A Tale of Two-Hybrids: Investigating the Interactomes of Cullin-Associated Proteins

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A Tale of Two-Hybrids: Investigating the Interactomes of Cullin-Associated Proteins

by

Elyse Reitter

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science
in
Biology

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ABSTRACT

Cul3 is the major component of an E3 ligase in human cells. Cul3 was initially identified in the Singer lab as a protein that binds and degrades cyclin E, and subsequent studies have shown it to be part of a complex that is involved in a multitude of biological functions. The nature of this complex, its constituents, its regulation, and its dynamics is just beginning to be understood. The research presented here utilizes a series of two hybrid screens to identify families of interactomes with Cul3 at the center. This information will complement other work in the lab in which proteomics has been utilized. In summary, this work identifies potential binding cohorts within the E3 ligase complex and suggests future work to unravel mechanistic pathways that are regulated by Cul3.
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INTRODUCTION AND BACKGROUND

Ubiquitination

Ubiquitination is a highly conserved process that primarily regulates protein degradation within eukaryotic cells, but is also involved in cell cycle regulation, cell signaling, and other processes (Pickart 2001). Errors in this process have been linked to many health problems, including cancer and neurodegenerative diseases (Krek 2003). Because of the wide range of potential human health applications, understanding the complexes involved in ubiquitination has become a rapidly growing area of research.

This process of ubiquitination occurs in several steps, ending with the 76 amino acid peptide ubiquitin covalently attaching to the targeted substrate protein. This results in the modification of the substrate (Ye & Rape 2009). Ubiquitin can be attached in several different ways: as a single ubiquitin (monoubiquitination), multiple single ubiquitins (multi-monoubiquitination), or as chains of ubiquitins (polyubiquitination) attached to each other through isopeptide bonds (Komander 2009, Ye & Rape 2009). Polyubiquitin chains can be straight or branched, and be attached to different lysine residues (K6, K11, K27, K29, K33, K48, K63) to form different types of chains (Komander 2009, Ye & Rape 2009). These varying attachment patterns signal different actions for the targeted substrate. The best understood example of this occurs when ubiquitin is attached as a K48 polyubiquitin.
chain, which flags the substrate for degradation by the 26S proteasome (Kerscher et al. 2006, Pickart 2001).

The binding of ubiquitin to a substrate occurs as a three-step process, as illustrated in Figure 1. Ubiquitination begins with ubiquitin binding to a ubiquitin activating enzyme (E1). E1 binding is an ATP-dependent reaction in which the Cys on the E1 forms a thioester bond to the carboxyl group on Gly76, the C-terminal end of ubiquitin (Pickart 2001, Ye & Rape 2008). After the ubiquitin is activated by E1, it is transferred to the Cys active site of a ubiquitin conjugating enzyme (E2) (Kerscher et al. 2006, Pickart 2001, Ye & Rape 2009). Then, a ubiquitin ligase (E3) interacts with E2 and the substrate to recognize and catalyze the attachment of ubiquitin to a lysine residue on the substrate—or to ubiquitin if a polyubiquitin chain is being formed (Krek 2003, Pickart 2001).

E3 Ubiquitin Ligases

Three types of E3 exist: the RING (Really Interesting New Gene) class, the HECT (Homologous to the E6-AP Carboxyl Terminus) class (Kerscher et al. 2006), and the RBR (RING-IBR (In between RING) -RING) class. The largest group of these is the RING class of E3s, with over 600 coded within the human genome. RING E3s have a 70-amino acid RING finger domain which contains two zinc cations (Kerscher et al. 2006). The cullin family of proteins belongs to this group of E3 RING ligases. Cullin-RING ligases (CRLs) are multisubunit complexes whose basic structure is composed
of the cullin monomer, a separate RING finger domain protein called Rbx, a covalently attached ubiquitin-like protein called Nedd8, and a variety of substrate selection subunits. The cullin structure is a “curved, yet rigid, N-terminal stalk that consists of three repeats of a five-helix bundle linked to a C-terminal globular domain” (Petroski & Deshaies 2005). Seven CRLs are expressed in humans—Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and Cul7 (Wimuttisuk et al. 2014). These CRLs form unique E3 ubiquitin ligase complexes that recognize and catalyze the attachment of ubiquitin to specific substrates, only some of which have been identified. Though there are important differences between these complexes, all contain a RING protein and a cullin protein. (Petroski & Deshaies 2005).

**BTB Proteins**

Bric-a-brac, tramtrack, and broad complex, or BTB domain proteins, are proteins that contain a BTB domain (also called a POZ domain). This domain, first identified in *Drosophila*, is a region of about 120 amino acids which is important for protein binding and transcriptional regulation (Bardwell & Treisman 1994, Stogios & Privé 2004, Zollman et al. 1994). There are several different families of BTB proteins, though the characteristic BTB fold region is conserved despite amino acid sequences varying between the families (Stogios et al. 2005).

One of the largest families are the BTB-ZF (zinc finger) proteins. In this family, the BTB domains often dimerize. The typical structure of a BTB-ZF protein includes a BTB domain at the N-terminus, and zinc fingers at the C-terminus. These two domains
are usually separated by a variable linker segment of 100-375 amino acids (Stogios et al. 2005).

The Skp1 proteins consist of the BTB fold with two C-terminal helices. These proteins are not closely related to the other BTB proteins, but they are necessary for recognizing substrates in the Cul1 complex. The ElonginC protein is similarly critical to the Cul2 complex and makes up its own category of BTB-related proteins. ElonginC is more closely related to other BTB proteins but is missing a terminal helix (Stogios et al. 2005).

Perhaps the most well-known of the BTB proteins is the family of BTB-BACK-Kelch proteins. The typical structure of this family includes a BTB domain at the N-terminal end, followed by a BACK domain and typically between 5 and 7 kelch repeats at the C-terminal end, each of which consist of about 50 amino acids (Stogios & Privé 2004). The exact function of the 130-amino acid BACK domain is currently unknown (Dhanoa et al. 2013), but it is speculated to be involved in with the function of the Cul3 complex (Stogios & Privé 2004). Together, the BTB and BACK domains interact with the N-terminal region of Cul3 (Ji & Privé 2013). The kelch repeats can result in five different possible protein structures: N-propeller, C-dimer proteins; N-propeller proteins; propeller proteins; N-dimer, C-propeller proteins; or C-propeller proteins (Dhanoa et al. 2013). Different kelch repeat patterns are able to bind different substrates. These BTB domain-containing proteins are involved in many crucial cellular processes, including organization of the cytoskeleton, ion channel regulation, and transcriptional suppression (Dhanoa et al. 2013).
In the Cul3 complex, BTB proteins function as linkers and substrate adaptors (Wimuttisuk et al. 2014). As stated in Canning et al. (2013), these proteins are unique because of the ability for the BTB adaptor domain to dimerize and recruit two Cul3 subunits. The BTB proteins also generally have a second protein-protein interaction domain. Because of this, a single subunit is able to recognize substrates and act as an adaptor.

**LRR Proteins**

LRR proteins are linked to BTB proteins within the Cul3 complex. LRR proteins contain a leucine rich repeat motif of 20-30 amino acids, which forms a solenoid structure. This structure both facilitates and mediates protein-protein interactions (Bella et al. 2008, Kobe & Kajava 2001, Schenková et al. 2012). Additionally, LRR proteins contain a B/C-like box. Within the Cul3 complex, LRR proteins can bind both Cul3 and BTB domain containing proteins, with BTB binding occurring using the LRR domain and the B/C-like box enabling Cul3 binding (Wimuttisuk et al. 2014).
Figure 1: The Ubiquitin Pathway

A ubiquitin protein binds to and activates ubiquitin activating enzyme (E1) in an ATP-dependent process. After the ubiquitin is activated by E1, it is transferred to a ubiquitin conjugating enzyme (E2). Then, a ubiquitin ligase (E3) interacts with E2 and the substrate to recognize the substrate and catalyze the attachment of ubiquitin.
BAIT PROTEINS

Since this thesis presents data obtained from several two-hybrid screens, the background will include a discussion of the proteins used as bait (the phrase often used to describe the protein used to screen for novel binding partners). The following six proteins were used as bait proteins in the yeast two-hybrid screens described here. Explanation of the screens and how they were performed is detailed in the Materials and Methods section.

**Cul3**

Cul3 is one of the best-characterized of the cullin-RING ligases. The Cul3 complex, illustrated in Figure 2, consists of Cul3, which functions as a scaffold protein, the E2 ubiquitin conjugating enzyme, the Rbx1 RING protein, and the BTB domain-containing protein. This complex is also known as the BCR complex - from BTB-Cul3-Rbx1. Within the BCR complex, leucine rich repeat (LRR) domain-containing proteins sometimes mediate protein interactions between Cul3 and the BTB domain-containing proteins through the B/C-like box (not shown) (Wimuttisuk et al. 2014).

Cul3 is made up of 768 amino acids. First identified in a yeast two-hybrid screen for cyclin E, it was found to play an integral role in cell cycle regulation through the ubiquitination of cyclin E in mammalian cells (Singer et al, 1999). Since its discovery, it has been found to ubiquitinate many additional proteins as part of the BCR complex, including Dishevelled, DNA topoisomerase I, Nrf2, Daxx, and RhoBTB2 (Angers et al. 2006, Zhang et al. 2004, Cullinan et al. 2004, Kwon et al. 2006, Wilkins
et al. 2004). The N terminal end of Cul3 is where the BTB protein binds, which allows for the binding of a specific substrate (Pintard et al. 2004). At the C terminal end of Cul3, Rbx1 attaches and aids in the recruitment of the E2 ubiquitin conjugating enzyme (Seol et al. 1999).

Cul3, as a primary focus of research in this lab, was a clear candidate for a yeast two-hybrid screen. Though many Cul3 interacting proteins are already known, new screens can discover new proteins, which will lead to new experiments and a greater understanding of the importance of the Cul3 complex and its role in human cell biology.

Mayven (KLHL2)

Mayven, also known as Kelch-like protein 2 (KLHL2), consists of 593 amino acids (Dhanoa et al. 2013). As a member of the KLHL protein family, it is a BTB-BACK-Kelch protein, which means that it consists of three different motifs, as shown in Figure 3. The first is the 115-amino acid N-terminal BTB domain. The third structure is the C-terminal kelch repeats motif (Soltysik-Espanola 1999). All of the KLHL family of proteins form N-dimer, C-propeller structures. In Mayven, the 6 kelch repeats take the form of a β-propeller (Dhanoa et al. 2013, Canning et al. 2013). The most recently identified structure is the 130-amino acid BACK domain, the function of which is still unknown. Much of the information about Mayven is still unknown, including the function of several of its structures, and, perhaps most importantly, what its binding partners are (Dhanoa et al. 2013). Though Mayven has been associated with the Cul3
complex, it is currently unknown which LRR proteins mediate the interaction between Mayven and Cul3 (Wimuttisuk et al. 2014).

Although Mayven has been found in many organs, including the heart, spleen, and kidney, it is primarily found in the brain (Dhana et al. 2013). Mayven has been found to function in many cell types as an actin-binding protein. This binding is involved in the creation of the actin cytoskeleton, which is necessary for the function and structure of all eukaryotic cells (Soltysik-Espanola 1999, Dhanoa et al. 2013).

Cul3, Mayven, and its binding proteins appear to be involved with the regulation of proteins in the postsynaptic density of neurons (Liebau et al. 2009). Shank3, a protein which has been implicated in the onset of autism spectrum disorder (ASD), also encodes scaffolding proteins in the postsynaptic density. (Sala et al. 2001). Shank3 has been found to bind to both Mayven and another BTB domain-containing adaptor called actinfilin (KLHL17). Previous work in our lab has demonstrated that Shank3 levels are regulated through ubiquitination by Cul3 (either directly or through a substrate adaptor protein). The goal of this screen was to discover more about Mayven’s interacting proteins and discover more about its potential role in ASD.

**KLHL3**

Kelch-like protein 3 (KLHL3) is another BTB-BACK-Kelch protein in the KLHL family and is the BTB protein most closely related to Mayven based on amino acid sequence similarity, as shown in Figure 4. It is similar to Mayven both in size and
structure, which can be seen in Figure 3, with a length of 587 residues and 6 Kelch repeats forming a β-propeller. In contrast to Mayven, KLHL3 is much better characterized in both its function and binding to Cul3. The role of KLHL3 and Cul3 in the regulation of blood pressure is especially notable, with mutations in KLHL3 or Cul3 disrupting the interaction between KLHL3 and WNK1/WNK4 proteins and resulting in familial hyperkalemic hypertension (Louis-Dit-Picard, et al. 2012, McCormick et al. 2014, Ohta et al. 2013, Wu & Peng 2013). KLHL3 is also associated with the actin cytoskeleton.

KLHL3 is well-known for its interactions with Cul3 and its importance in disease pathology in familial hyperkalemic hypertension. Recent work has also shown that KLHL3 and Cul3 are involved in other diseases, including type 1 diabetes mellitus (Guo et al. 2021) and congenital heart diseases (Wang et al. 2017). Because of this, a yeast two-hybrid screen was proposed to uncover more of the functions and interacting proteins associated with KLHL3, which could be help identify degradation substrates, and uncover further avenues of research on Cul3 regulation.

**CTB9**

CTB9, or Cullin-3-binding protein 9, is also part of the KLHL family with the alternative name of KLHL42. CTB9 is a smaller BTB protein, only 505 amino acids in size. Though it has the characteristic BTB domain and 6 kelch repeats, the BACK domain is smaller in size than other members of the KLHL protein family, as shown in Figure 3 (Dhanoa et al. 2013, Cummings et al. 2009). CTB9 was found to interact
with Cul3 in a yeast two-hybrid screen, and further experiments showed its role in regulation of cellular microtubule formation. For example, CTB9 in the Cul3 complex regulates the levels of the neuronal migration and microtubule-severing katanin protein KATNA1 (Cummings et al. 2009, Hatakeyama & Hayashi 2018). Additionally, CTB9 was recently found to mediate fibrotic signaling in systematic sclerosis associated interstitial lung disease through ubiquitination of the PPP2R5ɛ protein (Lear et al. 2020).

It is likely that CTB9 is involved in other cellular processes and diseases. This protein was primarily chosen as a candidate for a yeast two-hybrid screen due to the previous work done with it in the Singer lab (Cummings et al. 2009) as well as its role in the cell cycle process, via degradation of microtubule severing proteins.

**FMOD**

Fibromodulin, or FMOD, is a small leucine-rich repeat protein associated with assembly of the extracellular matrix. FMOD has links to cellular repair mechanisms and cancer, though it seems to have multiple functions within the cell. Following UV light exposure, FMOD levels were increased. Additionally, Fmod expression levels were found to be lower in senescent cells compared to growing cells (Iovine et al. 2011). In a glioblastoma study, FMOD was required for migration of glioma cells and also induced glioma cell migration (Mondal et al. 2017). In breast cancer, FMOD is necessary for cell migration and invasion (Khan et al. 2019).
FMOD is also known as LRR5. It contains an LRR domain which influences binding between a cullin and BTB protein (Wimuttisuk et al. 2014). In a previous Cul3 yeast two-hybrid screen performed in this lab, FMOD was found to bind Cul3. It was hypothesized that a yeast two-hybrid screen would uncover BTB proteins associated with both Cul3 and FMOD.

Muf1

Muf1 is a protein also known as LRRC41 (leucine-rich repeat containing 41). It is an LRR protein which contains an N-terminal SOCS-box and a C-terminal leucine-rich repeat region. The SOCS box contains the BC-box motif that mediates the interaction between a BTB protein and the cullin E3 ligase (Kamura et al. 2001, Wimuttisuk et al. 2014, Schenková et al. 2012). Unlike many of the other proteins used for the yeast two-hybrid screens mentioned in this paper, Muf1 is not associated only with Cul3. Instead, it has been found in complexes with Cul3 as well as Cul2 and Cul5 (Kamura et al. 2001, Schenková et al. 2012). In the context of the Cul3 complex, Muf1 is a substrate for the complex of Cul3 and the BTB protein RhoBTB3 and is able to bind RhoBTB3. Little is known about the role of Muf1 within the cell.

Because much is still unknown about Muf1, a yeast two-hybrid screen was performed to discover interactions between Muf1 and other proteins. This was anticipated to clarify the role that Muf1 plays not only within the Cul3 complex, but within the cell as a whole.
Figure 2: The Cul3 E3 Ubiquitin Ligase Complex

The general structure of the Cul3 E3 ligase complex: Cul3, shown in red, is the scaffold of the complex and facilitates bond formation between ubiquitin and a substrate. The BTB protein, in green, binds to the N terminal end of Cul3 and allows for the binding of a specific substrate. Rbx1, depicted in yellow, attaches to the C terminal end of Cul3 and aids in the binding of the E2 protein (blue). The LRR protein, in light blue, is not always present in this complex. If present, it helps to regulate the binding between the BTB protein and Cul3.
Figure 3: Structure of Bait BTB Proteins

This figure shows the structural similarities between the three BTB-BACK-Kelch proteins used as bait proteins in the yeast two-hybrid screens. The BTB domain is shown in pink, the BACK domain (truncated in CTB9) in orange, and multiple Kelch repeats in green.
Figure 4: Human BTB Protein Phylogenetic Tree

The phylogenetic tree shows amino acid sequence relatedness of human BTB domain-containing proteins, with proteins used as bait for yeast two-hybrid screens shown in red (CTB9, KLHL3, Mayven). The colored branches correspond to the family of BTB proteins - KLHL family in pink, zinc finger BTB proteins in blue, potassium channel BTB proteins in orange, and others (including RhoBTB, MATH-BTB, and BTB ankyrin repeat proteins) in black.
MATERIALS AND METHODS

Yeast Two-Hybrid Screen

Six separate yeast two-hybrid screens were performed using the following method to discover interacting proteins. First, the DNA for the protein of interest was amplified using PCR to prepare for cloning. All but one of the yeast two-hybrid screens used the pGBKT7 expression vector, which contains the GAL4 DNA binding domain (Figure 5). For the Ctb9 screen, the pGilda vector was used (Figure 6). The vector was cut using restriction enzymes, which varied for each bait protein as shown in Table 1. PCR DNA was cut using restriction enzymes, also detailed in Table 1. Ligation of DNA into the vector was performed using T4 DNA ligase, forming the “bait” construct.

The “prey” construct was made in a similar manner, with the human cDNA library of interest inserted into the pACT2 yeast vector, which contains the transcriptional activation domain. The cDNA libraries with each prey construct are shown in Table 1. These cDNA libraries were chosen due to accessibility, with the FMOD and CTB9 screens performed with the human testis library before the human kidney cDNA library was available. Illustrations of the bait and prey constructs can be seen in Figure 9.

An overview of the yeast selection process is shown in Figure 8. The bait and prey constructs (as a library) were separately inserted into the Y2HGold strain of yeast. Bait yeast was grown on SD plates lacking tryptophan to confirm the presence of the bait construct. Libraries were then introduced into the prey containing strains...
and plated on plates with no leucine or tryptophan (SD/-Leu/-Trp) to select for both plasmids. Colonies from these plates were struck on a test plate of double dropout media containing 40 µg/ml X-alpha-Gal and 200 ng/ml Aureobasidin A (SD/-Leu/-Trp). Any colonies which grew and were blue in color were then patched on SD-Leu plates to allow the cells to lose the prey plasmid. The colonies were transferred to SD-Trp plates using sterile velvet. When colonies grew on the original plate but not the replica plate, this yeast was struck onto a SD-Leu test plate and inoculated into 500 µL SD - Leu media with glucose.

**Bait-Prey Interactions**

The bait construct contains a DNA binding domain which binds a promoter region. For the screens using the pGBK7 vector, the bait protein was fused to the GAL4 DNA binding domain which binds to the GAL4 upstream activating sequence. The CTB9 screen utilizing the pGilda vector was fused to the LexA DNA binding domain which binds to the LexA upstream activating sequence. The prey constructs containing the cDNA library also contain the transcriptional activation domain. When there is a successful interaction between bait and prey, transcription of a reporter gene occurs. In both the pGilda and GAL4 systems, these reporter genes are HIS3 and lacZ. HIS3 is involved in histidine production and allows for growth on histidine-deficient media, and lacZ turns the colonies blue.
Plasmid Recovery and Sequencing

For each yeast-two hybrid screen, approximately 150 yeast colonies were obtained. From these, several were chosen at random for sequencing. DNA extracts were obtained from the yeast cultures using standard phenol-chloroform DNA extraction (Sambrook and Russell 2006). This DNA was transformed into electrocompetent *E. coli* using electroporation. Colonies from these bacteria were cultured for small (50 mL) maxipreps to prepare DNA for sequencing.

Sequence Analysis and Identification of Potential Interactors

Gal4-AD forward primer was used to sequence the proteins pulled out from the screen. The potential clones were sequenced using dideoxy sequencing method.

Gal4-AD forward primer sequence: AATACCACTACAATGGAT

Sequences were run using BLAST search to find protein identity from the sequence. Due to cost of sequencing, samples were sequenced until duplicate proteins began to appear. For each screen, between 10 and 20 samples were sequenced.

Data Analysis and Clustering

Amino acid sequences of all known human BTB proteins were acquired from UniProt. This information was used to construct a data table of BTB proteins, organizing them by category (kelch-like, zinc finger, etc.). This data was also used to
build a phylogenetic tree to demonstrate the relatedness of the proteins (Figure 4). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (-265429.94) is shown. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 179 amino acid sequences. There were a total of 2444 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Data was analyzed by the program MEGA X: Molecular Evolutionary Genetics Analysis (Kumar et al. 2018).
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<td>Human testes cDNA library</td>
<td>EcoR1-Sal1</td>
</tr>
</tbody>
</table>

Table 1: Bait and Prey Protein Assembly

This table summarizes the construction of the bait and prey plasmids for the yeast two-hybrid screen. Bait constructs were made using the pGBK7T vector, apart from CTB9, then cut with the restriction enzymes listed here. Prey constructs consisted of the pACT2 yeast vector with the cDNA library listed above inserted using the corresponding restriction enzymes.
Figure 5: pGBKT7 Vector Map

This vector map for pGBKT7 is adapted from Maple, J., & Møller, S. G. (2007). This two-hybrid expression vector was used to create the bait constructs in all the two-hybrid screens except for CTB9. It contains the GAL4 DNA binding domain as well as the TRP1 gene for tryptophan biosynthesis.
Figure 6: pGilda Vector Map

This vector map is adapted from Nova Lifetech Inc. (2021). This two-hybrid expression vector was used to create the bait construct for the CTB9 yeast two-hybrid screen. It contains the LexA DNA binding domain.
Figure 7: pACT2 Vector Map

This vector map is adapted from Takara Bio USA, Inc. (2021). This two-hybrid expression vector was used to create the prey construct for all the yeast two-hybrid screens performed. It contains the GAL4 transcription activation domain as well as the LEU2 gene for leucine biosynthesis.
Figure 8: Yeast Selection Process

An overview of how yeast was selected for colonies in which successful binding occurred between bait and prey constructs. Yeast with the bait construct was grown in -trp media, then combined with prey construct yeast and grown on -trp/-leu double dropout media. Blue colonies were grown on -leu plates, then replica plated to -trp plates. Successful colonies that grew on -leu but not -trp plates were grown in -leu media.
Figure 9: Bait and Prey Interactions

The bait construct contains both the bait protein of interest as well as a DNA binding domain, which is able to bind to an upstream activation sequence (GAL4 for all screens except the CTB9 screen, in which the vector contains the LexA DNA binding domain). The prey construct contains a cDNA library and a transcriptional activation domain. When a successful interaction occurs between bait and prey, RNA polymerase is recruited and transcription of the reporter genes (HIS3 and lacZ) proceeds.
RESULTS

The objective of these two-hybrid screens was to uncover previously unknown proteins which interact with cullin-associated proteins. Elucidation of these binding interactions help us to perceive the role of each of these proteins within cellular mechanisms, which also has potential to broaden understanding of human diseases associated with these complexes.

Cul3

Though other yeast two-hybrid screens have discovered a multitude of Cul3 interacting proteins of different types, in the case of this yeast two-hybrid screen, all Cul3 interacting proteins found were BTB domain-containing proteins. Unfortunately, none of these proteins were novel binding partners to Cul3, though some of the interactions are not well understood.

Kctd13, Ctb3, and Kctd10 are known as BACURD1, BACURD2, and BACURD3, which stands for BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation proteins 1 and 2. As the name suggests, both are involved in the degradation of the Rho family GTPase RhoA by Cul3 through ubiquitination. Knockdown of Kctd13 or Cul3 causes increased levels of RhoA, which results in actin stress fiber formation (Chen et al. 2009). Lin et al. (2015) found that Kctd13 and Cul3 are coexpressed during late mid-fetal period of cortical development and when levels of these proteins are low, “increase in RhoA levels leads to stress fiber formation, axon
growth inhibition, enhanced cell spreading, loss of dendritic spines, and neurite retraction, whereas the opposite effects are observed when RhoA levels are decreased.” These functions are hypothesized to be linked to the development of autism spectrum disorder (ASD) and other psychiatric diseases. Kctd10 is not as well-characterized as Kctd13 and Ctb9, though its regulation of RhoA by ubiquitination through the Cul3 pathway has been linked to the process of lipid droplet formation during adipogenesis (Dubiel et al. 2017).

Like Kctd10 and Kctd13, Kctd6 and Kctd9 are potassium channel domain containing BTB proteins. However, comparatively little is known about their cellular functions and potential disease relevance (Teng et al. 2019). Kctd6 binds and regulates levels of the protein sAnk1.5 in muscles as part of the Cul3 complex and may also be involved in erythrocyte spherocytosis through interactions with ANK1 (Lange et al. 2012). Additionally, Kctd6 (along with other members of the Kctd family) was linked to disruption of the Hedgehog signaling pathway which is associated with human glioblastoma tumor growth (De Smaele et al. 2012). Kctd9 was found to have functions associated with viral liver damage, specifically in the function of natural killer cells. In those cells, Kctd9 was upregulated and was found to be necessary for proliferation, though the mechanisms behind this are unknown (Ji et al. 2016, Zhang et al. 2019).

Though BTBD3 and BTBD10 are not zinc finger BTB proteins like Kctd10 and Kctd13, they are similarly involved in cancers and brain development. Chen et al. (2004) found BTBD10 to be downregulated in all eighteen glioma samples studied,
but not in hepatocellular carcinoma, lung cancer, or ovarian cancer. Though the glioma association with BTBD10 is similar to the glioblastoma connection observed with Kctd9, BTBD10 does not seem to have involvement with the Hedgehog signaling pathway. Instead, BTBD10 is involved with the Akt pathway. This is related to apoptosis: decreased levels of BTBD10 appeared to result in beta cell apoptosis in rat pancreatic beta cells (Wang et al. 2011), as well as contributing to apoptosis in rat neuronal cells following intracerebral hemorrhage (Zheng et al. 2013). BTBD3, on the other hand, does not seem to be associated with apoptosis. Instead, it is primarily involved in dendrite and neural circuit formation during mammalian brain development (Matsui et al. 2013, Thompson et al. 2019). This has been linked to OCD behaviors in mice and may be linked to other psychiatric disorders (Thompson et al. 2019). Also unlike BTBD10, BTBD3 is associated with the onset of hepatocellular carcinoma (HCC). In four HCC cell lines, it was found to be overexpressed. BTBD3 did not result in increased proliferation but appeared to promote cancer cell invasion and metastasis (Xiao et al. 2017).

BTBD13 was another protein found in the screen, though it is more commonly known as Germ cell-less protein-like 1 (GMCL1). Though it does not appear that any studies have researched this protein specifically, some broader studies have observed downregulation of the gene to conditions such as reduced sperm motility (Liu et al. 2018) and B cell lymphoma (Fournier et al. 2010). A 2017 study also noted the GMCL1 gene to be correlated with atrial fibrillation in Europeans. Interestingly, KLHL3 was also found in this study (Christopherson et al. 2017).
Mayven

Eleven different proteins were identified from the Mayven screen, all of which appear to be novel interactions. Unfortunately, the functions of many of these proteins are not well understood. Because of this, it is not clear how these proteins are involved with Mayven and the Cul3 complex.

The first, cystatin C, is a well-known marker of kidney function, though it is found throughout the body and is related to cardiovascular function through the regulation of cysteine proteases (Bökenkamp et al. 2006, Shi et al. 1999). In a proteomics screen by Wu et al. (2011), cystatin C and Mayven were both identified as genes that showed increased expression in the kidney disease membranous glomerulonephropathy, though the study did not note any interactions between the two.

One of the more interesting proteins discovered in this screen was cyclin A2. Cyclin A2 is a key cell cycle regulation protein which is necessary for replication of DNA during S phase of the cell cycle, as well as entry into mitosis (Pagano et al. 1992). Like Kctd13, Ctb3, and Kctd10 from the Cul3 screen, cyclin A2 also interacts with RhoA. Decreased expression of cyclin A2 causes lower levels of RhoA and is correlated with increased cell invasion. In renal, colorectal, and prostate cancers, low levels of cyclin A2 seem to be associated with higher likelihood of metastasis (Arsic et al. 2012, Loukil et al. 2015), Cyclin A2 also binds PLZF, a zinc finger protein associated with the Cul3 complex (Liu et al. 2016, Suliman et al. 2012,).
TGFBR2 is a transmembrane serine/threonine kinase protein which functions with TGFBR1. Though TGFBR1 was not found in this screen, both TGFBR1 and TGFBR2 were found in the KLHL3 screen. TGFBR1 and TGFBR2 are both important proteins in the regulation of cell signaling through the initiation of the Ras/RhoA, Smad, and Akt pathways (Moore-Smith and Pasche 2011). Because of this, these proteins are associated with cell proliferation and invasion in several cancers, most notably breast and pancreatic cancers (Moore-Smith and Pasche 2011, Zhou et al. 2018). It is not known how Mayven interacts with TGFBR1 and TGFBR2, as there is currently no data on how these proteins are associated with the Cul3 complex.

Very little is known about the protein ester hydrolase C11orf54. It is a nuclear protein with no current data about its biological function (Manjasetty et al. 2006). It has, however, been identified in two different proteomics screens for renal cell carcinoma and endometrial cancer (Giribaldi et al. 2013, Ura et al. 2017).

Hemoglobin subunit beta (β-globin) is another protein found in this screen that has unknown interactions with the Cul3 complex. However, the fetal γ-globin protein is regulated by the Cul3 complex with the BTB protein SPOP (Lan et al. 2019).

KCNE3 is a potassium channel protein. It has recently been discovered that it, along with KLHL14, inhibits epithelial to mesenchymal transition in amniotic epithelial cells. This has implications for embryonic development as well as cancer metastasis (Di Lollo et al. 2020).

The protein breast cancer overexpressed 1, or BCOX1, is also a novel binding protein which is not well-characterized, though it seems to be a cytoplasmic protein.
connected to breast cancer (Song et al. 2006). High levels of BCOX1 were found to correlate to poor prognosis in a study of breast cancer patients (Liu et al. 2014). In vitro, cells with increased BCOX1 showed increased cell adhesion and migration (Zhong et al. 2015). There are currently no known studies identifying the function of BCOX1 within the cell or how this protein might be regulated.

RACK7 (also known as ZMYND8) is another cancer-associated protein discovered. A study of prostate cancer cells both in vitro and in mice found that knockdown RACK7 led to increased cancer cell proliferation and migration. This is believed to be caused by the function of RACK7 as a transcriptional repressor through histone methylation (Li et al. 2016). However, in breast cancer cells, RACK7 was shown to have the opposite effect, with deletion of RACK7 decreasing tumor growth and metastasis (Chen et al. 2018).

Like several other proteins from this particular screen, the function of the mitochondrial optic atrophy 3 protein (OPA3) with regard to Mayven and the Cul3 complex as a whole is unclear. What is known is that expression of OPA3 can be regulated by the K-ras signaling pathway, which is also associated with TGFBR1 and TGFBR2 (found in this screen) (Meng et al. 2020, Ryu et al. 2013). OPA3 is expressed in high amounts in pancreatic cancer cells. Downregulation led to a decrease in energy metabolism of cells, as well as a decrease in cell proliferation (Meng et al. 2020).

Another mitochondrial protein of unknown function is the coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (CHCHD10). Very little is known
about this protein. Though mutations correlate with diseases such as ALS, dementia, and Parkinson’s disease, there is currently no known cause of this. Homozygote CHCHD10 knockout mice showed no visible effects from lack of protein function (Burstein et al. 2018).

Activator of 90 kDa heat shock protein ATPase homolog 1 (AHSA1) is the final interacting protein found in this screen. As its name suggests, AHSA1 functions as a cochaperone protein to control the activation of the chaperone protein HSP90 (Woodford et al. 2016). Function of AHSA1 is involved in the regulation of the Akt signalling pathway, like several other proteins noted in this screen (Okayama et al. 2014).

KLHL3

The KLHL3 screen found ten different interacting proteins, most of which were not previously known to be KLHL3 interacting partners. TGFBR2 was also identified in the Mayven screen, though TGFBR1 was not, despite being very similar in structure and function. This result suggests that the Cul3 complex may regulate the signaling pathway initiated by these proteins, though current data has not yet shown this.

ATP1B1 is a sodium/potassium transporting ATPase protein found throughout the body, though most research has focused on its role in renal function. Specifically, SNPs in ATP1B1 have been associated with human essential
hypertension in studies of African American and Chinese populations (Faruque et al. 2011, Xiao et al. 2009). In Milan hypertensive strain rats, expression and activity of Na/K-ATPase proteins including ATP1B1 were increased compared to wild type rats (Ferrandi et al. 1996). The KLHL3-Cul3 complex is well-known for its role in blood pressure regulation through the ubiquitination of WNK kinases regulating sodium chloride cotransporters (NCC) in the kidney (McCormick et al. 2014, Schumacher et al. 2014). The discovery of ATP1B1 in this screen could indicate an additional link between KLHL3 and hypertension.

RANBP2 is an E3 SUMO protein ligase responsible for catalyzing the attachment of a small ubiquitin-like modifying protein (SUMO1) to a substrate, a process known as SUMOylation. The E2 enzyme UBE2I binds RANBP2 and is responsible for attachment of SUMO to a substrate (Pichler et al. 2002). Disruption of SUMOylation through knockout of UBE2I causes errors in transcription regulation, nuclear transport, and other cellular processes, eventually resulting in apoptosis (Nacerddine et al. 2005, Wilkinson and Henley 2010). RANBP2 and interacting proteins have also been implicated in microtubule attachment, particularly during spindle formation in mitosis (Joseph et al. 2004).

Another protein found in this screen which is involved in SUMOylation is the E3 SUMO protein ligase PIASx. During brain development, a SUMOylated MEF2A protein represses transcription, which induces postsynaptic dendritic differentiation in the cerebellar cortex (Shalizi et al. 2006). Shalizi, et al. (2007) discovered PIASx to be the SUMO protein ligase responsible for initiation of this process. This shows the
PIASx is important in neural system development, and its discovery in this screen may mean that KLHL3 is also involved.

The Pleckstrin homology domain containing protein PHLDB3 is a member of the Pleckstrin class of proteins which have only recently begun to be investigated for their role in oncogenesis. One study indicated that PHLDB3 inactivates the tumor suppressor protein p53, resulting in increased tumor growth in lung and colon cancers (Chao et al. 2016). Like several other proteins found in these screens, PHLDB3 regulates the Akt signaling pathway (Fuselier and Lu 2020). More information about these proteins is needed in order to elucidate their involvement with the KLHL3-Cul3 complex.

The cell membrane protein mucin-20 (MUC20) was an additional novel binding protein found in this screen. Similar to others identified, MUC20 has been found to be overexpressed in colorectal, ovarian, and pancreatic cancers (Zheng et al. 2019). This is thought to be due to the role of MUC20 in the regulation of cell signaling pathways, most notably the HGF-induced Grb2-Ras pathway through the binding to Met (Higuchi et al. 2004).

Dynamitin, or p50, is a member of the dynein protein superfamily and acts as a scaffold subunit within the dynactin complex, as shown in Figure 10. This complex binds microtubules, and transports cargo within the cell (Jacquot et al. 2010). Dynamitin is especially interesting to our studies because both underexpression and overexpression of dynamitin have been shown to cause loss of function of this complex (Burkhardt et al. 1997). Overexpression is a phenotype that would be
expected if degradation were impaired. It is possible that this complex is required for 
p50/dynamitin correct placement of ion transporters at the membrane or for moving 
vesicles containing them to lysosomes. Consistent with this idea, a potassium channel, 
Kv1.5 is regulated by this complex by a mechanism of modulation of retrograde 
trafficking of newly endocytosed channels (Choi et al. 2005). In that study, they 
showed that over expression of p50 could disrupt this process and resulted in an 
increase in channel abundance. Cul3 has been shown to play a role in trafficking and 
it is tempting to speculate it could be via this pathway (Huotari et al. 2012).

Another protein discovered in this screen is the guanine nucleotide exchange 
protein Gef10. Gef10 is a member of the guanine nucleotide exchange factor proteins 
(RhoGEFs) which are involved in the regulation of RhoGTPases through the exchange 
of GDP and GTP causing conformational change (Mohl et al. 2006, Verhoeven et al. 
2003). Examination of transfected Gef10 activity demonstrated that Gef10 activates 
RhoB, as well as RhoA and RhoC to a lesser degree (Mohl et al. 2006).

KLHL26 is a KLHL family BTB protein that also appears to be a novel 
interacting partner with KLHL3. Compared to other KLHL proteins, the role of 
KLHL26 within the cell is currently not well understood. However, KLHL26 variants 
have been noted in several studies showing correlation with human disease, 
specifically the congenital heart defect Ebstein's anomaly and breast cancer 
(Samudrala et al. 2020, Zhang et al. 2014). In Ebstein's anomaly, mutant KLHL26 
results in altered cardiac muscle cell proliferation and differentiation (Samudrala et 
al. 2020). The breast cancer study identified three KLHL26 variants as breast cancer
risk factors in a genome-wide association study, along with eight TGFBR2 variants (Zhang et al. 2014). KLHL26 appears to also form heterodimers with other KLHL family proteins which may have cellular function. When KLHL heterodimers were studied in vitro, the KLHL26 dimers with KLHL12, KLHL21, and KLHL24 were not targeted by the regulatory E3 ligase SCF^{FBXL17} for degradation, implying that these are functional dimers which may be involved in cellular processes (Mena et al. 2018). As this screen identified KLHL3 as a binding partner for KLHL26, it is possible that KLHL3 may also form a heterodimer with KLHL26.

**CTB9**

The CTB9 screen found five different proteins, all of which are novel interactions. Perhaps the most interesting of these is FAZF, which was also found in the FMOD screen. FAZF is a BTB protein that contains a zinc finger domain. First discovered as an interacting partner with the Fanconi protein FANCC, it primarily functions as a transcriptional repressor. It has been shown to regulate cytokine production of T cells, as well as regulating the cell cycle (Beaulieu and Sant’Angelo 2011). Expression of FAZF in hematopoietic stem cells resulted in rapid proliferation. In contrast, expression of FAZF in a histiocytic lymphoma cell line caused arrest in the G1 phase of the cell cycle and increased apoptosis (Dai et al. 2002).

The next identified was adenylosuccinate synthetase, or ADSS1. ADSS1 is a cytoplasmic protein primarily found in the heart and skeletal muscle for the purpose
of AMP synthesis (Sun et al. 2005). Low levels of this protein have been linked to distal myopathy, exercise intolerance, and systemic sclerosis in humans. This was corroborated by a zebrafish knockdown of ADSS1, which resulted in malformation of muscle fibers (Park et al. 2016).

TRAP1, or tumor necrosis factor receptor-associated protein 1, is a mitochondrial protein belonging to the HSP90 family of chaperone proteins. TRAP1 has ATP-binding functions, protects cells against oxidative stress, and regulates cellular metabolism (Sciacovelli et al. 2013, Yoshida et al. 2013). It is also associated with tumorigenesis. TRAP1 has been found to be overexpressed in many different types of cancer cells, particularly breast cancer. Knockdown of TRAP1 was correlated with decreased tumor growth, an increase in apoptosis, and arrest of the cell cycle in G2/M phase (Im 2016).

Another protein found to bind CTB9 is the Ataxin-1 ubiquitin-like interacting protein A1U. As a ubiquitin-like protein, A1U is associated with protein degradation through the ubiquitin-proteasome system. Additionally, it facilitates cell cycle progression and apoptosis. This was demonstrated in Huang et al., where overexpression of A1U caused increased apoptosis and cell cycle arrest in G2/M phase (Huang et al. 2018).

The final protein identified in this screen was importin 4, which is a nuclear transport protein. It is known to be responsible for importing key proteins such as DNA repair protein FANCD2, ribosomal protein rpS3α, and vitamin D receptor protein (Miyauchi et al. 2005, Xu et al. 2009). Transportation into the nucleus has
been found to be disrupted in certain types of cancer cells and may be linked to poor prognosis. In gastric cancer cells, importin 4 was found to be overexpressed. This appeared to be related to increased cell proliferation and migration (Xu et al. 2019).

**FMOD**

After performing the yeast two-hybrid screen with FMOD as a bait, the only interacting protein that was discovered was FAZF. This result was unexpected, as it was hoped that there would be more variety to discover more about the function of the protein and other processes it may be involved in within the human body. FAZF, which was also found in the CTB9 screen, is closely related to PLZF, another BTB zinc finger protein. PLZF is involved in recruiting Cul3 to mediate remodeling of transcription complexes (Mathew et al. 2012).

Co-transfection with FMOD and FAZF and PLZF showed that FMOD binds specifically to FAZF and not to both FAZF and PLZF (Wimuttisuk et al. 2014). We have subsequently shown an inverse relationship between FMOD and FAZF levels indicating that FAZF may target FMOD for degradation, as shown in Figure 11. When coexpressing FMOD with varying levels of shRNA against FMOD, FAZF levels increased as FMOD levels decreased (Figure 11).
Muf1

The yeast two-hybrid screen for Muf1 did not show the results that were anticipated. Not only were there fewer unique proteins found than expected, but the proteins found are also not ones closely associated with the Cul3 complex. RANBP9 and JAB1 were the only proteins found in this screen. These two proteins, which interact with each other, are known to be part of a complex of proteins which also includes LRP, BACE1, and APP. This complex is involved with dendritic spine formation and processing of amyloid β peptides in the brain (Wang, et al. 2015). Additionally, a complex with RANBP9 and JAB1 is found along with the proteins Ran and Dyrk1b and is associated with epithelial cell migration in some forms of cancer (Zou et al. 2003). RANBP9 was also found to be one of the interacting proteins in the KLHL3 screen.
Figure 10: The Dynactin Complex

Dynactin consists of two molecules of the motor protein dynein that binds microtubules and moves cargo towards the minus ends (gold and pink), a complex called p150 glued (2 subunits, blue), a complex of actin like proteins (ARP1 -eight subunits, green, binds cargo) and p50, dynamitin (4 subunits, red, regulates assembly).
Figure 11: FMOD and FAZF Expression

Adapted from Wimuttisuk et al. (2014). Figure A shows that FMOD, named here as LRR5, does not bind PLZF (Lane 2) but does bind FAZF (Lane 4). In Figure B, when FMOD is coexpressed with FAZF, FAZF levels decrease. Figure C depicts the increase in endogenous FAZF levels when FMOD is knocked down using shRNA.
Figure 12: Overview of Protein Binding Interactions

Previously established and novel binding interactions between proteins are shown here, with proteins in larger bolded circles being the bait proteins used in the yeast two-hybrid screens. Lines between the circles represent binding interactions. Cul3, the only E3 ubiquitin ligase protein is depicted in red, while BTB proteins and LRR proteins are colored green and blue, respectively. All other proteins are shown in orange.
Table 2: Notable Functions of Interacting Proteins

The table above groups some of the proteins found in this screen by notable function, color coded by which screen(s) the protein was discovered in. Proteins that were discovered in two screens are listed with the two corresponding screen colors. Not all proteins and possible functions are listed here.
DISCUSSION

Overall, the general pattern observed in these protein interactions relates to involvement in cell cycle regulation. As summarized in Table 2, many of the proteins found have significant functional overlap, even those identified in different screens. Though not all screens discovered proteins which are directly related to cell cycle regulation, clearly cell signaling, transcriptional regulation, and cellular migration are processes that are closely involved with the initiation and progression of mitosis. The details of each of these interactions would be interesting to explore but are beyond the scope of this project.

Though many proteins were identified through these screens, it is by no means an exhaustive list of all interactions with the bait proteins. This may be due to several different things: first, certain proteins may be stabilized due to the presence of a protein complex, and therefore would not be found as a singular protein in this screen. Some proteins may also only be present under specific conditions or have a lower concentration in the cells used for the screens. Additionally, certain chaperone proteins may not have been present during the process, resulting in proteins that are not properly folded and therefore nonfunctional. These proteins would not have appeared in the screens.
For further research to be conducted, the first step would be to confirm the binding interactions between the proteins involved. These would be verified through Western blot analysis with endogenous or transfected proteins. Once the binding between the proteins is established through this method, further experiments could be performed through co-transfection with different proteins to determine how the novel binding fits into the Cul3 complex. Further Western blots utilizing a line of HEK293 Cul3 knockout cells could also be used to test whether Cul3 is involved in degradation of these proteins.

Some of the protein interactions discovered tie in well to ongoing research being conducted in our lab. Proteins found in the Cul3, Muf1, and KLHL3 screens are involved in brain development, particularly in the formation of dendrites. As mentioned in the background section, our lab has done some work looking into the protein Shank3 and its involvement in autism spectrum disorder. Previous work in the lab has shown that Shank3 is degraded by Cul3 and is also able to bind actinfilin (KLHL17) and Mayven. It would be interesting to cotransfect Shank3 with the brain-associated BTB proteins discovered in the Cul3 screen (Kctd13, Ctb3, Kctd10, and Btbd3) into HEK293 cells and SH-SY5Y neuroblastoma cells in order to determine whether these are also proteins which interact with Shank3. Muf1 could also be involved, perhaps through mediating the interactions between Cul3 and these BTB proteins. Both proteins found in the Muf1 screen (JAB1 and RANBP9) have been linked to dendritic spine formation, and RANBP9 was also discovered in the KLHL3 screen,
Another area of research that could be explored is KLHL3 and its involvement in NCC cotransporters in the kidney, resulting in hypertension. As discussed in the KLHL3 results section, the protein p50 is of particular interest. First, it would have to be determined whether endogenous p50 and KLHL3 are able to interact. This would be done using the mDCT15 distal convoluted tubule cell line and analyzed using co-immunoprecipitation assays. The next step would be to ascertain whether the degradation of p50 is controlled by the Cul3-KLHL3 complex. This would be tested through cotransfection of KLHL3 and Cul3 in mDCT cells, followed by a Western blot to observe changes in levels of endogenous p50. Previous work in the lab has shown that HEK293 Cul3 knockout cells have slightly elevated levels of p50. This would be expanded upon to determine if mouse embryonic fibroblast (MEF) and mouse kidney cells with knockout Cul3 also exhibit these increased p50 levels. KLHL3 knockout cells would also be tested to observe the involvement of KLHL3 in the regulation of p50 levels within the cell.

As discussed previously in the FMOD results section, interactions between FMOD and FAZF are an ongoing area of research in our lab that will be expanded upon in the future. First, we want to establish how the binding occurs and the involvement of Cul3 in this process. This would be done through introducing mutations in the BTB domain and proline rich region of FAZF, which previous work with a closely related protein has been found to be required for binding FMOD. We will then modify expression of endogenous FMOD using shRNA in cell lines and determine how expression levels of endogenous FAZF respond. We hypothesize that FMOD regulates
FAZF expression. Using cells that express both FMOD and FAZF we will determine if the proteins co-immunoprecipitate. We will measure this using co-immunoprecipitation of the two proteins followed by immunoblot analysis.

Following these experiments, the FMOD and FAZF interaction would be analyzed to determine its involvement in proliferation and apoptosis in cells. To do this, a B-cell line would be used. By downregulating FMOD in these cells using shRNA, we could observe if FAZF levels decrease and if apoptosis occurs. Thus, we can determine if the changes in FMOD expression have functional consequences dependent on downregulation of FAZF. In one example, when FMOD expression was down regulated in B-cells from CLL patients, the cells underwent apoptosis, whereas normal B-cells and normal human fibroblasts did not (Choudhury et al. 2010). We will determine if FAZF levels are inversely regulated, as predicted, in these cells using western blot analysis. We will then directly test our model by expressing shRNA against FAZF at the same time we downregulate FMOD.

These six yeast two-hybrid screens were quite successful in identifying novel interactions with proteins associated with the Cul3 complex. Many of the proteins that were found display involvement in significant cellular processes with potential or known implications for human disease. This research establishing the links between these proteins suggests potential involvement of the Cul3 complex in these areas, and therefore many new avenues of investigation that could be undertaken in the future.
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