construct, PTX-GFP, consists of NPCD exons 7-10 and contains the pentraxin domain (Figure 3.6F; green). The expression pattern of CD-GFP showed an exclusive nuclear localization, overlapping with nuclear Hoechst staining (Figure 3.6C; blue). The expression pattern of PTX-GFP (Figure 3.6F; green) was distinctly different, forming a dense, perinuclear structure characteristic of an aggresomes. PTX-GFP localization also overlapped with a dense region of perinuclear microtubule staining (Figure 3.6E; arrow) consistent with the microtubule organizing center (MTOC). These
results suggest that the region containing NPCD exons 7-10 is both sufficient and necessary for aggresome formation.

**NPCD Is Ubiquitylated**

As many of the proteins found in aggresomes are ubiquitylated, we asked whether NPCD is ubiquitylated. HEK293T cells were transfected with plasmids expressing HA-Ubiquitin and either Myc-Cherry (control) or Myc-NPCD (Figure 3.7). At 24h post-transfection, cell lysate was generated by boiling cells in SDS lysis buffer. The input fraction was prepared by the addition of 2x Laemmli sample buffer and directly loaded, and lysate for immunoprecipitation was diluted with lysis buffer without SDS before immunoprecipitation with Myc antibody. Our results showed that immunoprecipitated Myc-NPCD, but not Myc-Cherry, formed a high molecular weight smear, characteristic of polyubiquitylation. This finding suggests that NPCD is ubiquitylated.

Myc antibody revealed a band at the expected size of Myc-NPCD as well as a band at approximately 9 kDa above Myc-NPCD (Figure 3.7; asterisk). After Myc antibody immunoprecipitation, this band was more clearly seen (Figure 3.7; arrowhead) and most likely corresponds to monoubiquitylated NPCD. Consistent with this hypothesis, the high molecular weight smear detected by HA antibody began at this band.

**Mayven/KLHL2 Selectively Interacts with Cullin 3**

Our discovery of NPCD ubiquitylation and the reported E3 ubiquitin ligase function of other BTB-Kelch family members suggested a role of Mayven/KLHL2 as an E3 ubiquitin ligase for NPCD. All known BTB-Kelch family E3 ubiquitin ligases
interact with the structural Cullin 3 (CUL3) protein. Therefore, we tested whether Mayven/KLHL2 interacts with CUL3 using HEK293T cell co-immunoprecipitation. HEK293T cells were transfected with plasmids expressing Myc-Mayven/KLHL2 and one of three HA-tagged cullin proteins: HA-Cullin 1, HA-Cullin 2, or HA-Cullin 3 (HA-CUL1, HA-CUL2, or HA-CUL3). At 48h post-transfection, protein lysate was collected and directly loaded or first immunoprecipitated with Myc antibody before SDS-PAGE (Figure 3.8). After immunoprecipitation of Myc-Mayven/KLHL2, only HA-CUL3 was observed to co-immunoprecipitate. We observed no interaction between Mayven/KLHL2 and CUL1 or CUL2, consistent with the selective CUL3 binding of other BTB-Kelch E3 ubiquitin ligases. These results suggest a selective interaction of Mayven/KLHL2 with CUL3, and also suggest a novel E3 ubiquitin ligase function for Mayven/KLHL2.

HA antibody detected a band at the expected size of HA-CUL3 (Figure 3.8; asterisk) as well as a band approximately 9kDa higher. As the NEDD8 protein is 9kDa and neddylation has been reported for all cullin proteins, this band most likely represents the post-translational addition of NEDD8 protein to CUL3 (Figure 3.8; arrow).

To further test Mayven/KLHL2 interaction with CUL3, we observed whether expressed Mayven/KLHL2 interacts with endogenous CUL3 in mammalian cells. HEK293T cells were transfected with plasmids expressing Myc-Mayven/KLHL2 or Myc-Cherry. At 48h post-transfection, protein lysate was collected and directly loaded or first immunoprecipitated with Myc antibody before SDS-PAGE (Figure 3.9). Endogenous CUL3 was detected after immunoprecipitation of Myc-Mayven/KLHL2
(Figure 3.9; asterisk), but not with Myc-Cherry. This result further supports an E3 ubiquitin ligase function for Mayven/KLHL2.

**Mayven/KLHL2 Domains Involved in CUL3 Interaction**

To determine which domain(s) of Mayven/KLHL2 interact with CUL3, HEK293T cells were transfected with plasmids expressing HA-CUL3 and one of several Myc-tagged Mayven/KLHL2 domain truncation mutants (Figure 3.10). At 48h post-transfection, cell lysates were generated and directly loaded or first immunoprecipitated with Myc antibody before SDS-PAGE. HA-CUL3 was observed to co-immunoprecipitate with the BTB domain alone (Figure 3.10; lane 1), the BTB and BACK domain (Figure 3.10; lane 2), and the BTB deletion mutant (Figure 3.10; lane 3), as well as full length Mayven/KLHL2 (Figure 3.10; lane 5). CUL3 interaction with the BTB and the BTB-BACK domain truncation mutants was consistent with reports of the BTB domain of other BTB-Kelch proteins binding to CUL3. However, our result of CUL3 interaction with the Mayven/KLHL2 BTB domain deletion mutant mirrors reports of other BTB-Kelch protein BTB domain deletion mutants (such as KLHL12 and KEAP1) interacting with CUL3 (Kobayashi et al., 2004; Rondou, Haegeman, Vanhoenacker, & Van Craenenbroeck, 2008). Those findings are directly contradicted by reports from other groups that KLHL12 and KEAP1 BTB domain deletion mutants cannot interact with CUL3 (Angers et al., 2006; Furukawa & Xiong, 2005). Rondou et al. suggest that differences in the type of assay used (HEK293T cell co-immunoprecipitation vs. the in-vitro reticulocyte translation and immunoprecipitation assay used by Angers et al.) as well as differences in the amount of sequence included in
the BTB deletion mutants are possible explanations for the discrepancy in results. As we are the first to report Mayven/KLHL2 binding to CUL3, our result suggests that the BTB domain is not required for CUL3 interaction. Furthermore, it appears that the BACK domain may be capable of mediating CUL3 interaction. We speculate that the BTB and BACK domains both interact with CUL3. Deletion of one domain or the other does not result in the complete loss of CUL3 binding. Our observation that the BTB-BACK domain truncation mutant binds much more strongly than either the BTB domain alone or the BTB deletion mutant is consistent with our speculation.

**Mayven/KLHL2 Increases NPCD Ubiquitylation**

So far, our observations support an E3 ubiquitin ligase function for Mayven/KLHL2. An in-vivo ubiquitylation assay was performed to assess the effect of Mayven/KLHL2 overexpression on NPCD ubiquitylation. HEK293T cells were transfected with plasmids expressing Myc-Cherry (control substrate) or Myc-NPCD (experimental substrate) and either mVenus (control effector) or mVenus-Mayven/KLHL2 (experimental effector). At 24h post-transfection, expression of the “effector” or “effector control” (green fluorescence) was confirmed before protein lysate was harvested. Lysate was directly loaded or first immunoprecipitated with Myc antibody before SDS-PAGE (Figure 3.11A). After enrichment of Myc-NPCD by immunoprecipitation, we observed that coexpression of Mayven/KLHL2 increased the intensity of the high molecular weight smear compared to the mVenus effector control (Figure 3.11A; bottom right four blots). Not only was the high molecular weight smear of NPCD increased with Mayven/KLHL2 coexpression, but the amount of
monoubiquitylated NPCD was also increased (Figure 3.11A; open arrowhead). Our results demonstrate that Mayven/KLHL2 overexpression increases NPCD ubiquitylation, and strongly suggest that Mayven/KLHL2 acts as an E3 ubiquitin ligase for NPCD.

To quantify this result, densitometry was performed on western blots from three experiments. Quantification was performed on immunoprecipitated monoubiquitylated NPCD and levels were normalized to non-ubiquitylated NPCD from the same sample. Quantification of monoubiquitylated NPCD was used as an approximation of total ubiquitylation, as measurement of a single band was more accurate than the total high molecular weight smear. Statistical analysis showed a significant increase in the amount of monoubiquitylated NPCD in cells expressing mVenus-Mayven/KLHL2 vs. mVenus control (Figure 3.11B; * = p < 0.05; unpaired Student’s t-Test). This result suggests that Mayven/KLHL2 functions as an E3 ubiquitin ligase with NPCD as its substrate.

**Effect of Mayven/KLHL2 on NPCD Expression**

We observed that overexpression of Mayven/KLHL2 leads to increased NPCD ubiquitylation. To assess the effect of Mayven/KLHL2 overexpression on NPCD protein levels, western blot experiments were conducted, looking at soluble and insoluble levels of NPCD in response to Mayven/KLHL2 coexpression. HEK293T cells were transfected with plasmids expressing HA-NPCD and one of three effectors: pCMV-Myc (control), Myc-Mayven/KLHL2, or Myc-BTB-BACK (control). The Myc-BTB-BACK domain truncation mutant was used as a control protein unable to interact with NPCD. Protein lysate was harvested 10h and 20h post-transfection, and soluble and insoluble protein
fractions were prepared. Isolation of the insoluble fraction was performed to take advantage of the detergent-insoluble characteristic of aggresome-bound proteins.

We observed no appreciable difference in level of soluble NPCD protein in response to Mayven/KLHL2 coexpression at either time point (Figure 3.12A; Soluble, IB: HA). However, an increased level of insoluble NPCD protein was seen at 10h with Mayven/KLHL2 coexpression compared to both controls (Figure 3.12; asterisk). At 20h, we observed an increase in insoluble NPCD in all conditions, with cells coexpressing Mayven/KLHL2 having a slightly higher level of insoluble NPCD compared to controls. These results clearly demonstrate that coexpression of Mayven/KLHL2 with NPCD increased levels of insoluble NPCD at an earlier time point. Our results suggest that Mayven/KLHL2 increases the rate of NPCD aggresome formation.

To quantify these results, densitometry was performed on western blots from three experiments (Figure 3.12B). Levels of insoluble HA-NPCD were normalized to levels of soluble HA-NPCD from the same sample. Statistical analysis reveals a significant increase of insoluble NPCD at 10h compared to controls (N=3, one-way ANOVA with Tukey post-hoc tests; ** = p < 0.01). Levels of insoluble HA-NPCD in response to Mayven/KLHL2 coexpression were not significantly higher at 20h, however an increased trend was observed (N=3, one-way ANOVA with Tukey post-hoc tests; # = p = 0.053). These results suggest that Mayven/KLHL2 enhances NPCD aggresome formation, but that at later time points, levels of insoluble NPCD have reached a plateau or ceiling. The absence of an effect with the BTB-BACK mutant suggests that the effect on aggresome formation is mediated by ubiquitylation through Mayven/KLHL2.
Mayven/KLHL2 Increases NPCD Aggresome Formation

In our previous experiment, we showed Mayven/KLHL2 overexpression increases the rate of formation of insoluble NPCD. To visually assess whether this reflects an increase in aggresome formation, we used fluorescence microscopy to observe HEK293T cells transfected with plasmids expressing mVenus-Mayven/KLHL2 (Figure 3.13A-F; green) and either Myc-Mayven/KLHL2, pCMV-Myc (control), or Myc-BTB-BACK (control). At 10h post-transfection, we observed that NPCD aggresomes (Figure 3.13A-C; arrows) were larger in size compared to those seen with pCMV-Myc control or the Myc-BTB-BACK control. In addition, the percentage of cells with aggresomes (Figure 3.13G; left graph) was significantly increased with Mayven/KLHL2 coexpression compared to both controls (N=3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05). These findings suggest that the observed increase of insoluble NPCD with Mayven/KLHL2 coexpression is caused by both an increase in the size of aggresomes per cell as well as an increase in the number of cells with aggresomes. At 20h post-transfection, NPCD aggresomes were observed to be comparable in size (Figure 3.13D-F; arrows). However, quantification at 20h (Figure 3.13H) reveals a significant increase in the percentage of cells with aggresomes in response to Mayven/KLHL2 coexpression compared to pCMV-Myc or Myc-BTB-BACK controls (N=3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05).

Our results from the 10h time point suggest that Mayven/KLHL2 coexpression enhances NPCD aggresome formation, leading to larger aggresomes and an increased percentage of cells with aggresomes at an earlier time point. At 20h, we observed aggresomes of similar size with coexpression of all plasmids, but an increase in the
percentage of cells with aggresomes with Mayven/KLHL2 compared to controls. This result suggests that at later time points, the size of aggresomes has reached a maximum and the increased trend of insoluble NPCD is caused by an increase in the number of cells with aggresomes. Again, the absence of an effect with the BTB-BACK truncation mutant suggests that ubiquitylation by Mayven/KLHL2 may mediate these effects on aggresome formation.

Mayven/KLHL2 Localizes to Aggresomes with NPCD Coexpression

Mayven/KLHL2 and NPCD colocalize to aggresomes, and overexpression of Mayven/KLHL2 increases NPCD aggresome formation. While NPCD in the absence of Mayven/KLHL2 forms aggresomes (Figure 3.5B, E, H; arrows), it was unknown whether Mayven/KLHL2 forms aggresomes in the absence of NPCD. HEK293T cells were transfected with plasmids expressing mVenus-Mayven/KLHL2 (Figure 3.14A, C, E, G; green) and either mCherry control (Figure 3.14B, F; red) or mCherry-NPCD (Figure 3.14D, H; red). At 10h and 20h post-transfection, the percentage of cells with Mayven/KLHL2-containing aggresomes (green) was quantified (Figure 3.14I).

At both time points, cells expressing Mayven/KLHL2 (green) in the absence of NPCD showed Mayven/KLHL2 to be mostly cytosolic and non-aggresomal (Figure 3.14A, B, E, F), while cells coexpressing NPCD (red) showed strong aggresome localization of Mayven/KLHL2 (Figure 3.14C, D, E, F; arrows). Quantification of the percentage of cells with aggresomes (Figure 3.14I) demonstrated a significant increase in the percentage of cells with aggresomes with NPCD expression at both time points when compared to mCherry control (N=3, one-way ANOVA with Tukey post-hoc tests; * = p <
These results demonstrate that overexpression of NPCD leads to Mayven/KLHL2 localization in aggresomes. These findings suggest that either Mayven/KLHL2 binds to NPCD, resulting in the recruitment of both proteins to the aggresome, or that Mayven/KLHL2 is recruited to the aggresome after NPCD aggresome formation. Based on our previous finding that overexpression of Mayven/KLHL2 increases the rate of NPCD aggresome formation, we hypothesize that it is likely the former rather than the latter explanation. We speculate that Mayven/KLHL2 interacts with NPCD, leading to increased ubiquitylation, and increased ubiquitylation enhances recruitment to aggresomes.

**NPCD Expression in Neurons Causes Increased Cytotoxicity**

Aggresome formation is associated with a multitude of neurodegenerative diseases. Commonly, diseases-associated proteins with dysregulated expression or mutations that cause cytotoxic effects form aggresomes when overexpressed in cultured cells. As we have observed the formation of aggresomes with overexpression of NPCD, we tested whether NPCD overexpression in neurons causes cytotoxic effects. Rat E18 hippocampal neurons were transfected with control plasmids, plasmid expressing Myc-NPCD, or plasmids coexpressing Myc-NPCD and HA-Mayven/KLHL2. At 12h post-transfection, cell health was assessed by live/dead cell staining with calcein AM (Figure 3.15A, B, C; green, live) and ethidium homodimer-1 (Figure 3.15D, E, F; red, dead). Cultures transfected with control plasmids (Figure 3.15A, D) showed approximately 47% cell viability (47.3 ± 10.7%). Cultures transfected with plasmid expressing Myc-NPCD (Figure 3.15B, E) showed approximately 30% cell viability (29.7 ± 7.2%). Cultures
transfected with plasmids expressing both Myc-NPCD and HA-Mayven/KLHL2 (Figure 3.15C, F) showed approximately 11% cell viability (10.7 ± 5.1%). Statistical analysis revealed a significant decrease in cell viability with NPCD expression compared to control plasmids, and a significant decrease in cell viability with NPCD and Mayven/KLHL2 coexpression compared to both control plasmids and NPCD plasmid expression alone (N = 3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05, ** = p < 0.01). These results demonstrate increased cytotoxicity with the transfection of plasmids expressing NPCD or NPCD with Mayven/KLHL2 compared to control plasmids. This finding suggests that dysregulation of NPCD expression in neurons is cytotoxic, similar to other disease-associated aggregation-prone proteins. Our observation that Mayven/KLHL2 coexpression increased the cytotoxic effect of NPCD suggests that increased ubiquitylation may play a role in protein aggregation-associated cytotoxicity.

**Neuronal Apoptosis after NPCD and Mayven/KLHL2 Expression**

Our previous experiment demonstrated decreased neuronal cell viability with NPCD overexpression or NPCD with Mayven/KLHL2 coexpression. Overexpression of disease-associated proteins has been reported to lead to neuronal cytotoxicity through apoptotic cell death (Zhou, Hurlbert, Schaack, Prasad, & Freed, 2000). To examine for an apoptotic mechanism of NPCD-induced neuronal cytotoxicity, TUNEL staining of transfected neuronal cultures was performed. Rat E18 primary hippocampal neurons were transfected with control plasmids, plasmid expressing Myc-Mayven/KLHL2 alone, plasmid expressing HA-NPCD alone, plasmids expressing both Myc-Mayven/KLHL2
with HA-NPCD, or plasmids expressing Myc-BTB-BACK with HA-NPCD. At 8h post-transfection, neuronal cultures were fixed and TUNEL stained (Figure 3.16F-J; green). Cultures transfected with control plasmids (Figure 3.16A, F) showed a low level of TUNEL staining (11.6 ± 4.3%). Transfection of plasmid expressing Mayven/KLHL2 resulted in similar levels of TUNEL staining (13.8 ± 3%). Transfection of plasmid expressing NPCD (Figure 3.16C, H) revealed an increase in TUNEL staining (25.1 ± 3.8%) and cotransfection of plasmids expressing Mayven/KLHL2 and NPCD resulted in a high level of TUNEL staining (43.5 ± 9.3%). Cotransfection of plasmids expressing the BTB-BACK domain truncation mutant with NPCD revealed levels of TUNEL staining (21.7 ± 5.9%) similar to that of NPCD alone. Statistical analysis revealed no significant difference between transfection of control plasmids and Mayven/KLHL2. However, a significant increase in TUNEL staining was seen with NPCD plasmid transfection (N = 3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05). This effect was significantly increased with cotransfection of Mayven/KLHL2 and NPCD (N = 3, one-way ANOVA with Tukey post-hoc tests; ** = p < 0.01 compared to control and p < 0.05 compared to NPCD). These results suggest that NPCD overexpression causes neuronal apoptosis and that increased ubiquitylation by Mayven/KLHL2 may exacerbate this cytotoxic effect. Our finding that there was no significant difference between TUNEL staining of cells transfected with NPCD alone compared to NPCD with the Mayven/KLHL2 BTB-BACK truncation mutant further supports a role for Mayven/KLHL2 in regulating this NPCD-induced apoptotic effect.
Chapter III Figures

Figure 3.1 – NPCD Yeast Two-Hybrid Bait Protein Constructs. *Npcd* exons 1-5 were cloned into the pGBDU-C1 bait expression vector. This region of *Npcd* contains the chromo domain (CD). *Npcd* exons 7-10 were cloned into the pGBDU-C1 bait vector as well, with this region containing the pentraxin domain (PTX). Both cDNA fragments were joined in-frame and 3’ to the GAL4 DNA-binding domain sequence located in the pGBDU-C1 vector.
Table 3.1 – Yeast Two-Hybrid Screen Results for NPCD PTX Bait. A total of 42 clones were identified as potential prey interactions on nutritional selective media. Prey plasmids from these 42 clones were purified, restriction endonuclease digested, and sorted into 7 candidate families based on unique restriction digest patterns. The number of clones represented by each family is listed, with candidate family 1 and 7 having the most members. Two representative clones from each family were sequenced and compared to the NCBI Nucleotide Sequence Database. The gene names were identified, with 4 candidate families corresponding to kelch-like 2 (Klhl2). The sequenced and purified prey plasmids from each candidate family were cotransformed with the original NPCD PTX bait plasmid into the reporter yeast strain pJ69-4A and grown on nutritional selective media. Only two genes were identified as confirmed interactions: Klhl2 and Lims1.

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<th>Candidate Family</th>
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<td>6</td>
<td>1</td>
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<td>7</td>
<td>10</td>
<td>kelch-like 2 (Klhl2), Mayven</td>
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Figure 3.2 – Co-immunoprecipitation of NPCD with Mayven/KLHL2. HEK293T cells were transfected with plasmids expressing HA-NPCD and one of the following: Myc-Cherry, Myc-KEAP1, or Myc-Mayven/KLHL2. At 48h post-transfection, protein lysate was collected and directly loaded (Input) or first immunoprecipitated with Myc antibody (IP: Myc) before SDS-PAGE. Prior to immunoprecipitation, successful expression of all four constructs was seen (Input, IB: Myc, IB: HA). GAPDH antibody (IB: GAPDH) shows the presence of the ubiquitous metabolic protein GAPDH (asterisk). After immunoprecipitation, all three Myc-tagged constructs were seen (IP: Myc, IB: Myc). GAPDH immunostaining did not detect GAPDH in the precipitate. HA-NPCD co-immunoprecipitation was only seen with Myc-Mayven/KLHL2 but not with Myc-Cherry or Myc-KEAP1 (IP: Myc, IB: HA).
Figure 3.3 – Colocalization of NPCD and Mayven/KLHL2. HEK293T cells were transfected with plasmids expressing mCherry-NPCD (red) and mVenus-Mayven/KLHL2 (green). At 48h post-transfection, cells were fixed. (A–C) Confocal microscopy shows strong colocalization in bright structures (arrow). Observations using wide-field fluorescence microscopy and Hoechst dye (not visible using confocal microscopy, as no UV laser was equipped) showed the majority of these structures to be perinuclear. Scale bar = 10µM
Figure 3.4 – Mayven/KLHL2 Domain Analysis and Co-IP with NPCD. HEK293T cells were transfected with plasmids expressing HA-NPCD and one of several Myc expression constructs: (1) Myc-BTB, (2) Myc-BTB-BACK, (3) Myc-BACK-Kelch, (4) Myc-Kelch, (5) Myc-Mayven/KLHL2, or (6) Myc-Cherry. At 48h post-transfection, protein lysate was collected and directly loaded (Input) or first immunoprecipitated with Myc antibody (IP: Myc) before SDS-PAGE. Immunostaining with Myc antibody showed successful expression (Input, IB: Myc) and immunoprecipitation (IP: Myc, IB: Myc) of all Myc-tagged constructs. After immunoprecipitation, HA-NPCD was seen only to co-immunoprecipitate with Myc-BACK-Kelch, Myc-Kelch, and Myc-Mayven/KLHL2 (lanes 3, 4, and 5).
Figure 3.5 – Colocalization of NPCD with Aggresome Markers. HEK293T cells were transfected with plasmid expressing mVenus-NPCD (green; B, E, H). At 48h post-transfection, cells were fixed and stained with antibodies against (A) vimentin (red), (D) HSP70 (red), or (G) ubiquitin (red). (A-C) Confocal microscopy of an optical slice through the NPCD-containing structure (green) showed a vimentin ring (red) (arrow). A higher optical slice (inset) showed the cage-like appearance of vimentin (red) forming around the NPCD (green) structure. (D-F) HSP70 immunostaining (red) was colocalized with NPCD expression (green) (arrows). Cytosolic puncta were visible using both antibodies, with some colocalization among the puncta. (G-I) Ubiquitin immunostaining (red) showed strong colocalization with NPCD (green) (arrow). Scale bar = 10µM
Figure 3.6 – Distinct Localization of NPCD Domain Expression. COS-7 cells were transfected with constructs expressing fluorescently-tagged NPCD exons 1-5 containing the chromo domain (CD-GFP) or fluorescently-tagged NPCD exons 7-10 containing the pentraxin domain (PTX-GFP). 48h post-transfection, cells were fixed and stained with both an antibody against tubulin (red) and Hoechst nuclear dye (blue). (A-D) Wide-field fluorescence microscopy showed CD-GFP (green) expression overlapping with Hoechst staining (blue). (E-H) PTX-GFP (green) localized to a perinuclear aggresome (arrow), overlapping an area of dense tubulin staining (red) most likely corresponding to the MTOC. Scale bar = 10µM
Figure 3.7 – Ubiquitylation of NPCD. HEK293T cells were transfected with plasmids expressing HA-Ubiquitin and either Myc-Cherry or Myc-NPCD. At 24h post-transfection, protein lysate was collected by boiling cells in SDS lysis buffer. Samples were directly loaded (Input) or first immunoprecipitated overnight with Myc antibody (IP: Myc) before SDS-PAGE. Successful expression (Input, IB: Myc) and immunoprecipitation (IP: Myc, IB: Myc) of Myc-Cherry (30 kDa) and Myc-NPCD (45 kDa) was seen, resulting in bands of the expected sizes (arrows). A band 9 kDa above Myc-NPCD was seen (asterisk, arrowhead), corresponding to monoubiquitylated NPCD. While a high molecular weight smear was seen in both Myc-Cherry and Myc-NPCD expressing cells before immunoprecipitation (Input, IB: HA) corresponding to the total fraction of ubiquitylated proteins, immunoprecipitation and enrichment of Myc-Cherry and Myc-NPCD showed a high molecular weight smear only in cells expressing Myc-NPCD (IP: Myc, IB: HA).
Figure 3.8 – Selective Co-IP of Expressed Cullin 3 with Mayven/KLHL2. HEK293T cells were transfected with plasmids expressing Myc-Mayven/KLHL2 and one of the following: HA-Cullin 1, HA-Cullin 2, or HA-Cullin 3. At 48h post-transfection, protein lysates were collected and directly loaded (Input) or first immunoprecipitated with Myc antibody (IP: Myc) before SDS-PAGE. Successful expression of Myc-Mayven/KLHL2 and all three HA-Cullin constructs was seen (Input, IB: Myc, IB: HA). After immunoprecipitation, only HA-CUL3 was seen to co-immunoprecipitate with Myc-Mayven/KLHL2 (asterisk). Neddlylation (post-translational addition of the 9 kDa NEDD8 protein) of all cullin proteins is reported in the literature. The band 9kDa above CUL3 (arrow) as well as bands above all cullin proteins in the input fraction are predicted to be neddylated forms of the respective cullin proteins.
Figure 3.9 – Co-IP of Endogenous Cullin 3 with Mayven/KLHL2. HEK293T cells were transfected with plasmids expressing either Myc-Mayven/KLHL2 or Myc-Cherry control. At 48h post-transfection, protein lysate was collected and directly loaded (Input) or first immunoprecipitated with Myc antibody (IP: Myc) before SDS-PAGE. Both Myc-Mayven/KLHL2 and Myc-Cherry expressed well (Input, IB: Myc) and immunoprecipitated successfully (IP: Myc, IB: Myc). Before immunoprecipitation, Cullin 3 antibody detected similar levels of endogenous CUL3 protein; the lower band is CUL3, the upper band is due to neddylation (Input, IB: Cullin 3). After immunoprecipitation, endogenous CUL3 protein was seen only to co-immunoprecipitate with Myc-Mayven/KLHL2 (asterisk).
Figure 3.10 – Mayven/KLHL2 Domain Analysis and Co-IP with CUL3. HEK293T cells were transfected with plasmids expressing HA-CUL3 and one of several Myc expression constructs: (1) Myc-BTB, (2) Myc-BTB-BACK, (3) Myc-BACK-Kelch, (4) Myc-Kelch, (5) Myc-Mayven/KLHL2, or (6) Myc-Cherry. At 48h post-transfection, protein lysate was collected and directly loaded (Input) or first immunoprecipitated with Myc antibody (IP: Myc) before SDS-PAGE. All Myc-tagged constructs expressed successfully (Input, IB: Myc). A variable level of both neddylation (arrows) and expression was seen with HA-CUL3 (Input, IB: HA). After immunoprecipitation, all Myc-tagged constructs were immunoprecipitated successfully (IP: Myc, IB: Myc). HA-CUL3 was seen to weakly co-immunoprecipitate with Myc-BTB and Myc-BACK-Kelch (lanes 1 and 3), and strongly with Myc-BTB-BACK and Myc-Mayven/KLHL2 (lanes 2 and 5).
Figure 3.10

<table>
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</tr>
<tr>
<td>5</td>
<td>++++</td>
</tr>
<tr>
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</table>

**Input**

**IP: Myc**
Figure 3.11 – In-Vivo Ubiquitylation Assay of NPCD by Mayven/KLHL2. HEK293T cells were transfected with plasmids expressing HA-Ubiquitin, one of two substrates (Myc-Cherry or Myc-NPCD), and one of two effectors (mVenus control or mVenus-Mayven/KLHL2). At 24h post-transfection, protein lysate was harvested and directly loaded (Input) or first immunoprecipitated with Myc antibody (IP: Myc) before SDS-PAGE. **(A) (Top Left Blots)** Cells expressing the control substrate (Myc-Cherry) showed no difference in total ubiquitylation in response to either effector, either mVenus-Mayven/KLHL2 or mVenus control (IB: HA). **(Bottom Left Blots)** After immunoprecipitation of Myc-Cherry (IB: Myc), very little ubiquitylation was seen (IB: HA). **(Top Right Blots)** Cells expressing Myc-NPCD as the ubiquitylation substrate showed increased total ubiquitylation with coexpression of mVenus-Mayven/KLHL2 (IB: HA). **(Bottom Right Blots)** After immunoprecipitation of Myc-NPCD, increased ubiquitylation of Myc-NPCD was clearly visible (IB: HA) in response to mVenus-Mayven/KLHL2 coexpression. Ubiquitylation levels were subjectively assessed by observing the high molecular weight smear, and densitometry of the monoubiquitylated bands was quantified (open arrowhead). **(B)** Densitometry was performed on three in-vivo ubiquitylation assay western blots, normalizing the amount of immunoprecipitated monoubiquitylated Myc-NPCD (IB: HA) to the amount of immunoprecipitated non-ubiquitylated Myc-NPCD (IB: Myc). A significant increase in monoubiquitylated Myc-NPCD was observed in cells coexpressing mVenus-Mayven/KLHL2 compared to cells expressing the mVenus control. (N=3, unpaired Student’s t-test; * = p < 0.05)
Figure 3.11

A

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<th>Myc-NPCD</th>
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B

![Bar chart showing normalized intensity with statistical significance symbol (*)]
Figure 3.12 – NPCD Protein Expression Levels in Soluble / Insoluble Fractions. HEK293T cells were transfected with plasmids expressing HA-NPCD and one of three effectors: pCMV-Myc (control), Myc-Mayven/KLHL2, or Myc-BTB-BACK (control). At 10h and 20h post-transfection, cells were harvested and separated into soluble and insoluble fractions before SDS-PAGE. (A) GAPDH antibody detected GAPDH protein only in the soluble fraction (IB: GAPDH) at both time points. Myc antibody detected expression of soluble Myc-Mayven/KLHL2 and Myc-BTB-BACK at both 10h and 20h (Soluble, IB: Myc). Immunoblotting the insoluble fraction with Myc antibody revealed the presence of insoluble Myc-Mayven/KLHL2 but not Myc-BTB-BACK at 10h (Insoluble, IB: Myc, 10h). At 20h, Myc antibody detected both insoluble Myc-Mayven/KLHL2 and Myc-BTB-BACK (Insoluble, IB: Myc, 20h). HA antibody detected equivalent levels of soluble HA-NPCD at both time points, regardless of the coexpressed effector (Soluble, IB: HA). The amount of soluble HA-NPCD at the later time point appeared to be increased (Soluble, IB: HA, 10h vs. 20h). Immunoblotting of the insoluble fraction using HA antibody showed a larger amount of insoluble HA-NPCD in cells coexpressing Myc-Mayven/KLHL2 (center lane) when compared to pCMV-Myc (left lane) or Myc-BTB-BACK (right lane) coexpression conditions (Insoluble, IB: HA, 10h). At the 20h time point, HA-NPCD was found in high amounts in all coexpression conditions (Insoluble, IB: HA, 20h). (B) Densitometry readings from three western blot experiments were analyzed. The amount of insoluble NPCD was normalized to the amount of soluble NPCD. (Left graph) At 10h post-transfection, Myc-Mayven/KLHL2 coexpression led to significantly increased levels of insoluble HA-NPCD (N= 3, one-way ANOVA with Tukey post-hoc tests; ** = p < 0.01). No difference was seen between control and Myc-BTB-BACK coexpression conditions. (Right graph) 20h post-transfection, insoluble HA-NPCD was increased with Myc-Mayven/KLHL2 co-expression, but not significantly (middle bar). No significant difference was seen between control and Myc-BTB-BACK coexpression conditions (left, right bars). (N=3, one-way ANOVA with Tukey post-hoc tests; # = p = 0.053)
Figure 3.12

A

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</tr>
<tr>
<td>Myc-Mayven/KLHL2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Myc-BTB-BACK</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HA-NPCD</td>
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<td>+</td>
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</table>

**Graph**

- **Soluble**
  - IB: GAPDH
  - IB: Myc
  - IB: HA

- **Insoluble**
  - IB: GAPDH
  - IB: Myc
  - IB: HA

B

<table>
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<td>Myc-BTB-BACK</td>
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**Graph**

- **Normalized Insoluble HA-NPCD**
  - 0 to 5
  - 10h
  - 20h

- **Legend**
  - **:** p-value < 0.01
  - **#**: significant difference from 10h
Figure 3.13 –Expressed Mayven/KLHL2 Affects NPCD Aggresome Formation. HEK293T cells were transfected with plasmids expressing mVenus-NPCD (green) and one of three effectors: (A, D) pCMV-Myc (control), (B, E) Myc-Mayven/KLHL2, or (C, F) Myc-BTB-BACK (control). Cells were fixed 10h (top) or 20h (bottom) post-transfection and observed for the percentage of cells with mVenus-NPCD aggresomes (green). (A-C) At 10h post-transfection, cells transfected with control plasmids or Myc-BTB-BACK showed small punctate structures in a small percentage of cells (A, C; arrow). Cells transfected with Myc-Mayven/KLHL2 showed the formation of larger aggresomes (B; arrows) in a higher number of cells. (D-F) 20h post-transfection, larger aggresomes (arrows) were seen in all conditions. (G, H) Quantification and statistical analysis showed a significant increase in the percentage of cells with aggresomes in both time points with Myc-Mayven/KLHL2 transfection (N=3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05). No differences were seen between control plasmid and Myc-BTB-BACK transfection conditions. Scale bar = 10µM
Figure 3.14 – Mayven/KLHL2 Aggresome Formation Without NPCD. HEK293T cells were transfected with plasmids expressing mVenus-Mayven/KLHL2 (green; A, C, E, G) and either mCherry (control) (B, F; red) or mCherry-NPCD (D, H; red). Cells were examined using fluorescence microscopy for the propensity for Mayven/KLHL2 to form aggresomes. Cells were fixed and examined at (A-D) 10h or (E-F) 20h post-transfection. (A, B) Cells transfected with mVenus-Mayven/KLHL2 (green) and mCherry control (red) showed no aggresome formation in the majority of cells. (C, D) At the same time point, mCherry-NPCD (red) coexpression results in increased Mayven/KLHL2 aggresomes formation (arrows). (E, F) At 20h, cells expressing mVenus-Mayven/KLHL2 (green) and the mCherry control (red) did not show aggresome formation for Mayven/KLHL2 in the majority of cells. (G, H) With coexpression of mCherry-NPCD (red), increased localization of mVenus-Mayven/KLHL2 (green) in aggresomes at 20h was seen (arrows). In the same culture, cells expressing mVenus-Mayven/KLHL2 (green) but not mCherry-NPCD (red), Mayven/KLHL2 expression was cytosolic and non-aggresomal (asterisk). Scale bar = 25µM (I) Percentage of cells with aggresomes was quantified in three experiments. At 10h post-transfection, there was a significant increase in aggresome formation with mCherry-NPCD coexpression vs. mCherry control (N= 3, unpaired Student’s t-test; * = p < 0.05). At 20h, this difference was statistically significant as well. (N=3, unpaired Student’s t-test; ** = p < 0.01)
Figure 3.14

mVenus-Mayven/KLHL2
mCherry

mVenus-Mayven/KLHL2
mCherry-NPCD

10h

20h

I

% of cells wagglesomes

mCherry (10h)
mCherry-NPCD (10h)
mCherry (20h)
mCherry-NPCD (20h)
Figure 3.15 – Effect of NPCD and Mayven/KLHL2 on Neuronal Cell Health. Rat E18 hippocampal neurons were transfected with (A, D) control plasmids, (B, E) Myc-NPCD + pCMV-HA, or (C, F) Myc-NPCD with HA-Mayven/KLHL2. At 12h post-transfection, cells were stained with a live/dead cell stain (Calcein AM/Propidium Iodide) and observed using fluorescence microscopy. (A, D) Neurons transfected with control plasmids (pCMV-Myc, pCMV-HA) looked generally healthy and showed approximately equal numbers of live (green) and dead cells (red) per field. (B, E) Neuronal cultures transfected with Myc-NPCD and pCMV-HA showed an increased number of dead (red) cells. (C, F) Neuronal cultures transfected with Myc-NPCD and HA-Mayven/KLHL2 presented extremely unhealthy live cells (green) and an increased number of dead (red) cells. (G) Quantification of data from three experiments observing cell health in neuronal cultures. Control plasmids (pCMV-Myc, pCMV-HA) resulted in approximately 47% cell viability (left bar). Neurons transfected with Myc-NPCD and pCMV-HA showed a significant decrease in the percentage of live cells, with only 30% cell viability (center bar). With transfection of plasmids expressing Myc-NPCD and HA-Mayven/KLHL2, cell viability was seen at only 11%, a significant decrease from both control and NPCD alone. (N=3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05; ** = p < 0.01 compared to control, p < 0.05 compared to NPCD)
Figure 3.15

**Calcein AM (Live)**

A

**Ethidium homodimer-1 (Dead)**

D

B

E

C

F

G

% of live cells

Control

NPCD

NPCD Mayven/KLHL2

* *
Figure 3.16 – Effect of NPCD and Mayven/KLHL2 on Neuronal Apoptosis. Rat E18 hippocampal neurons were transfected with (A, F) control plasmids, (B, G) Myc-Mayven/KLHL2 with pCMV-HA, (C, H) HA-NPCD with pCMV-Myc, (D, I) Myc-Mayven/KLHL2 with HA-NPCD, and (E, J) Myc-BTB-BACK with HA-NPCD. At 8h post-transfection, cells were fixed, washed, and TUNEL stained. (A, F) Neuronal cultures transfected with control plasmids (pCMV-Myc, pCMV-HA) showed healthy cultures (top, bright field) with low levels of TUNEL staining (green) of dead or dying cells (bottom; arrow). (B, G) With expression of Myc-Mayven/KLHL2, there was no observable change. (C, H) However, with HA-NPCD plasmid transfection, the number of TUNEL positive cells (green) increased (bottom; arrows) and cell cultures looked less healthy (top, bright field). (D, I) Neuronal cultures transfected with plasmids expressing Myc-Mayven/KLHL2 and HA-NPCD showed unhealthy cultures (top, bright field) and an increased number of TUNEL positive (green) dead or dying cells (bottom; arrows). (E, J) Transfection with plasmids expressing Myc-BTB-BACK and HA-NPCD showed levels of TUNEL staining similar to that of HA-NPCD with pCMV-Myc. (H) Quantification and statistical analysis of three experiments showed no significant difference between control plasmids and Mayven/KLHL2 transfection (left two bars). A significant increase in the percentage of TUNEL positive cells was seen with NPCD, and Mayven/KLHL2 with NPCD. However, no significant difference was seen between NPCD, and BTB-BACK with NPCD (right bar). (N=3, one-way ANOVA with Tukey post-hoc tests; * = p < 0.05 compared to control; ** = p < 0.01 compared to control, p < 0.05 when compared to NPCD)
Chapter IV: Discussion

Summary

This study reports the identification and characterization of a novel protein-protein interaction between NPCD and Mayven/KLHL2. Furthermore, our findings describe a novel E3 ubiquitin ligase function for Mayven/KLHL2. We observed a propensity of overexpressed NPCD to form aggresome structures in cultured cells and colocalization with Mayven/KLHL2 in these structures. Mayven/KLHL2 coexpression did not affect soluble NPCD protein expression levels, but enhanced NPCD aggresome formation. NPCD overexpression in primary hippocampal neuron cultures was cytotoxic and induced neuronal apoptosis. Coexpression of Mayven/KLHL2 with NPCD increased this neurotoxic and apoptotic effect.

Identifying NPCD Protein-Protein Interactions

The NPR isoform of Npcd was first identified through binding to an affinity column with the snake neurotoxin taipoxin (Dodds, Omeis, Cushman, Helms, & Perin, 1997). It was proposed that NPR did not directly bind to taipoxin, but rather formed complexes with related neuronal pentraxin family proteins NP1 and NP2 that bound to taipoxin (Kirkpatrick, Matzuk, Dodds, & Perin, 2000). Further study of NPR described a role with NP1 and NP2 in AMPA-type glutamate receptor clustering and a related role in the formation and maintenance of synapses (Cho et al., 2008; O'Brien et al., 1999; Sia et al., 2007; Xu et al., 2003). However, the cytosolic isoforms of Npcd (Chen & Bixby,
2005a) are likely to have a unique function from that of the mostly extracellular NPR isoform.

All previously reported pentraxin domain-containing proteins are found in the extracellular space, such as the secreted classical short pentraxins CRP and SAP, involved in innate immunity, or neuronal pentraxins NP1 and NP2, involved in synaptogenesis and refinement (Agrawal, Singh, Bottazzi, Garlanda, & Mantovani, 2009; Deban, Bottazzi, Garlanda, de la Torre, & Mantovani, 2009; Kirkpatrick, et al., 2000; Yuste, Botto, Bottoms, & Brown, 2007). Even NPR, a type-II integral membrane protein, only presents 6 amino acids inside of the cell, with the majority of the protein, including the pentraxin domain, located in the extracellular space (Dodds, et al., 1997; Kirkpatrick, et al., 2000). Apexin, a long pentraxin found in the guinea pig spermatozoa acrosome, is found inside of the cell, however even it is processed as a secreted protein and localized inside of the Golgi-derived acrosome (Reid & Blobel, 1994). Its function inside of the acrosome is unknown, however its aggregation may have a role in retention of proteins to the acrosome through sequestration in these aggregates (Reid & Blobel, 1994).

The pentraxin domain in the extracellular context is reported to mediate calcium-dependent binding to a wide range of substrates; examples include the heteromultimerization of NPR with NP1 and NP2, taipoxin binding of NP1 and NP2, and a wide range of microbial moieties and immunoglobulins with SAP and CRP (Agrawal, et al., 2009; Deban, et al., 2009; Kirkpatrick, et al., 2000; Yuste, et al., 2007). However, extracellular functions of pentraxin-domain containing proteins may not be relevant to the cytosolic functions of NPCD and its pentraxin domain.
The function of the CBX6 isoform of \textit{Npcd} or other chromo domain-containing proteins may also be less than helpful in predicting a function for non-nuclear cytosolic NPCD isoforms. The CBX6 isoform has been reported in the literature as being localized to the nucleus and recruited to methylated histones (Bernstein et al., 2006; Vincenz & Kerppola, 2008). While a function of CBX6 has not been reported, family members with chromo domains regulate homeotic genes during development and may be involved in epigenetic transcriptional repression (Bernstein, et al., 2006; Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001; Ruddock-D'Cruz et al., 2008; Vincenz & Kerppola, 2008). These functions are best described in the nucleus where CBX6 and other chromobox and polycomb family proteins reside. A role of the NPCD chromo domain outside of the nucleus is unknown.

NPCD was identified in a yeast two-hybrid screen with the RPTP PTPRO (Chen & Bixby, 2005a). PTPRO is a neuronal guidance cue and involved in the development of the nervous system (Bodden & Bixby, 1996; Gonzalez-Brito & Bixby, 2009; Shintani et al., 2006; Stepanek, Stoker, Stoeckli, & Bixby, 2005; Stepanek, Sun, Wang, Wang, & Bixby, 2001). Using the intracellular phosphatase domain of PTPRO, cytosolic NPCD isoforms were identified (Chen & Bixby, 2005a). The interaction of NPCD with PTPRO, along with knockdown experiments in the neuronal-like PC12 cell line, suggested a role for NPCD in neurite outgrowth and neuronal development (Chen & Bixby, 2005b). As the domains of NPCD had no known cytosolic function, and NPCD was not known to interact with any additional proteins besides PTPRO, a protein-protein interaction screen was performed to identify NPCD binding partners in the hope of better understanding its function.
In our studies, we identified Mayven/KLHL2 in a yeast two-hybrid screen as interacting with the pentraxin domain region of NPCD (Figure 3.1, Table 3.1). This novel interaction was especially promising in the context of process outgrowth because Mayven/KLHL2 had been reported to be involved in actin cytoskeleton regulation through cross-linking of F-actin and involved in oligodendrocyte process outgrowth (Jiang et al., 2005; Williams et al., 2005). As knockdown of NPCD in PC12 cells resulted in a loss of process outgrowth, and knockdown of Mayven/KLHL2 in oligodendrocytes resulted in a loss of process outgrowth as well, a functional relationship of the interaction between Mayven/KLHL2 and NPCD in the context of process outgrowth seemed likely (Chen & Bixby, 2005b; Jiang, et al., 2005; Williams, et al., 2005). The other interaction identified by the yeast two-hybrid screen was between NPCD and PINCH1/LIMS1. Although our early results encouraged us to focus on the interaction between Mayven/KLHL2 and NPCD, PINCH1/LIMS1 is also interesting in that it acts as a component of the integrin signaling complex (Tu, Li, Goicoechea, & Wu, 1999). As regulation of integrin signaling is a classical pathway through which cell growth, migration, survival, and process outgrowth are regulated, further investigation to confirm and characterize the interaction between NPCD and PINCH1/LIMS1 seems warranted.

A Role of Aggresomes for NPCD and Mayven/KLHL2

We demonstrated an interaction between NPCD and Mayven/KLHL2 in HEK293T co-immunoprecipitation experiments, confirming our yeast two-hybrid screen results in mammalian cells (Figure 3.2). Interestingly, NPCD selectively interacted with
Mayven/KLHL2 but not another BTB-Kelch family E3 ubiquitin ligase, KEAP1. While E3 ubiquitin ligases commonly have multiple substrates, substrates can also be targeted by multiple E3 ubiquitin ligases (Liani et al., 2004). To further support an interaction between NPCD and Mayven/KLHL2, colocalization experiments with fluorescently-tagged NPCD and Mayven/KLHL2 were performed. Mayven/KLHL2 and NPCD were shown to strongly colocalize, and the discrete structure to which they were found in was distinctive (Figure 3.2). Mayven/KLHL2 localization has been described as being diffusely cytosolic and associated with actin fiber filaments in U373-MG glioblastoma cells and hippocampal neurons (Soltysik-Espanola et al., 1999) whereas NPCD antibody immunohistochemistry shows cytosolic staining associated with the inner face of the plasma membrane in cerebellar granule cells (Chen & Bixby, 2005a). Neither protein was previously described as being localized to a dense perinuclear structure. Expression of fluorescently-tagged Mayven/KLHL2 in the absence of NPCD is consistent with what is reported in the literature (Figure 3.14A, E). However, expression of NPCD in HEK293T cells in the absence of Mayven/KLHL2 showed the same strong, perinuclear localization (Figure 3.5B, E, and H). To determine which region of NPCD was responsible for this localization, fluorescently-tagged regions of NPCD containing the chromo or pentraxin domain were expressed in COS-7 cells. Not surprisingly, we found that the chromo domain region of NPCD localized to the nucleus, similar to reports of CBX6 localization and a function of the chromo domain in binding to histone proteins (Bernstein, et al., 2006). We also found that the pentraxin-domain containing region was both necessary and sufficient for this distinct localization in the perinuclear structure (Figure 3.6F; arrow). Tubulin staining and nuclear Hoechst staining place this structure near or at the
microtubule organizing center (MTOC) and adjacent to the nucleus (Figure 3.6E-H). As
Mayven/KLHL2 was identified in the yeast two-hybrid screen using this region of NPCD
as bait, and both are found to colocalize strongly to this structure, we hypothesized that
the functional significance of the interaction between Mayven/KLHL2 and NPCD is
related to the identity of this structure.

Aggresomes are cytosolic protein inclusion bodies that form when the proteasome
degradation machinery is impaired or overloaded (Kopito & Sitia, 2000; Markossian &
Kurganov, 2004). Accumulation of proteins targeted by ubiquitylation to the proteasome
sometimes leads to protein aggregation. These protein aggregates are actively
transported along the microtubule network using HDAC6 and the dynein/dynactin
motors, and are directed to a structure at the MTOC (Kawaguchi et al., 2003; Markossian
& Kurganov, 2004). The resulting dense and perinuclear structure is surrounded by a
cage of the intermediate filament vimentin, and contains a large number of protein-
folding and ubiquitin-proteasome components, including proteasome degradation
machinery, ubiquitin enzymes, chaperones such as HSP70, and a large concentration of
ubiquitylated proteins (Kopito & Sitia, 2000; Markossian & Kurganov, 2004). The
structure in which NPCD and Mayven/KLHL2 colocalized to was already observed to be
dense, perinuclear, and located at the MTOC. Subsequently, immunostaining with three
aggresomal markers (vimentin, HSP70, and ubiquitin) confirmed the aggresomal identity
of these structures (Figure 3.5). The function of the vimentin cage surrounding the
aggresome is unclear, however it is theorized that it sequesters the contained proteins
from interaction with other cellular components (Kopito & Sitia, 2000; Markossian &
Kurganov, 2004). HSP70 and other components of the protein folding pathway are
thought to be recruited to the aggresome as a cellular attempt to address protein aggregation, and the high level of ubiquitylated proteins could reflect either ubiquitylation of aggregated proteins or the presence of ubiquitin moieties attached to various recruited ubiquitin enzymes, or both (Kopito & Sitia, 2000; Markossian & Kurganov, 2004). These findings suggested that the interaction of Mayven/KLHL2 with NPCD may be related to their aggresome localization.

A Novel E3 Ubiquitin Ligase Function for Mayven/KLHL2

In addition to having a reported role in regulating actin dynamics, Mayven/KLHL2 has also been reported to have involvement in transcriptional regulation of certain oncogenic and growth promoting pathways in breast cancer cells (Bu et al., 2005). However, these two functions seemed unrelated to the localization in aggresomes. Mayven/KLHL2 belongs to a group known as the BTB-Kelch family of proteins. BTB-Kelch family proteins share a common domain composition: an N-terminal BTB domain, a BACK domain, and C-terminal kelch repeats. While many family members already have described functions in actin binding and transcriptional regulation, including Mayven/KLHL2 (Bu, et al., 2005; Jiang, et al., 2005; Williams, et al., 2005), KBTBD7 (Hu et al., 2010) and KLEIP (Hara et al., 2004), a role for this entire protein family in the ubiquitin pathway has been hypothesized.

Gigaxonin/KLHL16, KLHL12, and KEAP1 are the most well studied of all the BTB-Kelch family E3 ubiquitin ligases (Allen et al., 2005; Angers et al., 2006; Furukawa & Xiong, 2005; Rondou, Haegeman, Vanhoenacker, & Van Craenenbroeck, 2008). At least 13 other BTB-Kelch proteins have been implicated as having E3 ubiquitin ligase
activity. E3 ubiquitin ligases are involved in the ubiquitylation of protein substrates. BTB-Kelch family E3 ubiquitin ligases are components of a multi-protein cullin-ring ubiquitin ligase (CRL) complex. This complex contains the structural protein CUL3 as well as ROC1 (Figure 1.3C). CRLs are substrate adapters that recognize and bind to proteins substrates, bringing them into close proximity to the E2 ubiquitin-conjugating enzyme for ubiquitylation (Perez-Torrado, Yamada, & Defossez, 2006). As proteins targeted to aggresomes are often ubiquitylated, NPCD localization in the aggresome suggested an E3 ubiquitin ligase function for Mayven/KLHL2. Not only did we show that NPCD is a substrate for ubiquitylation (Figure 3.7), but we also demonstrated exogenous and endogenous CUL3 binding to Mayven/KLHL2 (Figure 3.8, 3.9). Consistent with reports of other BTB-Kelch E3 ubiquitin ligases, binding to CUL1 or CUL2 was not detected (Pintard, Willems, & Peter, 2004). Truncation domain analysis shows that the kelch repeats of Mayven/KLHL2 bind to NPCD, consistent with the substrate interaction function of the kelch repeats in other BTB-Kelch E3 ubiquitin ligases. The kelch repeats form a protein-protein interaction structural domain known as a beta-propeller, thought to mediate the ability to bind to a large range of proteins and allowing for diversity in substrate recognition (Prag & Adams, 2003). Truncation domain analysis also showed that the BTB domain is sufficient for binding to CUL3 (Figure 3.10). However, a combination of the BTB and BACK domain revealed a much stronger interaction with CUL3. Surprisingly, a BTB deletion mutant containing only the BACK domain and kelch repeats was able to interact with CUL3 (Figure 3.10). The kelch repeats alone were not able to interact with CUL3. These results demonstrate that the BTB domain is sufficient but not necessary for CUL3 interaction. These results also
suggest that the BACK domain is involved, although it is unclear whether it is sufficient for CUL3 interaction. While the BTB domain is found in a large number of proteins and has a diverse role in protein binding, the BACK domain is a consensus sequence motif with no known function (Stogios & Prive, 2004). Our findings may suggest a novel role for the Mayven/KLHL BACK domain in CUL3 interaction. Conflicting reports of the domains responsible for CUL3 binding are seen with KEAP1 and KLHL12 (Angers, et al., 2006; Furukawa & Xiong, 2005; Kobayashi et al., 2004; Rondou, et al., 2008). Some groups report the ability of BTB deletion mutants to bind to CUL3 (Kobayashi, et al., 2004; Rondou, et al., 2008). Other groups report the inability of BTB deletion mutants to interact with CUL3 (Angers, et al., 2006; Furukawa & Xiong, 2005). Speculated reasons as to why there are conflicting results include differing experimental methods (HEK293T cell co-immunoprecipitation vs. in-vitro transcription and immunoprecipitation) as well as differences in the amount of sequence removed for each group’s BTB deletion mutant (Rondou, et al., 2008). Interestingly, the BACK domain has structural homology to the SKP1 cullin binding protein; taken together with our Mayven/KLHL2 domain truncation results, we hypothesize that the BACK domain may be involved in binding to CUL3 (Stogios & Prive, 2004).

CUL3 binding and domain truncation analysis experiments suggest an E3 ubiquitin ligase function for Mayven/KLHL2. As we showed that NPCD itself is ubiquitylated, we investigated whether coexpression of Mayven/KLHL2 increased NPCD ubiquitylation. Our results showed that overexpression of Mayven/KLHL2 increased NPCD ubiquitylation in an in-vivo ubiquitylation assay (Figure 3.11). Therefore, we
propose that Mayven/KLHL2 is an E3 ubiquitin ligase and NPCD is a substrate for its ubiquitylation activity.

**NPCD and Aggresome Formation**

When we investigated the effect of Mayven/KLHL2 overexpression on soluble NPCD protein levels, we discovered that Mayven/KLHL2, unlike most other BTB-Kelch family E3 ubiquitin ligases (Angers, et al., 2006; Furukawa & Xiong, 2005; Lee et al., 2010), does not enhance degradation of its substrate (Figure 3.12). Instead, we observed that Mayven/KLHL2 overexpression leads to enhanced aggresome formation, as measured indirectly by increased levels of insoluble NPCD (Figure 3.12) as well as observed directly in cultured cells with fluorescently-tagged NPCD in time course experiments (Figure 3.13).

While Mayven/KLHL2 is the first BTB-Kelch E3 ubiquitin ligase to have this reported effect on enhancing ubiquitylation and aggresome formation of its substrate, one other example has been reported for a RING type E3 ubiquitin ligase. Overexpression of the E3 ubiquitin ligase, “seven in absentia homolog” (SIAH) promotes the ubiquitylation and aggresome formation of an alternatively-spliced isoform of synphilin-1 known as synphilin-1A (Eyal et al., 2006; Szargel et al., 2009). In many ways, overexpression of the alternatively-spliced cytosolic NPCD isoform mirrors that seen with synphilin-1A (Eyal, et al., 2006). Both NPCD and synphilin-1A form large aggresomes in cultured cells without proteasome inhibition, both show increased ubiquitylation with coexpression of their E3 ubiquitin ligases, and both respond to this increased ubiquitylation by increased aggresome formation rather than enhanced degradation (as is
commonly seen with other ubiquitylation substrates). Additionally, overexpression of synphilin-1A in neuronal cultures leads to increased apoptosis (Eyal, et al., 2006). In our studies, we also observed an increase in neuronal cytotoxicity and apoptotic cell death with NPCD overexpression in neuronal cultures (Figure 3.15, 3.16). We also demonstrated that Mayven/KLHL2 coexpression with NPCD increases neuronal apoptosis (Figure 3.16D, I). As Mayven/KLHL2 was shown to increase ubiquitylation of NPCD in cultured cells, these findings suggest that increased ubiquitylation and possibly enhanced aggregation or aggresome formation lead to increased cytotoxicity in neurons.

Whether promoting aggresome formation is cytoprotective or cytotoxic remains a hotly-contested issue. Cultured cells and neurons with large protein inclusions have been reported to show reduced levels of apoptosis (Eyal, et al., 2006; Tanaka et al., 2004). However, whether survival is the reason for or the result of that cell’s ability to form a large protein inclusion is unclear. Also supporting a cytoprotective role of aggresomes, disruption of aggresome formation leads to an increase in cytotoxicity, suggesting that the smaller aggregates that are recruited to the aggresome are the true cytotoxic elements (Taylor et al., 2003).

On the other side of the argument, disease-associated proteins with increased aggregation properties (such as longer polyglutamate expansions in trinucleotide repeat disorder proteins, such as mutant AR and mutant huntingtin) can lead to earlier onset and higher penetrance of neurodegenerative symptoms (Andrew et al., 1993; Rubinsztein & Carmichael, 2003; Snell et al., 1993). Additionally, prevention of aggregation and subsequent aggresome formation appears to be cytoprotective (Cummings et al., 2001; Katsuno et al., 2005). However, increased aggregation properties may be distinct from
the formation of aggresomes and prevention of aggregation may also decrease the formation of those smaller precursors to aggresomes. In our studies, we show that coexpression of an aggresome-promoting E3 ubiquitin ligase (Mayven/KLHL2) increases the cytotoxicity of its neurotoxic aggregation prone substrate (NPCD). Whether increased ubiquitylation increases the aggregation properties of proteins, or enhances aggresome targeting is unknown. If ubiquitylation increased the aggregation property of proteins, then a cytoprotective effect of aggresomes would be consistent. However, if ubiquitylation is responsible for increasing the rate of aggregate deposition into the aggresomes, than the process of aggresome formation may be cytotoxic. Unfortunately, due to the effectiveness of overexpressed NPCD in killing neurons, we could not observe or assess the effect of Mayven/KLHL2 on protein inclusion or aggregate formation in neurons. The relationship between aggregation, aggresomes and protein deposits, and neurodegeneration is a complex one that requires further study.

Our serendipitous discovery of an E3 ubiquitin ligase function for Mayven/KLHL2 came about through the overexpression and aggregation properties of NPCD. Whereas most proteins do not form aggresomes when overexpressed, including several substrates for ubiquitylation, such as the Wnt signaling pathway protein dishevelled (DSH) and the D4 dopamine receptor (Angers, et al., 2006; Rondou, et al., 2008), there is a subset of proteins targeted for ubiquitylation that do form aggresomes when overexpressed in cultured cells. Like synphilin-1A, these proteins are often associated with neurodegenerative diseases. One of the first examples reported was the cystic fibrosis transmembrane receptor (CFTR). Mutations in the CFTR protein were known to decrease protein folding efficiency and result in the aggregation and deposition
of CFTR in cystic fibrosis. CFTR proteins with these disease-associated mutations were shown to form aggresomes when overexpressed in HEK293 and CHO cells (Johnston, Ward, & Kopito, 1998). Other wild-type or mutant proteins that form aggresomes when overexpressed include: mutant huntingtin protein (HTT), cytosolic prion protein (PRNP), mutant androgen receptor (AR), presenilin-1 (PS1), and alpha-synuclein (SNCA) (Li et al., 2002; Liani, et al., 2004; Sheflin, Keegan, Zhang, & Spaulding, 2000; Waelter et al., 2001; Yedidia, Horonchik, Tzaban, Yanai, & Taraboulos, 2001). As mentioned above, CFTR is associated with cystic fibrosis, HTT is associated with Huntington's disease, a mutant form of PRNP (PrPSc) is associated with prion disease, mutant AR is associated with spinobulbar muscular atrophy, PS1 is associated with Alzheimer's disease, and SNCA is associated with both Alzheimer's and Parkinson's diseases. We showed that NPCD readily forms aggresomes when overexpressed in cultured cells and is cytotoxic when overexpressed in neurons. Therefore, it seems likely that NPCD may have a role in a neurodegenerative disease.

Unfortunately, the literature reveals very little concerning the role of NPCD in neurodegenerative diseases. Currently, only one reported association of NPCD with Alzheimer's disease is available: an increased level of the NPR isoform of Npcd has been reported in the cerebrospinal fluid (CSF) and serum of Alzheimer's disease patients (Yin, Lee, Cho, & Suk, 2009). However, two related neuronal pentraxin family members are more directly associated with neurodegenerative diseases and neuronal cell death. As the function of NP1, NP2, and the NPR isoform of Npcd are often tightly associated, it stands to reason that cytosolic NPCD isoforms transcribed from the same gene are promising proteins to investigate in these diseases. Neuronal pentraxin 1 (NP1) has been shown to
be involved in amyloid beta (Aβ) induced neuronal damage in Alzheimer brain (Abad, Enguita, DeGregorio-Rocasolano, Ferrer, & Trullas, 2006). Not only does exposure of Aβ to cultured cortical neurons result in increased NP1 expression, but this increased expression leads to an increase in apoptotic cell death (Abad, et al., 2006). NP1 is found to be increased in brains of patients with sporadic late-onset Alzheimer's disease; not only is it found in tau protein deposits, but expression is increased in areas surrounding amyloid plaques (Abad, et al., 2006). Similarly, neuronal pentraxin 2 (NP2) expression is found to be upregulated in the substantia nigra of Parkisonian brains (Moran et al., 2008). Found to accumulate in Lewy bodies, a structure thought to be related to aggresomes, NP2 also associates with alpha-synuclein aggregates (Moran, et al., 2008). For NP1 and NP2, both increased expression and accumulation in deposits and plaques have been reported in disease states. Whether NPCD is similarly affected in disease states or associated with protein deposits has not been investigated.

The results of our project suggest that dysregulation of endogenous NPCD expression (mimicked by overexpression of NPCD in hippocampal neurons) may result in neuronal cytotoxicity. If NPCD expression is found to be increased in disease states, then our findings related to NPCD aggregation and ubiquitylation may represent a mechanism for disease progression, and a target for therapeutic development. Additionally, we showed that cytotoxic effects of overexpressed NPCD were modulated by effects on ubiquitylation, further supporting the idea that components of the ubiquitylation system, specifically E3 ubiquitin ligases, are an important area of study in the process of protein aggregation in the context of neurodegenerative diseases.
The Function of NPCD and Future Directions

The nature of the Mayven/KLHL2 and NPCD interaction suggested a role for Mayven/KLHL2 in the ubiquitylation of NPCD. Whether Mayven/KLHL2 acts on endogenous levels of NPCD as an E3 ubiquitin ligase, or has additional and uncharacterized functions with NPCD in the context of transcriptional regulation or actin binding is unknown. The possibility remains that regulation of the actin cytoskeleton by Mayven/KLHL2 is related to the process outgrowth phenotype seen with knockdown of NPCD in PC12 cells. If this were so, one could imagine signals from the extracellular space affecting PTPRO function, which may in turn affect NPCD function. NPCD has been reported to be phosphorylated in-vivo and a substrate for PTPRO in-vitro (Chen & Bixby, 2005b). Effects on NPCD phosphorylation may affect its interaction with other proteins such as Mayven/KLHL2, and may represent a signaling pathway of PTPRO through NPCD and Mayven/KLHL2 to the actin cytoskeleton and neurite outgrowth. Early attempts to characterize this pathway were stymied by the limitation of our experimental toolkit and approaches, as well as the unique aggregation properties of overexpressed NPCD. Overexpression of NPCD clearly results in the formation of aggresomes, unlikely to represent endogenous NPCD function. Overexpression studies in neurons leads to immediate cytotoxicity and cell death. Difficulties in the detection of endogenous NPCD or RNAi knockdown of NPCD in neurons prevented this line of experimentation from proceeding (unpublished observations of S. Bingham). As expression of NPCD in HEK293T or COS-7 cells has not been reported, knockdown of endogenous NPCD in neurons may be the best hope in uncovering NPCD function.
Attempts to generate a stably-transfected cell line with lowered NPCD expression were unsuccessful, and characterization of a potential NPCD gene trap knockout mouse was not completed. Preliminary studies with another cytosolic isoform of NPCD (Isoform V, 1.4kb variant) showed a similar phenotype of aggresome expression in cultured cells. Based on our results from the NPCD domain expression experiments (Figure 3.6) we suspect that cytosolic NPCD isoforms with a pentraxin domain may share similar aggregation properties. An interesting experiment would be the targeted mutagenesis or truncation of the pentraxin domain to identify if calcium-dependent protein binding is required for aggresome formation. The alternative possibility is that its aggregation properties are independent of pentraxin domain binding, and its propensity to form aggresomes is a structural characteristic.

Early attempts to identify additional protein-protein interaction of NPCD using tandem affinity purification in neurons were unsuccessful as overexpression of the TAP-tag NPCD construct resulted in neuronal cell death. However, identifying other NPCD protein-protein interactions and identifying other potential substrates for Mayven/KLHL2 are avenues for investigation. NPCD may have some endogenous function that requires binding to a protein other than Mayven/KLHL2. Likewise, Mayven/KLHL2 may target other substrates for ubiquitylation. The exploration of functions for both proteins in a “normal” context may yield interesting results in the areas of axonal growth and guidance and neuronal development.

However, the difficulties in working with NPCD and its aggregation properties may have been the reason for the discovery of the novel E3 ubiquitin ligase function of Mayven/KLHL2. With aggregation, NPCD and Mayven/KLHL2 colocalization was
easily observed, and the localization to aggresomes led our investigation into the study of the ubiquitylation machinery. Furthermore, our discovery of NPCD aggresome formation and role of its E3 ubiquitin ligase may prove to be an important mechanism of disease progression if NPCD is identified in protein deposits or is shown to have increased expression in neurodegenerative diseases. As NP1 and NP2 expression levels are reported to be increased in PD and AD, and both proteins are shown to be associated with protein deposits in these neurodegenerative diseases, we believe that NPCD is a promising protein to investigate in this context. Even if NPCD is not immediately discovered to be associated with any relevant disease models, the involvement of the ubiquitylation machinery with protein aggregation and neurodegeneration is still an important issue to study.

Although we did not identify a function for endogenous NPCD, we do report a novel E3 ubiquitin ligase function for Mayven/KLHL2. While many other BTB-Kelch family members share this function, the identification of substrates appears to be the limiting factor in characterizing this large family. Ironically, the original kelch-domain containing protein in *Drosophila*, kelch (KEL), was only recently shown to function as an E3 ubiquitin ligase (Hudson & Cooley, 2010). Originally described as an actin-binding protein involved in ring canal formation (Xue & Cooley, 1993), recent reports identified a dual role for KEL in both actin cross-linking and ubiquitin ligase function (Hudson & Cooley, 2010). This trend of multiple functions in addition to a role as an E3 ubiquitin ligase is seen in Mayven/KLHL2 and most likely many other members of the BTB-Kelch family. As previously mentioned, investigation into additional ubiquitylation substrates
and protein-protein interactions of Mayven/KLHL2 using either the yeast two-hybrid system or tandem affinity purification may identify novel functions or novel substrates.

Clearly, investigation of whether NPCD is upregulated in AD, PD, and HD animal disease models or diseased brains, or whether NPCD is found to be associated with any disease-associated protein deposits is a relevant future direction. Questions regarding the effect of NPCD ubiquitylation on aggregation and recruitment to aggresomes remain. Does ubiquitylation affect the aggregation properties of NPCD or simply the rate with which NPCD is shuttled to aggresomes? Why do cytosolic NPCD isoforms, like synphilin-1A and other disease-associated proteins, form aggresomes when overexpressed? How does overexpressed NPCD cause cytotoxicity and apoptosis in neurons? The questions regarding NPCD aggregation and aggresome formation are the same questions being asked of other proteins involved in neurodegenerative diseases. Hopefully by answering these same questions with NPCD, the general disease mechanism of other proteins can be resolved as well.

Elucidation of the mechanism of protein aggregation and aggregation-mediated cellular dysfunction is a critical component of understanding the disease process of these profoundly impactful diseases. The CDC National Center for Health Statistics reported in 2009 that Alzheimer’s disease was the 7th leading cause of death in the US, roughly equaling deaths associated with diabetes (Heron et al., 2009). Together with Parkinson’s disease, over 90,000 deaths in 2006 alone were reported. As the average lifespan increases over time, resulting in a growing older population, the development of treatment for these often age-related disorders is essential in the coming years. Initial treatments focused on the removal of visible protein deposits and plaques. However,
results from recent clinical studies, including those involving the AN1792 vaccine, demonstrated that the removal of Aβ plaques is not the answer (Holmes et al., 2008). These findings suggest that the visible protein plaques and deposits are a symptom but not necessary the cause of neurotoxicity, and that targeting treatments to the process of protein aggregation and/or aggresome/plaque/deposit formation, such as the ubiquitylation machinery, may be the key to the successful development of cures.
References


Cho RW, et al. 2008. mGluR1/5-dependent long-term depression requires the regulated ectodomain cleavage of neuronal pentraxin NPR by TACE. Neuron 57: 858-871.


Lee YR, Yuan WC, Ho HC, Chen CH, Shih HM, Chen RH. 2010. The Cullin 3 substrate adaptor KLHL20 mediates DAPK ubiquitination to control interferon responses. EMBO J.


