

Genetic and Molecular Characterization of Roc-Cullin Interactions in *Drosophila melanogaster* E3 Ubiquitin Ligases

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ABSTRACT

Patrick J. Reynolds: Genetic and Molecular Characterization of Roc-Cullin Interactions
in *Drosophila melanogaster* E3 Ubiquitin Ligases
(Under the direction of Robert J. Duronio)

Cullin-dependent E3 ubiquitin ligases (CDL) are key regulators of protein destruction that participate in a wide range of cell biological processes. The Roc subunit of CDL contains an evolutionarily conserved RING domain that recruits ubiquitin charged E2 and is essential for ubiquitylation. *Drosophila melanogaster* contains three highly related Roc proteins: Roc1a, Roc1b, and Roc2. We previously reported that mutation of *Roc1a* and *Roc1b* causes lethality and male sterility, respectively. Here we show that these disparate phenotypes are partially due to the ability of the Rocs to bind specific Cullins. Roc1a binds Cul1-4, Roc1b binds Cul3, and Roc2 binds Cul5. Through domain swapping experiments, we demonstrate that specific Cullin binding is strongly influenced by the Roc NH₂-terminal domain, which forms an inter-molecular β -sheet with the Cullin. Substitution of the Roc1a RING domain with that of Roc1b results in a protein with similar Cullin binding properties to Roc1a but that cannot complement *Roc1a* mutant lethality, indicating that the identity of the RING domain is an important determinant of CDL function as well. We also identified null mutations of *Roc2* and *Cul5* and show that they cause no overt developmental phenotype, consistent with our observation that Roc2 and Cul5 proteins are exclusive binding partners.

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LIST OF ABBREVIATIONS

2NAR	Fusion of the Roc2 NH ₂ -terminus and Roc1a RING
AN2R	Fusion of the Roc1a NH ₂ -terminus and Roc2 RING
ANBR	Fusion of the Roc1a NH ₂ -terminus and Roc1b RING
APC	Anaphase Promoting Complex
BARD1	BRCA1-Associated Ring Domain 1
BNAR	Fusion of the Roc1a NH ₂ -terminus and Roc1a RING
BRCA1	Breast Cancer 1
BTB	Broad complex, Tramtrack, and Bric-a-brac
Ci	Cubitus Interruptus
CKI	Cyclin dependent Kinase Inhibitor
CNS	Central Nervous System
<i>D.mel</i>	<i>Drosophila melanogaster</i>
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GRF	Genomic Rescue Fragment
GST	Glutathione S-Transferase
HECT	Homologous to E6AP C-Terminus
KLH	Keyhole Limpet Hemocyanin
MALS	Macroautophagy Lysosome System
NF-κB	Nuclear Factor-κB
PMSF	Phenylmethanesulphonylfluoride
Rbx1	Ring Box 1

ROC	Regulator Of Cullins
RING	Really Interesting New Gene
rp49	ribosomal protein 49
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Sb	Stubble
sn	singed
S2	Schneider 2
SCF	Skp1-Cul1-Fbox
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Skp1	S phase Kinase-associated Protein 1
SOCS	Suppressor of Cytokine Signalling
TRAF	TNF Receptor-Associated Factor
Ub	Ubiquitin
Uba	Ubiquitin Activating Enzyme (E1)
Ubc	Ubiquitin Conjugating Enzyme (E2)
Ubl	Ubiquitin Ligase (E3)
UPS	Ubiquitin Proteasome System
UTR	Untranslated Region
VHL	Von-Hippel Lindau
Vif	Virion Infectivity Factor
WT	Wild Type

CHAPTER I

Introduction

Protein Degradation Pathways

Many aspects of cell and developmental biology require precise timing and regulation of protein destruction. There are two major protein destruction systems in a cell: the ubiquitin-proteasome system (UPS) and the macroautophagy-lysosome system (MALS) [1-3]. Many components of these two pathways overlap, but their mechanisms are distinct. The UPS targets soluble, ubiquitin-tagged proteins for destruction or cleavage by the 26S proteasome. Macroautophagy degrades proteins in bulk (soluble or insoluble) during nutrient starvation [4,5]. Both pathways can be used to degrade misfolded proteins, but it is thought that most *specific* protein degradation occurs via the UPS.

E1-E2-E3 Enzymatic Cascade

Protein ubiquitylation requires the action of three families of proteins: E1 Ubiquitin Activators (Uba), E2 Ubiquitin Conjugators (Ubc), and E3 Ubiquitin Ligases (Ubl) [1,3]. Ubiquitin monomers are activated through conjugation via a thiolester linkage to an internal cysteine in E1, which then transfers the ubiquitin to a cysteine residue of an E2 protein. The E2 interacts with an E3 to mediate covalent attachment of ubiquitin onto substrate proteins. Repeated rounds of E2/E3-mediated ubiquitin transfer

result in polyubiquitylation, allowing substrate proteins to be recognized and destroyed by the proteasome (Figure 1.1). For proper proteasomal degradation of some substrates *in vivo*, another factor termed E4 is required [6]. E4 binds the ubiquitin conjugates and helps form multi-ubiquitin chains.

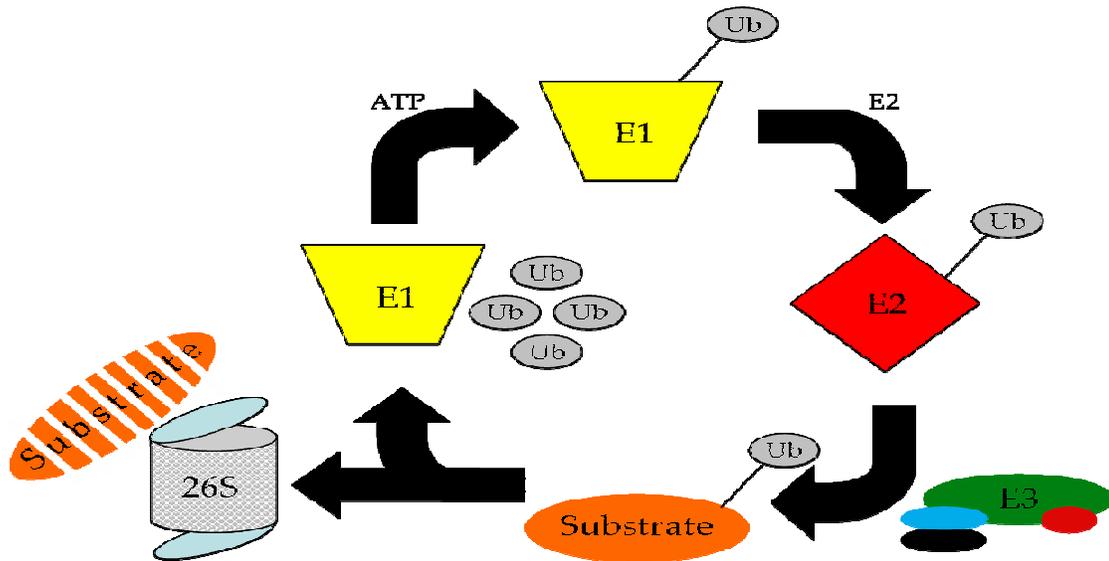


Figure 1.1: Ubiquitin Enzymatic Cascade. Polyubiquitylation of a substrate is achieved through several sequential steps. Ub is first activated by binding to an internal cysteine of an E1 (Uba). It is then transferred to an E2 (Ubc). The E2 then interacts with an E3 (Ubl) and mediates transfer of the Ub onto the substrate. Several rounds of this create a poly-ubiquitin chain, which then targets the substrate for degradation by the 26S proteasome.

Vertebrates and sea urchins have two distinct E1 enzymes, and most other organisms are thought to only have a single E1[7,8]. While there are considerably more E2's (twelve in *Drosophila*), much of the modularity of the ubiquitin-proteasome pathway comes from the large number of different E3 ligases (on the order of hundreds). There is also variation in the way the ubiquitin chains are assembled. The first ubiquitin monomer is added to a lysine residue in the substrate, and subsequent monomers are

added to lysines in the previous ubiquitin. Different branching sites for the ubiquitin chain have been shown to have different outcomes. For example, K48 linked ubiquitin chains of four or greater target proteins for proteasomal degradation [9]. K63 linked chains do not target the substrate to the proteasome, but instead are involved in activating proteins for diverse functions such as DNA repair and receptor internalization [10,11].

E3 Ligase Components

E3's can be broadly categorized as either HECT domain or RING domain. Ubiquitin is directly conjugated to an internal cysteine residue of HECT domain E3's before being transferred onto the substrate protein [12]. RING E3's do not conjugate ubiquitin, but rather stimulate its transfer from the E2 to the substrate [13,14]. RING domains contain conserved cysteine and histidine residues that chelate zinc ions to provide a structure that interacts with an E2 [15,16]. Several RING domain structures have been solved, and they share extensive structural conservation. At least two of these RING proteins, c-Cbl and Rbx1/Roc1, use a similar hydrophobic groove in the protein to bind an E2. All of the Zn^{++} chelating residues of the RING domain (as well as the Zn^{++} ions) are required for proper folding and function of the protein [14,16-19]. The importance to E3 ligase function of residues or domains outside of the RING domain is not currently understood.

RING E3's can be further categorized as either single or multi-protein complexes. Single protein E3's, like c-Cbl, perform the entire function of the E3 within the context of one polypeptide [20]. Other single protein RINGs, like BRCA1, can heterodimerize. While BRCA-1 has ligase activity on its own, this activity is greatly increased when it

heterodimerizes with another RING finger protein BARD-1[21,22]. Multi-protein RING E3 ligases, like the Anaphase Promoting Complex (APC) and Cullin-Dependent Ligases (CDL), use a complex of many different proteins to facilitate ubiquitin transfer [20]. CDL

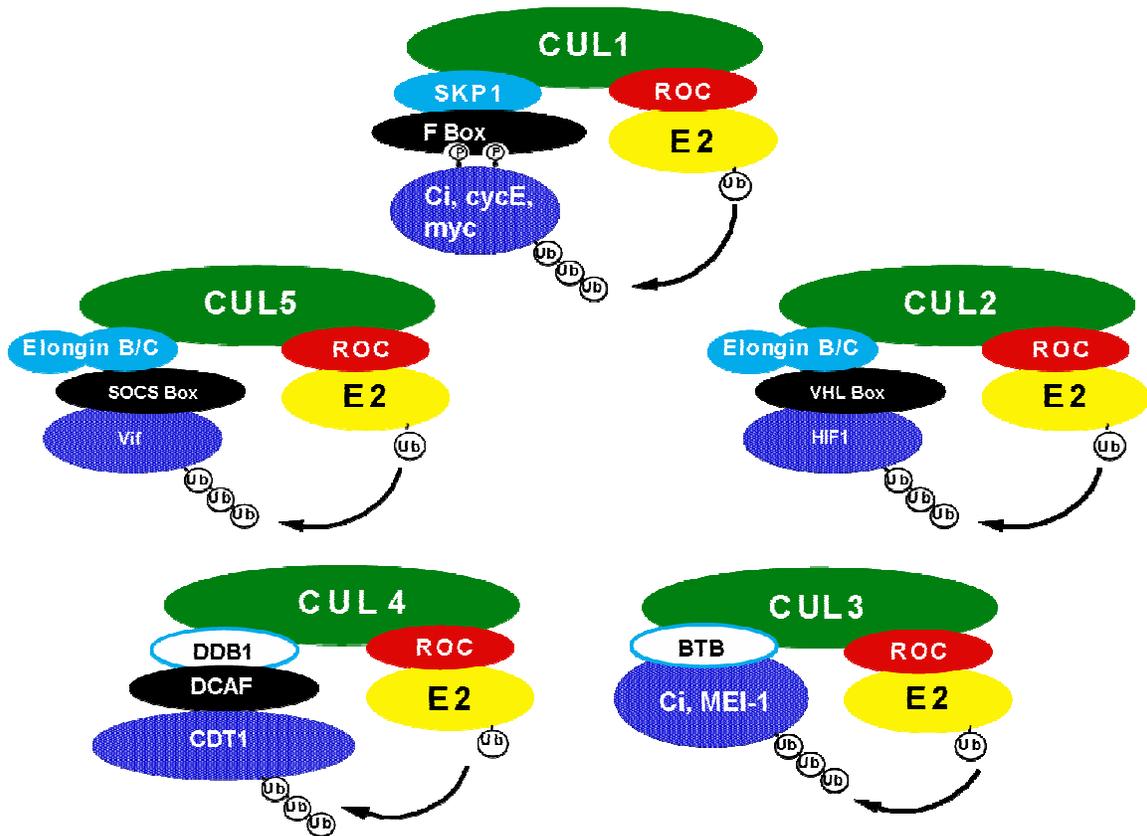


Figure 1.2: Components of Roc-Cullin E3 Ligases. Each CDL consists of a Cullin protein, a Roc protein, and one or several substrate adapter proteins. In this figure, the components bound to the left end of the Cullin are the substrate adapters. The proteins that have been drawn as polyubiquitinated are example substrates of each CDL.

are composed of three modules (Cullin, Roc, substrate adapter/receptor), each with a distinct role (Figure 1.2) [23]. The Cullin serves as a scaffold, binding a Roc protein at its COOH-terminus and a substrate adapter/receptor module at its NH₂-terminus. The Roc

protein provides an interaction surface for ubiquitin-bound E2, and thereby recruits charged ubiquitin to the E3 ligase machinery. The substrate adapter/receptor module (a single protein in Cul3 CDL and multiple proteins in all other CDL) binds directly and specifically to one or a small subset of proteins targeted for polyubiquitylation and destruction. Large gene families encode these substrate adapter/receptor modules, which are thought to provide most of the CDL substrate specificity [24,25]. For instance, the F-box family (33 members in *Drosophila* [26]) contains a diverse group of proteins that recruit specific substrates to the Cul1 E3 ligase via the Skp1 adaptor, which binds both the NH₂-terminus of Cul1 and the F-box domain [27,28]. For most F-box proteins, the substrate is not recognized by the E3 until it is phosphorylated on one or several specific residues. This F-box/substrate binding is highly specific. An example of this is the SCF^{Cdc4} (a Cul1-based E3 ligase with Cdc4 as the F-box protein) ubiquitin ligase. Sic1 is a CKI (cyclin-dependent kinase inhibitor) that must be ubiquitinated and destroyed late in G1 to allow for proper onset of DNA replication. In order for Sic1 to be destroyed, it must be phosphorylated on each of six residues [29]. Mutation of any one of these residues does not allow for recognition by CDC4 and fails to cause proper proteasomal degradation of Sic1 [29,30]. This reveals a high level of regulation for precise timing of protein degradation.

RING Proteins

The RING-finger motif was first described in the human *Really Interesting New Gene 1* (*RING-1*) [31,32]. It is similar to the zinc finger, but it adopts a different cross-brace structure consisting of eight zinc-chelating residues [31,33]. The motif has an

ordered structured even in the absence of zinc ions [31], but the zinc ions are necessary for the Roc1 protein to form the tertiary structure that is recognized by the E2 [28].

There are over 200 RING proteins that have been reported [15], yet very few of them have been studied in the context of ubiquitination. The RING finger was first suggested to be involved in ubiquitylation by a study where Potuschak *et al.* noticed the PRT1 gene in *Arabidopsis* (an N-end rule E3) contained a motif that was also present in several other proteins known to be involved in ubiquitylation [34]. The consensus RING finger sequence is $CX^2CX^{(9-39)}CX^{(1-3)}HX^{(2-3)}C/HX^2CX^{(4-48)}$, where C and H represent cysteines and histidines respectively, and X represents any amino acid [35]. RINGS are defined by the cross-braced pattern of eight cysteine and histidine residues [15,20,31]. These cysteine and histidine residues bind the zinc ions, and other metal-binding residues can be substituted. Interestingly, Rbx1 (as well as *Drosophila melanogaster* Roc1a and Roc1b-see Figure 2.2), has an aspartic acid at the eighth zinc coordination site. It has been shown that in this protein, substitution of the aspartic acid with any other amino acid (including cysteine and histidine) produces a protein that is less efficient in promoting global cullin neddylation [36]. The RING finger proteins are further sub-categorized as RING-HC or RING H2 depending on the identity of the fifth zinc-binding residue [37]. The *Drosophila* Rocs are therefore defined as RING-H2 proteins, as they contain a histidine residue at this position.

Drosophila Roc Proteins

Rocs were the last component found to be a part of the SCF complex [38]. These Roc proteins are an essential component of E3 ubiquitin ligases, and they are encoded by

a gene family [23]. Humans and *C. elegans* contain two Roc proteins, Roc1 and Roc2, while there has been a radiation of the Roc1 family in *Drosophilids* (see Figure 4.1). For instance, *Drosophila melanogaster* (*D. melanogaster*) encodes three Roc proteins named *Roc1a*, *Roc1b* and *Roc2* [39]. The level of functional redundancy among metazoan Roc proteins has remained largely unexplored. We have been addressing this issue in *D. melanogaster* by generating and characterizing mutations in the *Roc* genes. We previously showed that *Roc1a* mutants are lethal, while *Roc1b* mutants are male sterile [39,40]. These different phenotypes suggest that the Rocs are not redundant in function, even though *Roc1a* and *Roc1b* are 78% identical in the RING domain (Figure 2.2). Moreover, this lack of redundancy is not a result of tissue specific expression, since full compensation of *Roc1a* mutant phenotypes in wing imaginal cells cannot be achieved via over-expression of either *Roc1b* or *Roc2* [40]. These data suggest that there exist intrinsic differences in the highly related Roc proteins. Here we show that each *Drosophila* Roc protein binds a distinct set of Cullin proteins.

Roc-E2 Interactions

The Roc component of CDL's provides the bridge for ubiquitin transfer from E2 to substrate. Although Roc's are not known to form any covalent bonds with either ubiquitin or any Ubc, their interaction with an E2 is an essential part of the ubiquitylation reaction. The E2 interacts with the Roc and transfers the ubiquitin monomer onto the substrate (bound on the other end of the E3 by the substrate adapter). One of the ongoing mysteries with E2-E3 interaction is that there is a very large molecular distance of ~50Å between the substrate and the E2 active site [28,41,42]. Most of this distance is made up

by the large scaffold protein Cul1. Cul1 has a rigid cylindrical structure, and it has been shown that this rigidity is essential for SCF function, eliminating the notion that the cullin may bend and bring the E2 and substrate together in the middle [28]. Interestingly, the ubiquitin-bound E2 must disengage from the Roc1 before transferring the ubiquitin onto the substrate [43]. It can then be recharged with another ubiquitin monomer and begin the cycle again. Several rounds of this create a poly-ubiquitin chain and target the substrate to the proteasome. It is also known that the E2 cannot interact with the E1 and E3 simultaneously [44]. This is consistent with a model whereby the E1 transfers the ubiquitin onto the E2, the E2 then interacts with the E3 to transfer the ubiquitin onto the substrate, and then the E2-E1 interaction occurs again.

Dissertation Goals

In this thesis, I will describe my work characterizing the specific interactions between Roc and Cullin proteins in *Drosophila melanogaster*. In Chapter II, I show that Roc-Cullin interactions are specific when expressed at endogenous levels in S2 cells as well as in *D.melanogaster* embryos. There are several distinct Rocs and Cullins, and they are not all functionally interchangeable. In brief, I show that Roc1a binds Cul1-4, Roc1b binds Cul3, and Roc2 binds Cul5. Rocs can broadly be broken down into two domains: the NH₂-terminus and the COOH-terminal RING domain. We created and analyzed chimeras between the three Roc proteins to map binding determinants and demonstrate that both the RING and NH₂-terminus of the Roc proteins can influence Cullin binding. For example, a construct that contains the NH₂-terminus of Roc1a fused to the RING domain of Roc1b can still bind all the same cullins as Roc1a, and it is functional as an E3

ligase. Surprisingly, fusing the Roc1a NH₂-terminus with the Roc2 RING domain creates a protein that cannot bind any cullins and is completely inactive as an E3 ligase. This suggests that rather than simply providing an E2 binding interface for Cullin proteins, the Roc proteins are structurally distinct, and specific RING domains play an important role in determining overall CDL function during development. In Chapter III, I show these results are relevant *in vivo*. By creating transgenic fly lines expressing each of these constructs, I show that although ANBR can bind the same panel of cullins as Roc1a, it is not able to functionally substitute for Roc1a. When expressed under control of the Roc1a promoter, ANBR cannot rescue the lethality of Roc1a mutation. Conversely, I reveal that BNAR protein, which binds all the same cullins as Roc1b, is able to functionally substitute for Roc1b *in vivo*. Several independent transgenic BNAR lines rescue the Roc1b mutant male sterile phenotype as well as control Roc1b transgene. Interestingly, BNAR cannot rescue the sterility when expressed under control of the Roc1a promoter, suggesting complex regulation of Roc expression in the male germline. Finally, in Chapter IV I provide the first detailed analysis of the Roc2 and Cul5 loci, including characterization of mutants. Surprisingly, Roc2 null mutants are viable and only display a slight reduction in female fecundity. We could observe no other overt morphological defect in the mutants. We also identified and characterized Cul5 mutant flies. Consistent with Roc2 and Cul5 being exclusive binding partners, we also could not detect any obvious phenotypes in the Cul5 mutant flies. We provide evidence that knocking down the level of either protein greatly reduces the level of the other. Furthermore, we show that Roc1a cannot bind Cul5 even in a Roc2 deficient background. Thus, these are the

first proteins of the Roc or Cullin family shown to be dispensable for viability and fertility. In Chapter V, all of these results are summarized and discussed broadly.

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CHAPTER II

Molecular Characterization of Roc-Cullin Interactions

Preface

Most of this work has been submitted for publication in PLoS One. I performed all the published experiments, except for the analysis of AN2R and 2NAR cullin interactions in transgenic flies (performed by Jeff Simms). Jeff Simms also contributed transgenic stocks. I wrote the paper, and Robert Duronio designed the project (along with Tim Donaldson) and finalized the manuscript.

Background

The RING domain has been known to mediate ligase activity for a decade [1]. Besides the functional activity of the RING domain, little is known about the function of other regions of RING-containing Roc proteins. The crystal structure of the SCF complex revealed that the NH₂-terminus of Roc1 forms a β -sheet with several β -strands of the Cul1 protein [2]. It also showed that the RING domain forms a globular structure, with several E2-interacting residues present on the outer solvent-exposed side of the protein. Another report has shown that deletions into the NH₂-terminus of Roc1 eliminate cullin binding, but the resulting protein still retains its ligase activity [3]. The same paper showed that the reciprocal is also true: deletions into the COOH-terminal RING domain abolish ligase activity, but the resulting protein is still able to bind cullin. This suggests a

model whereby the NH₂-terminus of the Roc binds cullin, while the COOH-terminus binds E2.

Summary

To investigate the interactions between Rocs and Cullins, we began with a molecular/biochemical approach. After discovering that each Roc binds a distinct subset of Cullins *in vivo*, we sought to determine what region of the Roc is responsible for this. Since creating transgenic fly lines is time consuming, we tested transgenic constructs for expression and binding in S2 cells by transient transfection. This system is much faster, and I was able to recapitulate the binding data I observed in the whole organism (for Roc1a, Roc1b, and Roc2). Thus, this seemed like a reasonable model in which to test for molecular interactions and ligase activity. This rapid “interaction screening” allowed us to return to *Drosophila* after interesting results were obtained and see if they also occurred *in vivo*. We created a series of mutants including RING swap constructs that join the NH₂-terminus of one Roc protein with the COOH-terminal RING domain of another. We found that while the NH₂-terminus is responsible for most of the specific cullin binding, the RING domain also makes an important contribution. Swapping the NH₂-termini and RING domains between Roc1a and Roc1b created proteins that still displayed cullin binding specificity and were active as Ubl’s. Chimeras between Roc1a and Roc2, however, displayed different results. A chimera consisting of the Roc1a NH₂-terminus bound to the Roc2 RING was unable to bind cullin and it was inactive as a Ubl. The reverse chimera consisting of the Roc2 NH₂-terminus and Roc1a RING domain was

able to bind cullin and function as an E3 ligase, suggesting that the Roc1a RING makes more of a contribution to cullin binding than the Roc2 RING.

Materials and Methods

Cell Culture and Transfection

S2 cells were maintained in Schneider's medium supplemented with 10% FBS and 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were transfected using Effectene according to the manufacturer's instructions (Qiagen), and protein lysates were obtained 48 hours later. All transfected DNA's were cloned into the pCaSpeR-4 vector.

Cloning

The RING swap constructs were made by using the CAICR protein sequence that is common to all *Drosophila* Roc proteins (see Fig. 1B) as a region of overlap for primer design. Chimeras were expressed with either a *Roc1a*- or *Roc1b*-grf (genomic rescue fragment). The *Roc1a*-grf was previously described [4] and contains 980 bp upstream of the Start codon and 620 bp downstream of the stop codon. A FLAG- or V5- tag was inserted in frame immediately downstream of the initiating methionine. The *Roc1b*-grf, containing 840 bp upstream from the Start codon and 330 bp downstream from the Stop codon, was also previously described [5], and here we inserted an in frame V5 tag downstream of the Start codon. The A>2, 2>A, and all "Δ" constructs were made using the Stratagene Quikchange kit. The following primer sequences were used:

A>2 alpha: 5'-GGCGATAAGAAGCGCTTCACGGTGAAGAAGTGAACGCC-3'

A>2 beta: 5'-GAACCAGGCGTCCGCGGTTAGCGAGGAGTGCACCGTG-3'

A>2 gamma: 5'-TGCAACCACGCCTTCCATCATCACTGCATCTCTCGCTGG-3'

A>2 delta: 5'-GAGTGGGATTTCCAGAAGATGGGCCACTAAAATGACGTC-3'

2>A alpha: 5'-GGCAAACCGGAAAAAATGTTTGAGCTGAAGAAATGGAACGC-3'

2>A beta: 5'-GCGGACAACAAGCGGGATACCATGGGTCGCCAGGACTGC-3'

2>A gamma: 5'-TGCAACCACTCCTTTCCTTCTGCTGCATGTCGCTGTGG-3'

2>A delta: 5'-GAGTGGTCCATTCAGCGCTACGGAAAATAAGACGTCCTT-3'

1a^ΔF: 5'-CAGGCGAACCAGGCGCGGGATGTGATGGGTAGCGAGGAGTGCACC-3'

1a^ΔR: 5'-GGTGCCTCCTCGCTACCCATCACATCCCGCGCCTGGTTCGCCTG-3'

2^ΔF: 5'-CAGGCGGACAACAAGTCCGCCACTCGCCAGGACTGCGTG-3'

2^ΔR: 5'-CACGCAGTCCTGGCGAGTGGCGGACTTGTTGTCCGCCTG-3'

Creation of GST-fusion Proteins

Using the pCaSpeR-4 Roc constructs as template [5], PCR products were made using primers that added EcoR1 sites on either side, and the product was then cloned into pGEX-1 (GE Healthcare) and confirmed by sequencing. Primers used are as follows.

Roc1a 5'Eco: 5'-CAGAGGAATTCGAAGTCGACGAGGATGGATAC-3'

Roc1a 3'Eco: 5'-CAGAGGAATTCTTAGTGGCCGTACTTC-3'

Roc1b 5'Eco: 5'-TCATTAGAATTCGCCGAGGAGATAGAGGTTG-3'

Roc1b 3'Eco: 5'-CAGAGGAATTCACCGGTCTAGCGGCCGTACTTC-3'

Roc2 5'Eco: 5'-TGACAGGAATTCGCTGATGATCCAGAAACTC-3'

Roc2 3'Eco: 5'-CAGAGGAATTCACCGGTTTATTTTCCCATGCG-3'.

Protocol used for isolation of GST fusion proteins was previously described [3].

Constructs were transformed into BL21DE3 bacteria and induced by adding IPTG to

0.4mM. GST-Roc proteins were purified using Glutathione-Sepharose 4B beads (GE Healthcare). Protein concentration was determined by Coomassie stain using BSA standards.

Ubiquitin Ligase Assay

Ligase assays were performed essentially as described [4] for 45 minutes at 37⁰C with the following components: 50mM Tris-HCl pH 7.5, 5mM MgCl₂, 2mM NaF, 0.6 mM DTT, 2mM ATP, 10nM Okadaic Acid, 40 ng rabbit Ube1 (Boston Biochem), 300 ng UbcH5, 12 µg bovine Ub (Sigma), and 250 ng GST-Roc. Samples were run by SDS-PAGE on a 12% gel followed by Western blotting for Ubiquitin.

Virtual Alanine Scanning

The ClustalX program was used to align the amino acid sequences of 3 ROC proteins from *Drosophila melanogaster* with the sequence of the Human Rbx1 protein. Residues in this alignment were classified as 1) invariant and highly-conserved, or 2) non-conserved. This classification was mapped onto the experimentally determined structure 1LDJ (PDB accession code) of Human RBX1 in complex with Human Cullin 1 obtained from the Protein Data Bank (www.rcsb.org) [6]. The InsightII molecular modeling system from Accelrys Inc., (www.accelrys.com) was used to identify residues in the Human Cullin 1 protein that interact with the invariant/conserved residues and with the non-conserved residues in Rbx1. The crystal structure of human Cul1-Rbx1a (PDB: 1U6G) was used for analysis. The sequence of *Drosophila* Cul1, Rbx1a and Rbx2 were threaded on 1U6G using Rosetta design software. The residues responsible for *drosophila*

Cul1-Rbx1a binding were identified by virtual Alanine scanning (<http://rosetta.org/>). The residues at the interface which were different in the cases of Rbx1a and Rbx2 were probed further for binding analysis using Rosetta software for binding energy. The residues E23, V24, L32, T64, F81 and Y106 of Rbx1a at the Cul1-Rbx1a interface did not match with corresponding residues T26, L27, M35, V67, H86, M111 of Rbx2 and were probed. We analyzed the change in binding energy (Δ Binding Energy in Table 1a) of Cul1-Rbx1a when residues in Rbx1a were mutated to correspond to Rbx2. Since Cul1 does not bind Rbx2, we looked for mutations predicted to weaken binding, a positive Δ Binding Energy (Table 1a). Similarly, we analyzed the change in binding energy (Table 1b) of Cul1-Rbx2 when residues in Rbx2 were mutated to correspond to Rbx1a. In this case, we looked for mutations predicted to enhance binding, a negative Δ Binding Energy (Table 1b).

Results

Roc proteins are thought to bridge the Cullin and E2, thus allowing Ubiquitin to be transferred onto the substrate. Human Roc1 has been shown to interact with all cullins by yeast two-hybrid analysis and when overexpressed in tissue culture [7,8]. Other experiments have indicated that there may be some specific binding between Rocs and Cullins[3,5,9]. We tested this *in vivo* by creating transgenic fly lines that express each of the individual *Drosophila* Roc proteins (Roc1a, Roc1b, and Roc2) under control of the ubiquitously expressed Roc1a promoter [4]. We used the same promoter for each to reduce false negatives caused by lack of expression of a Roc protein in a particular tissue. All of the transgenic proteins contained an in-frame FLAG epitope at the NH₂-terminus.

Embryo protein lysates were made and subjected to immunoprecipitation with an anti-FLAG antibody. After washing, the beads were boiled in Laemmli buffer and SDS-PAGE was performed on the supernatant. To visualize bands, the gel was stained with either Coomassie, Silver Stain (Figure 1.2), or Sypro Stain. Based on the known molecular weight of the Cullin proteins (~90-100kDa), we narrowed our analysis to bands that were present in this range in the transgenic lanes but absent in the control non-transgenic lane. These bands were then identified by mass spectrometry (Figure 2.1). Roc1a binds Cul1, Cul2, and Cul3. Roc1b binds Cul3, and Roc2 binds Cul5. This revealed that the Rocs do indeed have specific binding partners when expressed at physiological levels. While differential transcriptional regulation of the Rocs certainly occurs [4], this result suggested that there is also something physically different about the Roc proteins that causes these specific interactions. To further this analysis, I obtained antibodies for all five of the cullins (and created the Cul5 antibody, see Chapter IV). I then performed the same immunoprecipitation, this time followed by Western blotting for each cullin (Figure 2.1). Roc1a binds Cul1-4, Roc1b binds Cul3, and Roc2 binds Cul5. Thus, the results were consistent between experiments, and they displayed a clear binding preference between specific rocs and cullins.

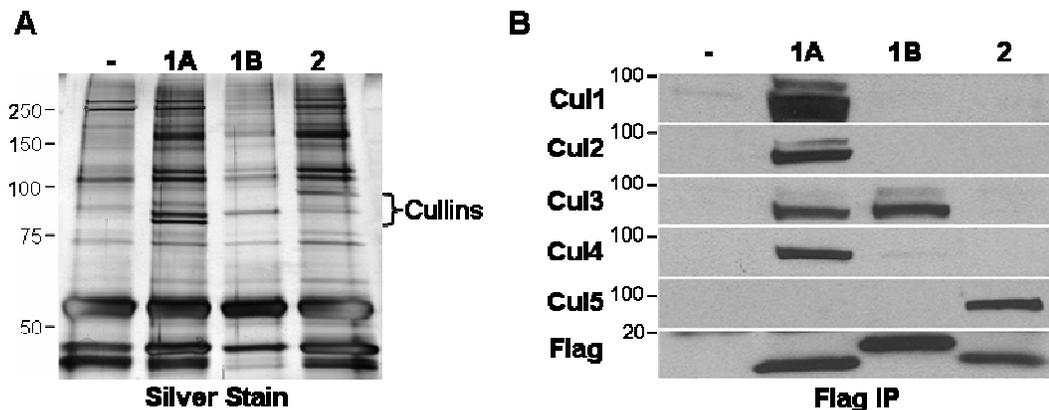


Figure 2.1: Rocs Display Unique Cullin Binding Profiles. (A) Silver stained SDS-PAGE gel of FLAG-

Roc immunoprecipitates. Bands present in the transgenic lanes but absent in the control non-transgenic lane were identified by mass spectrometry. Bands were identified as follows: Roc1a lane-Cul1, Cul2, Cul3. Roc1b lane-Cul3. Roc2 lane-Cul5. (B) FLAG-Roc immunoprecipitates were probed with antibodies to all of the cullins. Roc1a binds Cul1-4, Roc1b binds Cul3, and Roc2 binds Cul5.

After observing this network of Roc-Cullin interactions, we wanted to determine what regulates the binding. The crystal structure for a partial SCF complex consisting of Roc1, Cul1, and Skp1 has been solved [2]. It shows that the Roc protein is made up of an NH₂-terminal β -strand and a COOH-terminal RING domain. The NH₂-terminal β -strand residues interact with the Cul1 protein, and the RING domain is thought to mediate interaction with the E2 (the E2 is not present in the crystal structure). Since Roc1a and Roc2 are the most divergent in sequence (Figure 2.2) and have opposite patterns of Cullin binding, we began our analysis with these two proteins. In collaboration with Ramesh Jha in Brian Kuhlman's lab (UNC-Chapel Hill), we performed "virtual alanine scanning" to identify amino acids that significantly reduced binding between Roc1 and Cul1 when converted to alanine (thereby removing the side chain). The purpose of this was to identify all residues that are important for Roc-Cullin interaction, regardless of specificity. Of these amino acids, we looked for residues that were significantly different between Roc1a and Roc2, and thus could contribute the most to specific cullin binding. The Roc2 sequence was superimposed over the Roc1-Cul1 crystal structure to compare Roc-Cullin interactions between these two complexes. By measuring changes in binding energy (Table 2.1) when the individual residues were changed from Roc1a to Roc2 sequence (and vice versa), we narrowed our analysis to E²³, T⁶⁴, F⁸¹, and Y¹⁰⁶ in Roc1a (T²⁶, V⁶⁷, H⁸⁶, and M¹¹¹ in Roc 2). We were initially surprised that three of the four

residues were located in the COOH-terminal half of the protein, while the crystal structure suggested that most of the interactions occur in the NH₂-terminal β -strand. It is possible that the majority of *all* Roc-Cullin contacts are in the NH₂-terminus of the Roc, but some of the *specific* contacts are in the RING domain.

```

                                NH2-terminus
ROC1A  M----EVDE-----DG- YEVPS SSS---KGDKKRFEVKKWNAVALWAWDIVVDN
ROC1B  MAEEIEVEETEDFDHMDFNDEEPS CSGGAVQARTERFVKKWVAHAMWGWDVAVDN
ROC2   M----ADDP-----ENSVDRPTDDG-DAGKPEKMF^TLKKWNAVAMWSWDVECDI
                                ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

                                RING Domain
* *   CA.ICRNHIMDLCECQANQAS--ATSEECTVANGVCNHAFFHC^ISRWLKTRQVC^PLDNREWFQRYGH
* *   CA.ICRNHIMNLCIECQADFN---ANQDECTVANGECNHAFFHYHC^IARWLKTRLVC^PLDNKEWVYQRYGR
* *   CA.ICRVQVMDSCLRCQADNKRDMGRQDCVVVWGECNHSFHHCCMSLWVKQNNRCLCQ^Q^EW^SIQRMGK

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Figure 2.2: ClustalW Alignment of *Drosophila* Rocs. The protein sequences of Roc1a, Roc1b, and Roc2 were aligned using ClustalW and separated into the NH₂-terminus and RING domain. The carats indicate Roc1a residues that interact with Cul1[2]. The asterisks indicate the essential RING domain residues that chelate Zn⁺⁺ ions. Residues of RING insertion are underlined for Roc1a.

A		B	
A>2 Mutation	Δ Binding Energy	2>A Mutation	Δ Binding Energy
WT	0	WT	0
E23T	0.62	T26E	-4.37
T64V	2.01	V67T	-1.97
F81H	0.36	H86F	-2.37
Y106M	-0.53	M111Y	-3.18

Table 2.1: Roc point mutations change Roc-Cullin Binding Energy. WT (no amino acid change) is set at zero. Change in binding energy was determined in software (see Methods) when changing the specified residues. A. Roc1a residues were changed to the corresponding Roc2 residues. A positive (or very low negative) change in binding energy suggests the Roc1a residue forms a more favorable interaction with Cul1 than the Roc2 residue. B. Roc2 residues were changed to the corresponding Roc 1a residues. In this

case, a negative change in binding energy suggests the Roc1a residue forms a more favorable interaction with Cul1 than the Roc2 residue.

I created constructs (Figure 2.3) that mutated all four of the Roc1a residues to Roc2 and vice versa. These constructs are named A>2 (4 residues mutated to Roc2 sequence) and 2>A (four residues mutated to Roc1a). If these residues are responsible for mediating specific cullin binding, these constructs should switch the cullin binding partners. As Figure 2.4a shows, this did not alter the binding specificity of the Roc-Cullin interactions: A>2 behaved just like Roc1a, and 2>A behaved just like Roc2. Thus, these residues *alone* are not responsible for cullin binding specificity. They may still be important for binding in the context of a larger region.

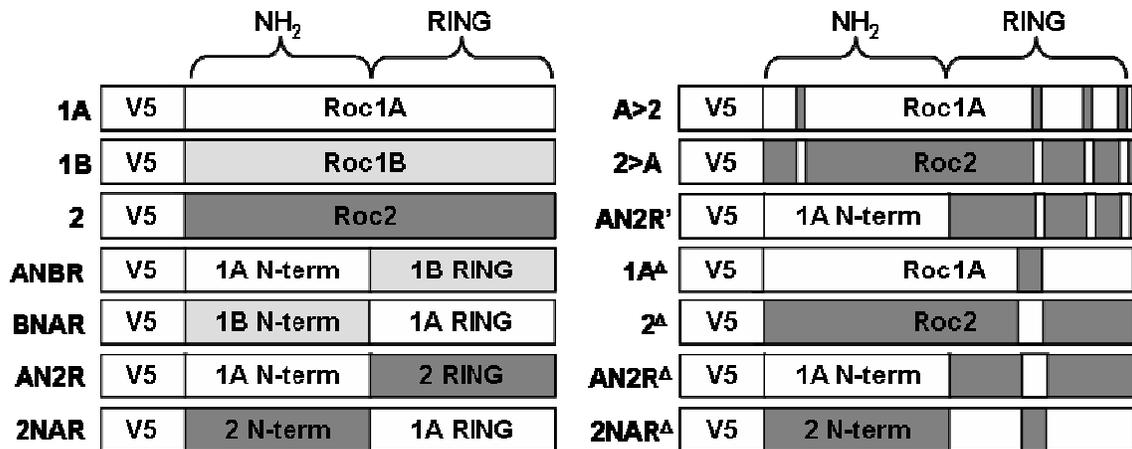


Figure 2.3: Schematic of All Transgenic Constructs. All constructs contained an N-terminal V5 tag. Roc1a sequence is indicated by white, Roc1b by light gray, and Roc2 by dark gray. The brackets above the constructs indicate the NH₂-terminus and RING domain (same as in Figure 1.3).

Although these individual point mutations were not able to alter the interactions, it is possible that a larger stretch of amino acids is responsible for this. The SCF crystal

structure also reveals that the Roc proteins have a RING insertion between the S1 and S2 β -strands that is not found in other RING proteins [2]. This region is in close proximity to the cullin protein, and the sequence is highly divergent between Roc1a and Roc2 (Figure 2.2). After initial attempts to swap this entire sequence between the Rocs proved unsuccessful (too large for efficient mutagenesis with Stratagene Quikchange Kit), we narrowed our analysis to the most divergent portion of the insertion. To test if this region mediates binding specificity, I mutated the S⁶²A⁶³T⁶⁴ residues of the Roc1a RING insertion to the corresponding R⁶⁵D⁶⁶V⁶⁷M⁶⁸G⁶⁹ residues of Roc2, and vice versa. I named the constructs 1A^Δ and 2A^Δ to indicate that this region is “changed.” As Figure 2.4b shows, this also failed to alter cullin binding. Although these amino acids may be important for Roc-Cullin binding, they are not responsible for the majority of the specificity.

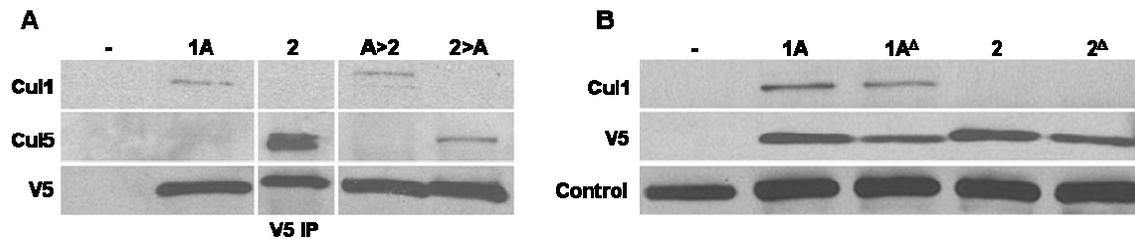


Figure 2.4: Point Mutants and Delta Constructs. A. All constructs are expressed with the *Roc1a* promoter and regulatory regions, and the chimeric proteins contain an amino-terminal V5 tag. A. Control Roc1a and Roc1b or A>2 and 2>A constructs were transfected into S2 cells and cellular extracts were immunoprecipitated with anti-V5 antibodies and probed for the presence of Cul1 and Cul5 by immunoblotting. B. Control Roc1a and Roc2 or A^Δ and 2^Δ constructs were transfected into S2 cells and cellular extracts were immunoprecipitated with anti-V5 antibodies and probed for the presence of Cul1 by immunoblotting. Control is a non-specific band for loading control.

It is likely that a larger region of the Roc protein interacts with the Cullin. We widened our search even further and broke the Roc into two domains: the NH₂-terminal β -strand and the COOH-terminal RING domain (Figure 2.2 and 2.3). If the NH₂-terminus alone is sufficient for Cullin binding, then the identity of the RING domain should not influence this interaction. To test this, I created a series of “RING Swap” constructs that contain the NH₂-terminus of one Roc connected to the RING domain of another. For example, ANBR consists of the Roc1a NH₂-terminus and the Roc1b RING domain. To determine if the N-terminus or the RING domain imparts the specificity, we began with the AN2R and 2NAR constructs. Surprisingly, AN2R failed to bind any cullin, although it was stably expressed at levels comparable to the other RING Swap proteins (Figure 2.5a). In the reciprocal experiment, however, 2NAR bound to Cul5 just as Roc2 alone does. It is likely that the AN2R protein does not fold properly and is completely inactive as a ligase. Interestingly, 2NAR also bounds Cul1 to a much lesser degree. This result was surprising, considering Roc2 does not bind Cul1. This suggests that while the NH₂-terminus plays the larger role in cullin binding, the RING domain (at least in the case of Roc1a’s RING domain) also makes a contribution. Consistent with this idea, we found that the 2NAR construct also interacted with Cul3 (Figure 2.5c), another binding partner of Roc1a. We also examined the cullin binding for ANBR and BNAR (Figure 2.5b). In this case, the interaction appeared to be solely mediated by the N-terminus: ANBR binds the same cullins as Roc1a, and BNAR binds the same cullins as Roc1b.

To confirm some of these observations *in vivo*, we generated multiple AN2R and 2NAR transgenic lines and analyzed embryo extracts by IP-Western analysis as in Figure 2.1. We consistently obtained similar results as in S2 cells; AN2R bound no Cullin, while

2NAR bound Cul5, as did normal Roc2 (Figure 2.6a). In addition, 2NAR also bound Cul3 (Figure 2.6b). Thus, the Roc NH₂-terminus provides a strong determinant for Cullin binding specificity, but is not always sufficient. In addition, since full length Roc2 does not bind either Cul1 or Cul3, our data suggest that the Roc1a RING domain plays a more important role in Cullin binding specificity than the Roc2 RING domain.

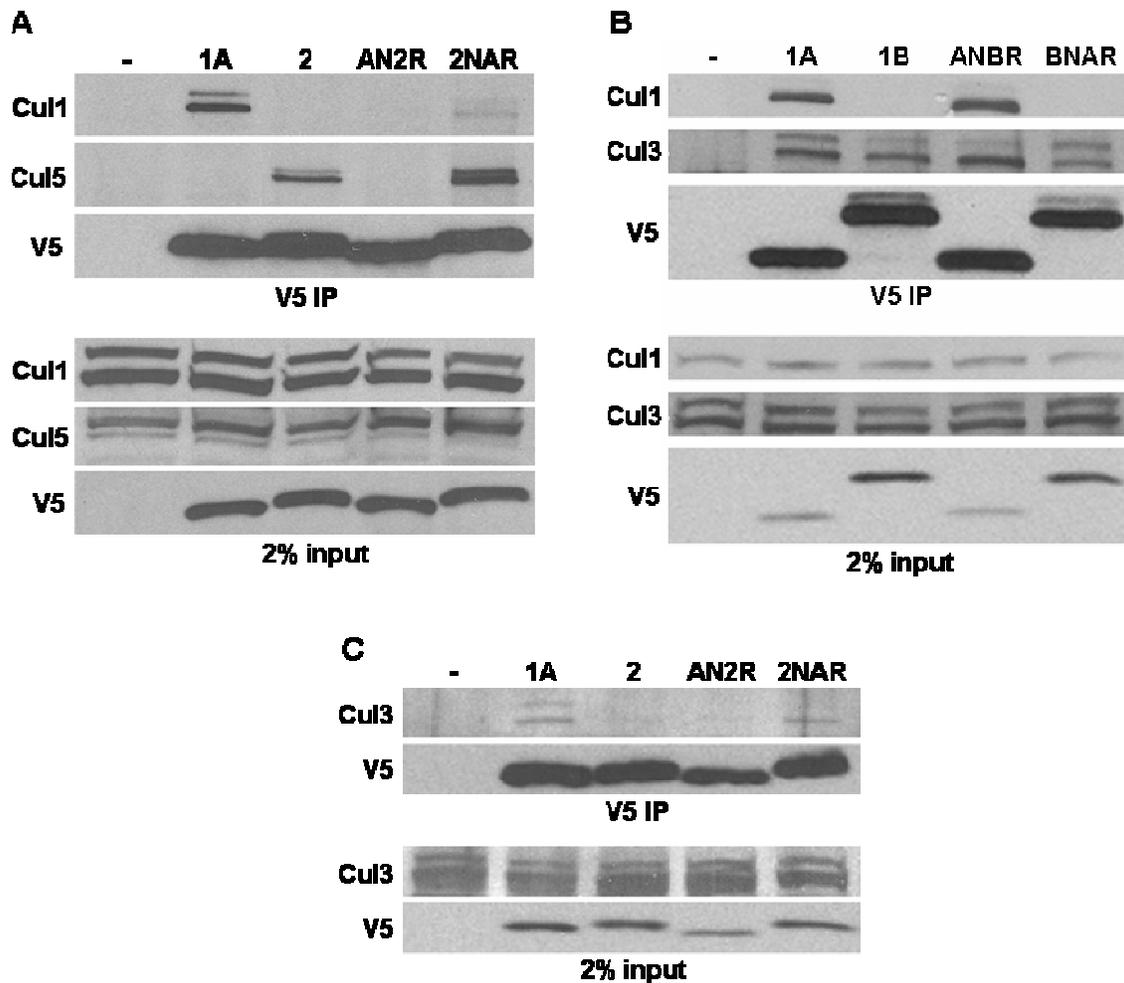


Figure 2.5: RING Swap Constructs and Cullin Binding in S2 Cells: A. Control Roc1a and Roc1b or ANBR and BNAR constructs were transfected into S2 cells and cellular extracts were immunoprecipitated with anti-V5 antibodies and probed for the presence of Cul1 and Cul3 by immunoblotting. B. Control Roc1a and Roc2 or AN2R and 2NAR constructs were transfected into S2 cells and cellular extracts were

immunoprecipitated with anti-V5 antibodies and probed for the presence of Cul1 and Cul5 by immunoblotting.

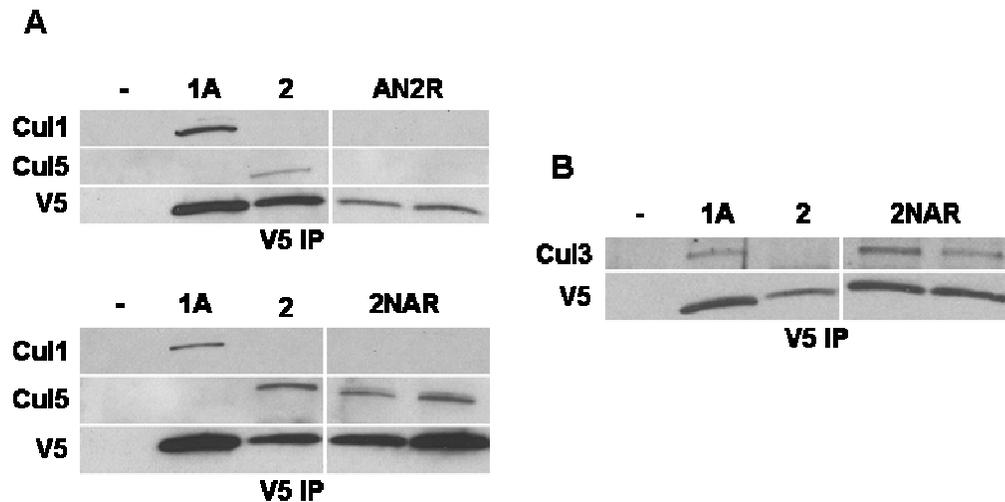


Figure 2.6: RING Swap constructs and Cullin binding in embryos. A. RING-swap chimeric proteins were immunoprecipitated from transgenic embryo extracts and tested for interaction with Cul1 and Cul5 by immunoblotting. Two independent transgenic lines of AN2R and 2NAR are shown. B. Two 2NAR transgenic lines were tested for interaction with Cul3.

To determine if the RING domain could actually inhibit some Roc-Cullin interactions in the context of a chimeric protein, I also created constructs consisting only of the NH₂-terminus. These constructs, however, were completely unstable in S2 cells and could not be detected by Western blot (data not shown). Therefore I could not determine if the NH₂-terminus alone is sufficient for some of the cullin interactions. This instability may be caused by an inability to form a protective Roc-Cullin-CAND1 complex, as several Roc COOH-terminal residues are known to make important contacts with CAND1 [10]. Finally, I created RING Swap constructs that also contain the “Δ” region of the opposite Roc in the RING insertion (AN2R^Δ and 2NAR^Δ, See Figure 2.3).

For example, AN2R^Δ is the same as AN2R, but with the RING insertion sequence of Roc1a. These proteins displayed the same cullin binding as the normal RING Swap proteins (data not shown), further suggesting that this RING insertion does not play a major role in cullin binding specificity.

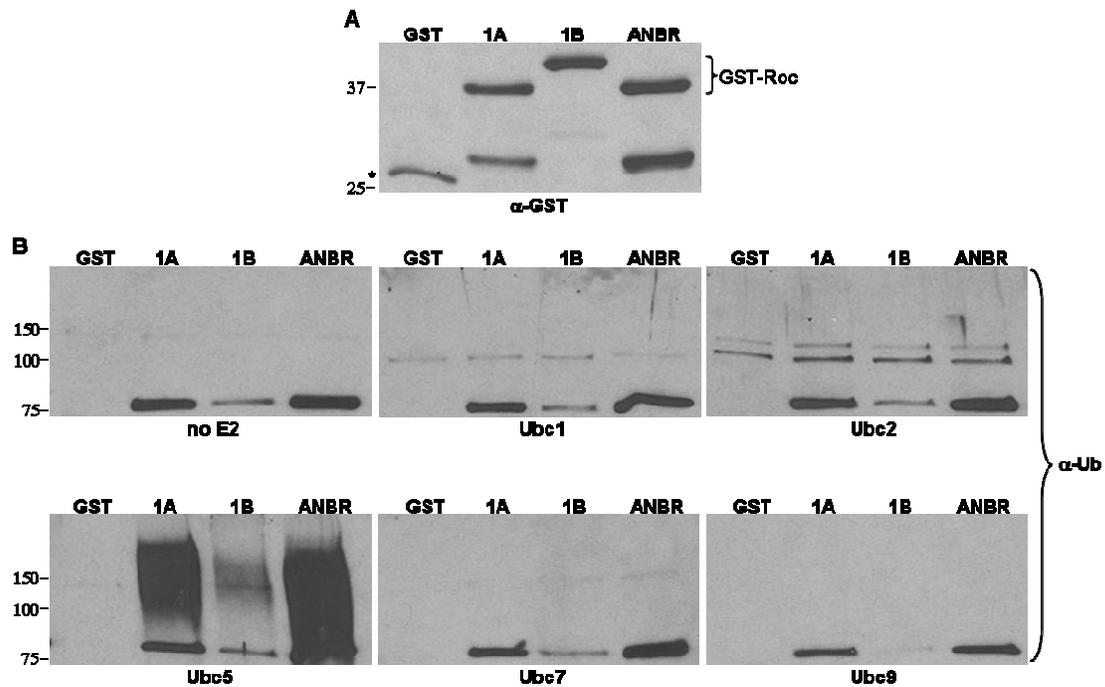


Figure 2.7: Activity of Rocs with Various E2 Enzymes. A. Loading control for GST-Roc proteins. Ligation reactions containing either control (GST alone), GST-Roc1a, GST-Roc1b, or GST-ANBR were separated by SDS-PAGE and blotted for GST. Bracket indicates GST-Roc proteins. Asterisk indicates GST. Lower bands in transgenic lanes are degradation products. B. Ligation reactions were run (see Methods) with control (no E2) or one of several Ubc enzymes and blotted for Ub to detect ubiquitin conjugates. High molecular weight conjugates are visualized as smearing (see Ubc5 panel).

Although some of these chimeric Rocs are capable of binding cullins, we did not know if they were functional as E3 ligases. It has been shown previously that RING proteins have ligase activity even in the absence of cullins and other associated factors

[3,4,8,11-15]. We were interested in knowing if the RING Swap Rocs were active ligases, and if this activity was E2 specific. Therefore, I created GST-fusions of all the Rocs and RING Swaps and tested them for ligase activity (see Methods). We were unable to detect polyubiquitylation with Ubc1, Ubc2, Ubc4, Ubc7, Ubc9, or CDC34 as the E2 (Figure 2.7). Since this result is completely negative, the Rocs *may* still interact with different E2's *in vivo*. We did not have a proper positive control to test for the activity of each E2. All of the Rocs, however, had varying amounts of ligase activity in the presence of UbcH5. While Roc1a and Roc2 displayed high ligase activity with UbcH5, Roc1b showed a weaker ability to promote poly-ubiquitination (Figures 2.7 and 2.8). This suggests that differential tissue expression of Rocs or E2's is not the sole determinant of their interaction, since all components are expressed at equal levels in this experiment.

We next examined the ability of the RING Swap proteins to promote poly-ubiquitylation. Interestingly, BNAR showed an almost identical pattern of laddering as Roc1b (Figure 2.8). Looking at the poly-ubiquitin laddering, a pattern emerges: Roc1a and ANBR have high levels, and Roc1b and BNAR have low levels. Both of these groups have the same NH₂-terminus. Thus, even though the RING domain is known to mediate most of the physical interaction with the E2 [2], the N-terminus of the protein appears to play a significant role in determining efficiency of poly-ubiquitination with a particular E2. AN2R protein, which was unable to bind any cullin (Figure 2.5), is also non-functional as a ligase in this assay. It is likely that this chimera does not fold properly and is completely inactive. 2NAR, which we showed is able to bind to Cul5 and Cul3, is an active ligase. It is interesting that these reciprocal protein chimeras have completely different activities.

We also observed that the pattern of poly-ubiquitination is different amongst the Roc proteins. Roc1a promotes formation of lower molecular weight ubiquitin conjugates, and Roc2 forms higher molecular weight conjugates. 2NAR, however, appears to form conjugates that span the entire range of the other two individual Rocs. What this means biologically remains to be determined, but it does suggest another level of complexity above a simple “on-off” switch for poly-ubiquitination.

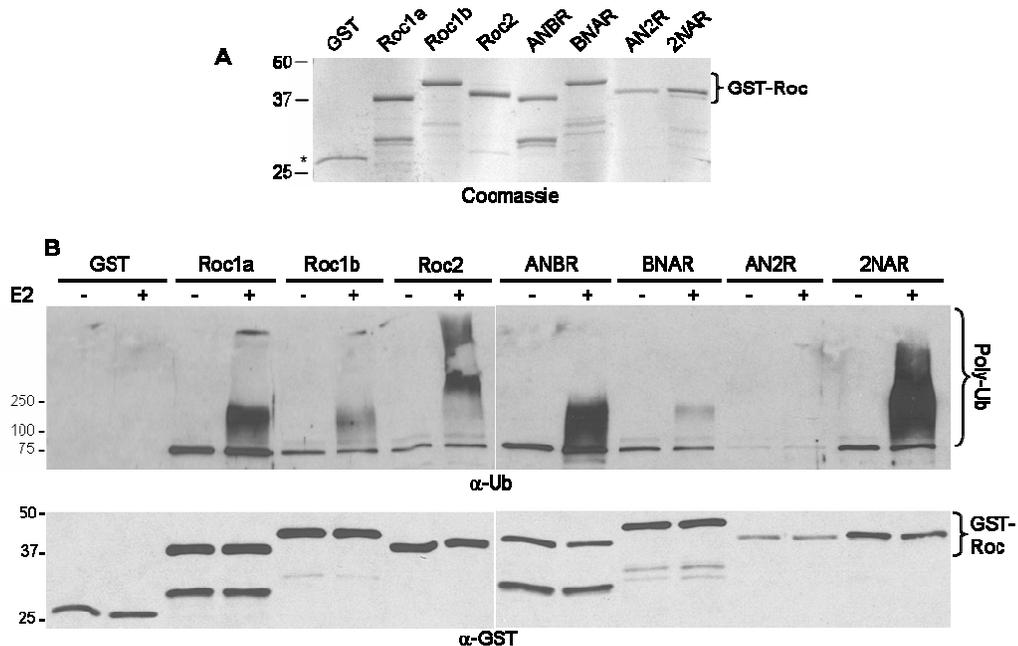


Figure 2.8: Ligase activity of RING swap proteins. A. GST-fusions of all Roc proteins and chimeras were made. Coomassie stain showing even levels of Roc protein were used in the ligase assay. Bracket indicates GST-Roc proteins. Lower bands are degradation products. B. All *Drosophila* Roc proteins and chimeras were assessed for ligase activity in a substrate free assay. Briefly, 250 ng of Roc protein (or control GST) were added to a ubiquitin ligase mixture containing UbcH5, ubiquitin, and ligase buffer and incubated for 45 minutes. “-” indicates that no E2 was added to the reaction, and “+” indicates it was. Ubiquitin conjugates were detected by Western using an anti-Ub antibody (bracket indicates poly-ubiquitin chains).

Discussion

In this study we show that the Roc proteins play a part in the functional modularity of Cullin-dependent E3 ligases. Our data show that selective Roc-Cullin interactions occur *in vivo* in *D. melanogaster*, and that the highly conserved Roc proteins serve distinct roles as members of different Cullin E3 ligase complexes.

Roc-Cullin interaction determinants

The Roc NH₂-terminus is necessary for binding to Cullin protein [3] and forms a β -strand that makes an inter-molecular β -sheet with the Cullin protein [2,16]. Our analysis of “RING swap” protein chimeras indicates that in some instances the Roc NH₂-terminal β -strand is the primary contributor to Roc-Cullin binding *specificity*. Both fusing the Roc2 NH₂ terminus to the Roc1a RING domain (2NAR) and fusing the Roc1a NH₂-terminus to Roc1b (ANBR) results in proteins that primarily display the Cullin binding preferences of Roc2 and Roc1a, respectively. However, our data clearly indicate that the RING domain also makes a contribution to Cullin binding preference. While a full length Roc1a construct bound Cul1-4 (Figure 2.1), a construct consisting of the Roc1a NH₂-terminus fused to the RING domain of the more distantly related Roc2 (AN2R) was unable to bind any Cullin. Substituting in the Roc1b RING domain, however, was able to restore Cullin binding, and the resulting ANBR protein displayed a Cullin binding profile identical to Roc1a. This suggests that a region of the RING domain that is more similar between Roc1a and Roc1b than Roc2 participates in Roc1a-Cullin binding preferences. Roc1a is 78% identical to Roc1b across the RING domain, while it only shares 45% identity with Roc2 in this region. In addition, the 2NAR protein bound detectably to Cul3

while normal Roc2 protein did not, again suggesting that the Roc1a RING domain influences Cullin selection. Taken together, we conclude from our data that the Roc NH₂-terminal β -strand makes a relatively stronger contribution to Cullin binding preference than the RING domain.

The lethality of *Roc1a* mutants is presumably caused by the inappropriate accumulation of at least one target of a Roc1a-containing Cullin dependent E3 ligase [4]. The ANBR protein cannot rescue the lethality of *Roc1a* mutants even though it binds all of the Cullins that Roc1a does. Thus, even though ANBR can activate polyubiquitin chain formation *in vitro* (Figure 2.7 and 2.8), fusion of the Roc1a NH₂ terminus with the Roc1b RING domain does not create a fully biologically active Roc1a protein. One possible interpretation of this result is that *in vivo* the Roc1b RING domain cannot productively interact (i.e. stimulate ubiquitylation of critical targets) with all of the E2s that Roc1a does. This may occur at the level of direct binding, such that there are some E2s that only bind the RING domain of Roc1a and not Roc1b, or at the level of stimulating ubiquitin transfer to substrate in the context of a fully assembled CDL. However, we show here that neither Roc1a nor ANBR can stimulate poly-ubiquitin chain formation using Ubc1, Ubc2, Ubc7, or Ubc9 in a substrate free *in vitro* ligase assay.

A similar observation was previously reported for TRAF3 and TRAF5 RING domain proteins [17]. TRAF5 can activate the NF- κ B pathway when overexpressed, while TRAF3 cannot. This is dependent on the RING domain, as mutation of a key RING cysteine inhibits this activation. A construct that fuses portions of the RING domains of TRAF3 and TRAF5 is also incapable of NF- κ B activation. The authors proposed that these chimeric proteins might not be able to chelate zinc ions or fold properly. This may

explain why AN2R is unable to bind any Cullin. However, the other Roc chimeras we constructed were able to bind Cullin and were active as E3 ligases. Moreover, the rescue of male sterility by the ANBR chimera (see below) indicates that Roc protein chimeras *can* be functional *in vivo*.

In the *Drosophila* male germline, Roc1b forms a complex with a testes-specific Cul3 isoform and the BTB protein KLH10 to regulate caspase activation during spermatid differentiation [18]. Consequently, *Roc1b* mutants are male sterile [5]. Interestingly, the BNAR chimera, which binds well to Cul3, effectively rescues Roc1b mutant male sterility. This is consistent with our previous data showing that Roc1a can partially rescue the male sterility of *Roc1b* mutants when expressed from the Roc1b promoter [5]. These data suggest that in the context of the testes specific Cul3 complex, the RING domains of Roc1a and Roc1b can productively interact with a similar set of E2s. Thus, our genetic rescue experiments with *Roc1a* and *Roc1b* mutants can be explained if Roc1b interacts with a subset of all the E2s that interact with Roc1a. It is possible that Roc1a can also interact with other E2's we did not test *in vivo*. Finally, the functional redundancy of Roc1a and Roc1b in the male germ line is only detected with the *Roc1b* promoter, suggesting that the *Roc1a* and *Roc1b* genes are expressed differently during spermatogenesis.

The structure of another RING-containing protein Cbl in complex with UbcH7 provides more interesting clues as to the nature of the E2-E3 interaction [19]. The authors showed that the F⁶³ residue of UbcH7 interacts with the W¹⁰⁸ and I³⁸³ residues of the Cbl “RING groove.” They suggest that this interaction contributes to the specificity amongst other E2-E3 pairs. However, these residues are identical across all three *Drosophila*

Rocs. Thus, if the *Drosophila* Rocs do bind different E2's, this is not accounted for by these residues. It is possible that while all eukaryotes are thought to use an E1-E2-E3 enzymatic cascade, the exact nature of the system may have evolved differently in certain organisms.

Acknowledgements:

I would like to thank Tim Donaldson for creative input in helping design the initial stages of the RING Swap project. I also thank Jeff Simms for cloning assistance, general fly maintenance, and *in vivo* analysis of RING Swap cullin binding. Lisa Wolff also assisted in creation of transgenic fly lines. Thanks to Ramesh Jha and Brian Kuhlman for their assistance in physical modeling of Roc-Cullin interactions. Finally, I thank Zhijun Li for technical advice regarding the ubiquitin ligase assays.

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CHAPTER III

Genetic Characterization of Roc-Cullin Interactions

Preface

The work presented in this chapter has been submitted for publication in PLoS One. I performed all the experiments and wrote the manuscript. Robert Duronio finalized the manuscript. Max Nourredine and Tim Donaldson created the Roc1a and Roc1b alleles (respectively) used in this chapter.

Background

Roc1a mutants are inviable [1]. This is presumably caused by improper accumulation of at least one target of a Roc1a-based E3 ligase. Considering the large amount of work published on different Roc1 complexes (and the fact that Roc1a can bind cullins 1-4), there are most likely many substrates that accumulate in the absence of Roc1a. This lethality shows that neither Roc1b nor Roc2 can participate in all the same complexes as Roc1a when under the control of their endogenous promoters. However, Roc1b can partially fulfill *some* Roc1a function when driven by the Roc1a promoter (1a::1b) [2]. While 1a::1b cannot rescue lethality, Ci (a known Roc1a-based Ubl target) accumulation is partially rescued with this transgene in a Roc1a mutant background. Roc2, however, cannot function analogously to Roc1a even when overexpressed [2].

Roc1b mutants are viable, but male sterile [2]. Thus, there are no essential targets of a Roc1b-based E3 ligase. This sterility suggests that during normal development,

neither Roc1a nor Roc2 can substitute for Roc1b. Sperm from Roc1b^{dc3} males are completely immotile, even though the sperm bundles are individualized properly. Interestingly, Roc1a can partially rescue the Roc1b mutant male sterility when driven by the Roc1b promoter, whereas Roc2 cannot [2]. In this chapter, we characterize which portions of the Roc proteins mediate their specific functions in the developing fly.

Summary

In the previous chapter, I established that the ANBR and BNAR chimeras bind cullins and have ligase activity as measured by a substrate free *in vitro* ligase assay. In this chapter, I examine the functionality of these proteins *in vivo*. I show that the ANBR protein is unable to rescue the lethality of Roc1a mutation, even though it binds the same cullins as Roc1a and has ligase activity (see Chapter II). This is not due to a lower level of expression, as several transgenic ANBR lines expressed protein at levels comparable to Roc1a (which *did* rescue Roc1a mutant lethality). BNAR, however, *is* able to rescue the male sterility induced by Roc1b mutation when expressed under control of the Roc1b promoter (1b::BNAR). Interestingly, BNAR expression induced by the Roc1a promoter (1a::BNAR) is not able to rescue Roc1b mutant male sterility, even though it is expressed at comparable levels to 1b::BNAR. This suggests that spatial expression of Roc1a and Roc1b in the male germline is very tightly regulated.

Materials and Methods

Immunoprecipitations and Western Blotting

For embryo lysates, overnight egg collections (0-16hrs) were dechorionated for 3 minutes in 50% bleach and dounce homogenized with 10 volumes of NP-40 lysis buffer (50mM Tris pH8.3, 150 mM NaCl, 0.5% NP-40, 1 ng/ml leupeptin, 0.5 ng/ml pepstatinA, 1mM PMSF). Lysates were centrifuged at 14,000 rpm for 10 min at 4⁰C and the supernatant was collected. For immunoprecipitations, 25 µl protein-A beads were washed 3x with 1 ml NP-40 lysis buffer, and then pre-incubated with antibody for 2h before adding to 1ml of lysate (1mg/ml protein). Immunoprecipitations were performed overnight at 4⁰C.

Antibodies

The following antibodies were used: rabbit anti-Cul1 (Zymed), rabbit anti-Cul2 (gift of Dr. Yue Xiong, UNC), guinea pig anti-Cul3 (gift of Dr. Jim Skeath, Washington University), rabbit anti-Cul4 [5], affinity purified rabbit anti-Cul5, guinea pig anti-Roc2 whole serum, mouse anti-FLAG (Invitrogen), mouse anti-V5 (Invitrogen), and Rabbit anti-Ub (Covance). Anti-peptide antibodies against Roc2 and Cul5 were generated using synthetic peptides (Invitrogen) coupled to KLH (Pierce). The peptide sequences are CADDPENSVD RPTDD (Roc2) and CKRDRDIFEEVWPDK (Cul5). Injections and serum withdrawal were performed at Pocono Rabbit Farm & Laboratory, Inc. High titer bleeds of anti-Cul5 were purified with the same peptide using the Sulfolink Kit (Pierce).

Stocks and Genetics

The *Roc1a*^{G1} and *Roc1b*^{dc3} alleles have been described previously [1,2]. The *Roc2*^{KG07982}, *Roc2*^{pBacf00911}, and *Cul5*^{EY21463} alleles were obtained from the Bloomington stock center. To test for rescue of *Roc1a* lethality, *Roc1a*^{G1}, *sn*, *FRT*/*FM7*, *Act-GFP* females were mated to males that expressed a specific transgene (V5-Roc1a or V5-ANBR) under control of the *Roc1a* promoter. Rescue was scored by the presence of *sn* males in the progeny. To test for rescue of *Roc1b* male sterility, males of the genotype *Roc1b*^{dc3}/*TM3*, *Sb* and containing a transgene insertion on the second chromosome (V5-Roc1b, V5-ANBR, or V5-BNAR) were mated to *Roc1b*^{dc3}/*TM3*, *Sb* females. *w*⁺ (indicating the presence of the transgene), *Sb*⁺, *Roc1b*^{dc3}/*Roc1b*^{dc3} male progeny were then mated with *w*¹¹¹⁸ virgin females to assay for rescue of sterility. Five batches of 100 eggs from this cross were transferred onto individual grape juice plates, and the numbers of hatched eggs quantified 36 h later.

Results

After we created chimeric proteins that could still bind specific cullins (ANBR and BNAR-see Figure 2.5), we wanted to test if they were functional *in vivo*. I showed in Chapter II that both are functional in an *in vitro* ligase assay with UbcH5 (Figure 2.7), but it is not known if these different RING domains interact with other Ubc's *in vivo* to mediate substrate degradation. Several transgenic lines of ANBR and BNAR were created. In this case, ANBR was put under control of the *Roc1a* promoter, and BNAR was put under control of the *Roc1b* promoter. This was to ensure they were expressed at the same time and in the same tissues as the endogenous proteins (*Roc1a* and *Roc1b*

respectively), as Roc1a and Roc1b have been shown to be differentially expressed developmentally [1].

The Roc1b RING domain cannot provide all Roc1a function

Since the ANBR protein displays the same binding specificity as Roc1a, we wanted to determine whether it could rescue the lethality caused by the null *Roc1a*^{G1} allele[1]. *Roc1a* is located on the X chromosome, and thus *Roc1a*^{G1} males are not viable. We set up an experiment where fathers carrying an autosomally located Roc1a (as control) or ANBR transgene (expressed with the *Roc1a* promoter) were crossed to *Roc1a*^{G1}/FM7 mothers and scored for the presence of rescued *Roc1a*^{G1} males (Figure 3.1).

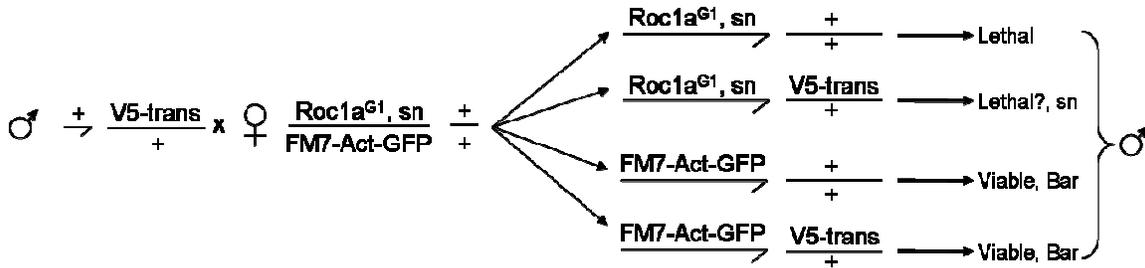


Figure 3.1: Roc1a mutant rescue crossing scheme. Males carrying a single copy of the transgene were crossed to females heterozygous for the *Roc1a* null allele (*Roc1a*^{G1}). Four possible genotypes are possible in the progeny, as shown. FM7-Act-GFP is a balancer chromosome expressing GFP under control of the Actin promoter. *sn* is a recessive bristle marker. Bar is a dominant eye marker. V5-trans indicates a transgene (*Roc1a* or ANBR) with a V5 epitope tag.

While the wild type *Roc1a* transgene was able to rescue the lethality of the *Roc1a*^{G1} mutation, none of 5 different ANBR transgenic insertions were able to do so

(Figure 3.2a). This was not a result of expression level differences, because all five of the ANBR proteins accumulated to a level comparable to the control Roc1a transgenic protein (Figure 3.2b). Thus, while ANBR binds Cul1 (Figure 2.4) and Cul2-4 (data not shown) like Roc1a, it is unable to function the same as Roc1a. This was somewhat surprising, considering that Roc1a and Roc1b share 78% protein identity in the RING domain. One possibility is that the Roc1b RING domain is unable to interact with the same E2's as the Roc1a RING domain when assembled into Cullin complexes *in vivo*, which would suggest that at least one essential target of a Roc1a E3 ligase is dependent on the specific RING domain sequence of Roc1a. The AN2R construct was also unable to rescue *Roc1a^{G1}* lethality (data not shown), consistent with the failure of the AN2R protein to bind Cullin. In several of these rescue experiment crosses we detected a small number of unbalanced male progeny that did not display the *sn* phenotype (see “% non-FM7 male” column), and thus did not contain the *Roc1a^{G1}* mutant chromosome. We do not unambiguously know the origin of these males, but since they were invariably sterile they are likely XO male progeny resulting from meiotic non-disjunction events in the *Roc1a^{G1}/FM7* females. This raises the possibility that reduction of *Roc1a* gene dose in females affects meiotic chromosome segregation in *Drosophila*. Consistent with this, Rbx1 has been shown to be essential for meiosis and proper chromosome condensation and segregation in *C. elegans* [3].

Chimeric Roc proteins can function in vivo

Recently, Arama et al. showed that a testes-specific Cul3 isoform forms an E3 ligase with Roc1b in the testes, and *Roc1b* mutant males are sterile because of a failure to

complete the late stages of sperm differentiation[4]. Since the BNAR construct displays the same binding specificity as Roc1b, we tested whether the BNAR chimera could rescue the male sterility caused by the *Roc1b^{dc3}* null mutation (Figure 3.3) [2]. Male fertility was measured by determining the proportion of eggs that hatched into first instar larvae after mating to wild type females.

A

Genotype	% <i>sn</i> male	% FM7 male	% non-FM7 male	% FM7 female	% non-FM7 female	n
(-)	0	21	6	36	37	177
Roc1a	22	16	0	31	31	387
Roc1b	0	25	2	47	26	163
ANBR ⁴²	0	20	0	40	40	303
ANBR ⁴³	0	14	0	46	40	199
ANBR ⁵¹	0	18	8	38	36	446
ANBR ⁷¹	0	15	7	37	41	390
ANBR ⁷⁶	0	18	1	39	42	475

B

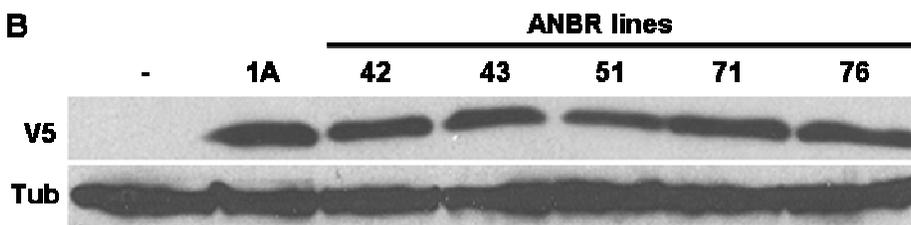


Figure 3.2 ANBR does not rescue the lethality of *Roc1a* mutation. A. Transgenes expressing chimeric ANBR proteins were tested for rescue of *Roc1a^{G1}* lethality (see *Methods* for genetics). All F1 progeny contain a single copy of the transgene expressed under control of the *Roc1a* regulatory sequences. The percentage of progeny with each genotype is indicated, as is the total number of progeny scored (n). B. V5 immunoblot of extracts prepared from adult males containing the indicated transgenes. (-) indicates non-transgenic wild type control.

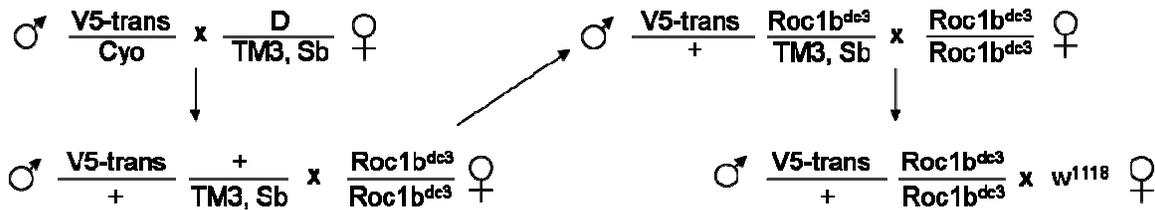


Figure 3.3: Roc1b Mutant Rescue Crossing Scheme. This crossing scheme was used to obtain a male fly that carried a transgene in a *Roc1b* mutant background (*Roc1b^{dc3}*). V5-trans denotes a V5-tagged transgene (1a, 1b, or BNAR). *Cyo* and *TM3* are second and third chromosome balancers, respectively. *D* is a dominant wing marker. *w¹¹¹⁸* is a *w* wild-type strain.

Three different transgenic lines expressing V5 epitope-tagged *Roc1b* under the control of the *Roc1b* promoter were able to rescue the male sterile phenotype (Figure 3.4a). Six different BNAR lines rescued the male sterility defect, five of them to the level of the control *Roc1b* transgenes. The BNAR chimeric proteins were expressed from the *Roc1b* promoter at levels comparable to normal *Roc1b* (Figure 3.4b). Because BNAR rescues the *Roc1b⁻* male sterility, we conclude that the *Roc1a* RING domain can provide *Roc1b* function during spermatogenesis. This finding is consistent with our previous observations indicating that forced expression of normal *Roc1a* from the *Roc1b* promoter can partially rescue the *Roc1b* male fertility defect [2]. When considered together with the failure of ANBR to rescue the *Roc1a* mutant, these results suggest that, within the context of the male germ line-specific Cul3 E3 ligase complex, the *Roc1a* RING domain can productively interact with the same E2s as *Roc1b*, while *Roc1b* cannot do so with all of the E2s that function with *Roc1a in vivo*.

Interestingly, expression of wild type *Roc1b* from the *Roc1a* promoter was unable to rescue male sterility (Figure 3.4a). This suggests that while the *Roc1a* promoter is active in the male body [1], it is not expressed in the male germline in a manner appropriate to provide *Roc1b* function. Thus, the *Roc1b* regulatory sequence appears to confer expression in the testes that cannot be duplicated by the *Roc1a* promoter.

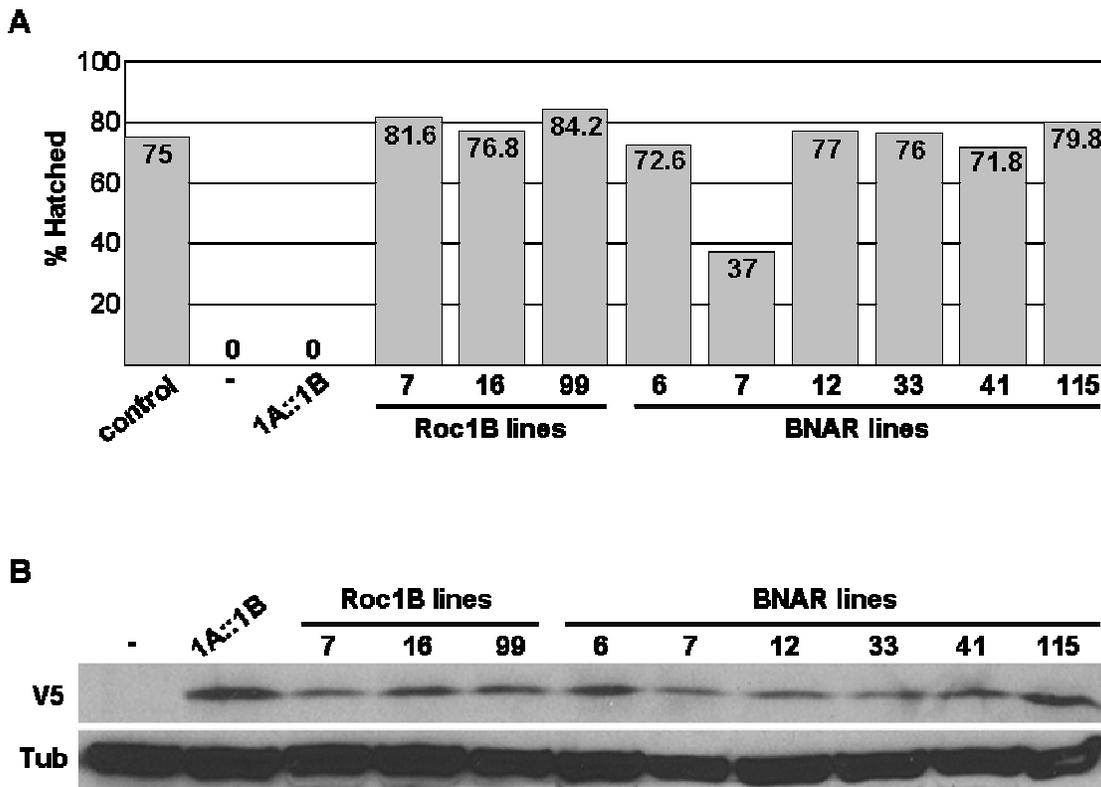


Figure 3.4: BNAR rescues the *Roc1b* mutant male sterility. A. Egg hatching was assessed for progeny of *Roc1b^{dc3}* homozygous mutant males containing the indicated transgene. “Control” indicates *Roc1b^{dc3}/+* genotype. (-) indicates no transgene; i.e. a *Roc1b^{dc3}* homozygous mutant. *1A::1B* indicates *Roc1b* driven by the *Roc1a* regulatory sequences. All other lines are under control of the *Roc1b* regulatory sequences. 500 eggs were analyzed for each line. B. V5 immunoblot of extracts prepared from adult males containing the indicated transgenes.

Discussion

The lethality of *Roc1a* mutants is presumably caused by the inappropriate accumulation of at least one target of a Roc1a-containing Cullin dependent E3 ligase [1]. The ANBR protein cannot rescue the lethality of *Roc1a* mutants even though it binds all of the Cullins that Roc1a does. Thus, even though ANBR can activate polyubiquitin formation *in vitro*, fusion of the Roc1a NH₂-terminus with the Roc1b RING domain does not create a fully biologically active Roc1a protein. One possible interpretation of this result is that *in vivo* the Roc1b RING domain cannot productively interact (i.e. stimulate ubiquitylation of critical targets) with the same set of E2's as Roc1a. This may occur at the level of direct binding, such that there are some E2s that only bind the RING domain of Roc1a and not Roc1b, or at the level of stimulating ubiquitin transfer to substrate in the context of a fully assembled CDL. The fact that different *Drosophila* E2's have been found to be involved in different developmental processes (including apoptosis, spermatogenesis, and telomere maintenance) suggests that they are not redundant in function [6-9]. We tested various E2's for interaction with Roc proteins (Chapter III), and were only able to detect an interaction with Ubc5. This does not, however, rule out that other Roc-E2 interactions occur *in vivo*.

In the *Drosophila* male germline, Roc1b forms a complex with a testes-specific Cul3 isoform and the BTB protein KLH10 to regulate caspase activation during spermatid differentiation [4]. The testes-specific Cul3 isoform is almost identical to the predominant somatic isoform, but it has a different NH₂-terminal extension and 5' UTR. Null mutations of Cul3 are lethal, and this lethality is presumably caused by improper accumulation or at least one Roc1a-Cul3 E3 ligase substrate [10,11]. *Roc1b* mutants are

male sterile [2]. Interestingly, the BNAR chimera, which binds well to Cul3, effectively rescues *Roc1b* mutant male sterility. This is consistent with our previous data showing that normal Roc1a can partially rescue the male sterility of *Roc1b* mutants when expressed from the *Roc1b* promoter [2]. These data suggest that in the context of the testes specific Cul3 complex, the RING domains of Roc1a and Roc1b can productively interact with a similar set of E2s. Finally, the functional redundancy of Roc1a and Roc1b in the male germ line is only detected with the *Roc1b* promoter, suggesting that the *Roc1a* and *Roc1b* genes are expressed differently during spermatogenesis. While endogenous Roc1a is expressed in the male body [1], it may not be expressed at the same developmental time or in the same part of the germline. This provides a glimpse at the amazing level of regulation in assembling Roc-Cullin E3 ligases.

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CHAPTER IV

Characterization of Roc2 and Cul5

Preface

Most of the work in this chapter has been submitted for publication to PLoS One. I performed all the experiments and wrote the manuscript. Robert Duronio finalized the manuscript.

Background

Drosophila melanogaster is unique in that it has three members of the Roc family, while all other non-drosophilid metazoans have two. Previous studies have examined Roc1a and Roc1b mutants [1,2], but Roc2 has remained largely unanalyzed in a multi-cellular organism. It should be noted that *D. melanogaster* Roc2 is more similar in sequence to Roc2 from other species than to any of the other *D. melanogaster* Rocs. Thus, it appears that the Roc1 family undergone a divergence in *Drosophilids*, while Roc2 has maintained its existence as the sole member of its class. Interestingly, another *Drosophilid* (*Drosophila pseudoobscura*) has undergone an even further divergence, possessing four Roc1 family members, while still maintaining a single Roc2 gene (Figure 4.1).

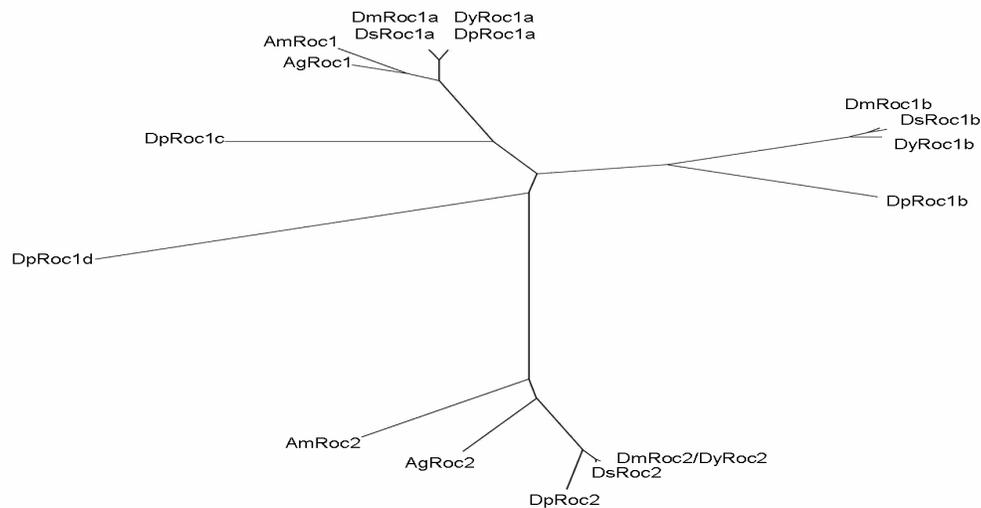


Figure 4.1: Clustering Diagram of Insect Roc proteins. ClustalX was used to create a branched clustering diagram of insect Roc proteins. Ag=*Anopheles gambia*, Am=*Apis mellifera*, Dm=*Drosophila melanogaster*, Dp=*Drosophila pseudoobscura*, Ds=*Drosophila simulans*, Dy=*Drosophila yakuba*.

Roc2 has been evolutionarily conserved, yet very little is known about its function. Several reports have suggested that it has a role in inhibiting apoptosis [3-6], but none of these studies have been carried out using mutational analysis in a multicellular organism (thereby making the physiological relevance difficult to interpret). Indeed, these papers often refer to Roc2 as SAG (sensitive to apoptosis gene). SAG was cloned as a redox-inducible early response gene in mouse tumor cell lines [7]. It was subsequently found to have antioxidant properties (free radical scavenging) when overexpressed after induction of redox stress. Further, SAG has been reported to bind pro-caspase-3 and induce its degradation [5]. This report is conflicting, however, as it shows SAG performing this function through the F-box protein β -TrCP, and only Roc1a is known to be part of a complex containing an F-box protein. Thus, it appears that

overexpression of a Roc protein can cause it to perform a function that it may not do *in vivo* at physiological levels. Consistent with this, another report showed SAG to be part of a feedback loop with HIF-1 α [6]. Tan *et al.* claim that HIF-1 α activates SAG expression in response to hypoxia, and that a SAG-Cul5-VHL complex then ubiquitinates HIF-1 α , leading to its degradation. But other reports have shown that only Roc1-Cul2 and not Roc2-Cul5 complexes can bind to VHL and other “VHL-box” proteins [8]. Since Roc1-Cul2 and Roc2-Cul5 both use elongins B and C to bind substrate adapters, it may be that these complexes can be forced into substituting for each other at higher expression levels. In another paper, Sasagawa *et al.* suggested that Roc1 and Roc2 may be redundant for some targets in *C. elegans* [9]. Interestingly, these studies showed that the rocs perform their overlapping function while still maintaining cullin binding specificity. Thus, Roc1-Cul2 and Roc2-Cul5 complexes, while unique, may be able to target some of the same proteins for degradation.

I have provided evidence that Roc2 and Cul5 are exclusive binding partners *in vivo* (Figure 2.1). This preferential binding appears to be conserved in humans as well [8], yet little is known about the role of this Roc2-Cul5 complex. It has been shown that several lentiviruses hijack the cell’s Cul5- or Cul4-based E3 ligase machinery to degrade the host’s family of defense proteins (APOBEC family) [10-14]. APOBEC3G is an antiviral factor with cytidine deaminase activity. When it is packaged into newly replicated virions, it induces C->U mutations during reverse transcription [15-22], thereby rendering the viral DNA unreadable.

Feng *et al.* recently identified a role for Cul5 in neuronal development [23]. Reelin is a secreted molecule that helps guide neuronal precursor cells to their final

positions. The authors show that Reelin phosphorylates the intracellular signaling protein Dab1 and leads to its degradation by a Cul5-based E3 ligase, thus suggesting that substrate adapters besides the F-box proteins may use specific phosphorylation as a way of targeting substrates to the E3 ligase machinery. Cul5 has also been shown to be highly expressed in the brain, although its function there is not well known [24,25].

Summary

In this chapter, I identify molecular null alleles of both Roc2 and Cul5. The presence of a single member of the Roc2 family has been well conserved throughout evolution. I show that mutation of Roc2 does not cause any overt phenotype. Rather, it only causes a slight reduction in female fecundity. This does not mean that Roc2 mutants have no phenotype, but it does reveal that Roc2 does not have any essential role during development. In Chapter II, I showed that Roc2 and Cul5 exclusively bind each other. In this chapter, I identify a null allele of Cul5 as well. Consistent with this exclusive binding, I also found that Cul5 mutants are viable and fertile. I created antibodies to both Roc2 and Cul5 and show that the mutant alleles of both proteins are devoid of protein expression. Interestingly, mutation of either Roc2 or Cul5 greatly reduces expression of the other. Finally, I show that Roc1a cannot bind the low level of Cul5 that remains in a Roc2 mutant. Thus, it is likely that Roc2 and Cul5 form an exclusive complex with a non-essential role during development.

Materials and Methods

RT-PCR Analysis

RNA was extracted from embryos using TRIzol (Sigma-Aldrich) according to the manufacturer's instructions. RT-PCR was previously described [1].

Primer sequences used for RT-PCR are as follows:

CG8234F: 5'-CACCCATGTGTCCTTCTCCGT-3'

CG8234R: 5'-TGACCACGGTTCACAAACCAG-3'

CG30035F: 5'-GAGAACATCCGTCATGCGGTG-3'

CG30035R: 5'-GAGCAGGATGCCTATGTTACC-3'

Cul5F: 5'-CACAAAGTTCATTTGACGAGGCG-3'

Cul5R: 5'-TGTGGCCAGGCGGAGATTCTC-3'

Roc2F: 5'-CAGAGACCGGTATGGCTGATGATCCAGAA-3'

Roc2R: 5'-CCCATGCGCTGAATGGACCA-3'

Immunoprecipitations and Western Blotting

For embryo lysates, overnight egg collections (0-16hrs) were dechorionated for 3 minutes in 50% bleach and dounce homogenized with 10 volumes of NP-40 lysis buffer (50mM Tris pH8.3, 150 mM NaCl, 0.5% NP-40, 1 ng/ml leupeptin, 0.5 ng/ml pepstatinA, 1mM PMSF). Lysates were centrifuged at 14,000 rpm for 10 min at 4⁰C and the supernatant was collected. For immunoprecipitations, 25 µl protein-A beads were washed 3x with 1 ml NP-40 lysis buffer, and then pre-incubated with antibody for 2h before adding to 1ml of lysate (1mg/ml protein). Immunoprecipitations were performed overnight at 4⁰C.

Antibodies

The following antibodies were used: rabbit anti-Cul1 (Zymed), affinity purified rabbit anti-Cul5, guinea pig anti-Roc2 whole serum, mouse anti-FLAG (Invitrogen), and mouse anti-V5 (Invitrogen). Anti-peptide antibodies against Roc2 and Cul5 were generated using synthetic peptides (Invitrogen) coupled to KLH (Pierce). The peptide sequences are CADDPENSVDPRPTDD (Roc2) and CKRDRDIFEEVWPKD (Cul5). Injections and serum withdrawal were performed at Pocono Rabbit Farm & Laboratory, Inc. High titer bleeds of anti-Cul5 were purified with the same peptide using the Sulfolink Kit (Pierce).

Results

To gain a better understanding of the Roc2 and Cul5 proteins, I identified alleles of each gene. I initially performed an imprecise P-element excision screen using the Roc2²⁴⁸⁷ allele (Figure 3.2). The Roc2²⁴⁸⁷ insertion is lethal, although this lethality is not caused by mutation of Roc2. The Roc2²⁴⁸⁷ insertion in trans to a deficiency that removes the Roc2 locus is not lethal, suggesting that the lethality is caused by another insertion on the chromosome. After an unsuccessful excision screen, I discovered that several new alleles were available: Roc2^{pBac} and Roc2^{KG} (Figure 4.2a). The pBac insertion is in the 5' UTR, just upstream of the first coding exon. The KG allele is in a large (~25kb) intron separating the first and second coding exons. I also acquired a single allele of Cul5, termed Cul5^{EY}. This Cul5 allele is in the middle of the second coding exon.

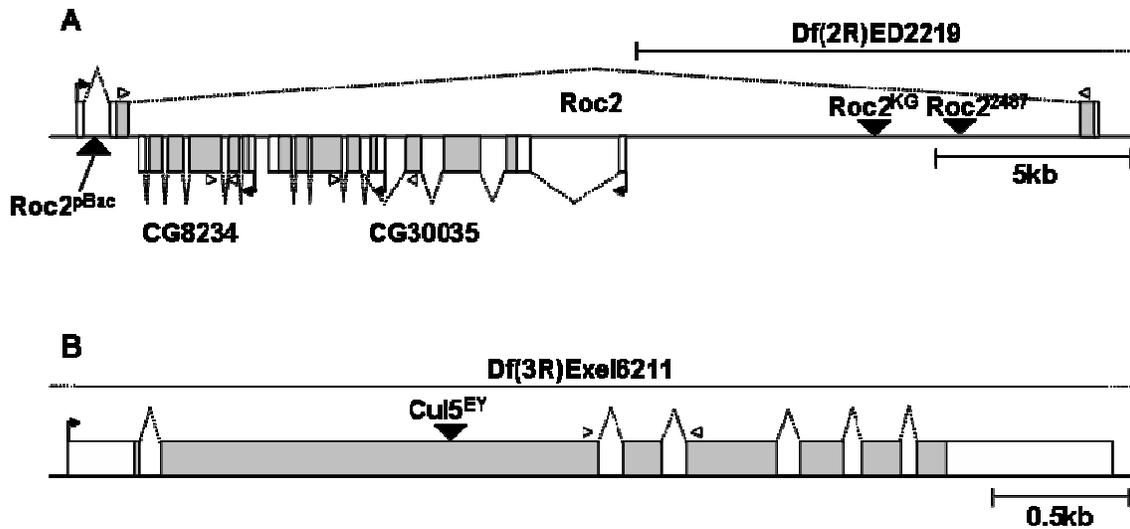


Figure 4.2: Schematics of *Roc2* and *Cul5* Loci. *A*, Schematic of the *Roc2* locus. *CG8234* and *CG30035* are genes of unknown function as annotated by FlyBase (putative sugar transporters). *B*, Schematic of the *Cul5* locus. Right angle arrows indicate start of transcription. Open arrow heads show the position of primers used for RT-PCR. Larger black triangles are P-element or piggyBac insertions. The boxes indicate exons, and the shaded regions represent the open reading frame. Dotted line indicates splicing.

I tested the new alleles by RT-PCR and found that the *Roc2^{pBac}* allele is hypomorphic and the *Roc2^{KG}* allele is null at the level of mRNA detection (Figure 4.3a). Neither allele down regulates the expression of nearby sugar transporter genes *CG30035* and *CG8234*. Also, the *Cul5^{EY}* allele does not express a detectable level of *Cul5* mRNA (Figure 4.3b).

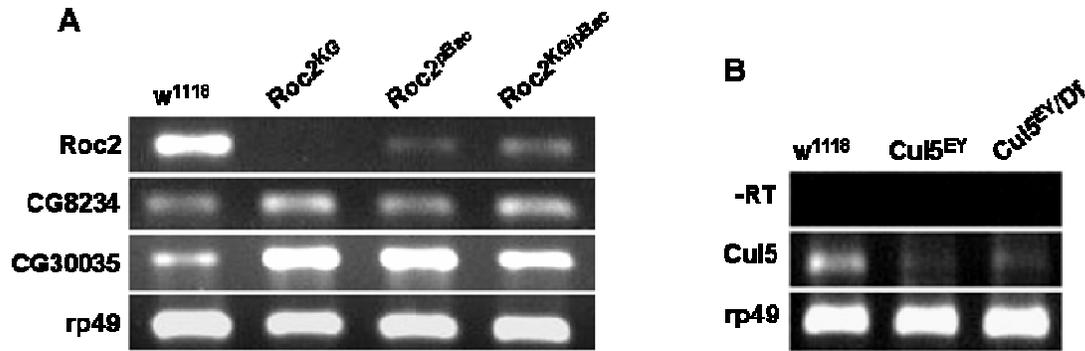


Figure 4.3: RT-PCR of *Roc2* and *Cul5* Mutants. A. RT-PCR analysis of the *Roc2* alleles. KG and pBac are homozygous for the insertions, and KG/pBac is a transheterozygote. Ribosomal protein 49 (rp49) was used as a positive control. B. RT-PCR analysis of the *Cul5* allele. -RT indicates that no reverse transcriptase was added.

To further establish the severity of these alleles, I created antibodies against both *Roc2* and *Cul5*. In both cases, I designed a peptide corresponding to the NH₂-terminus of the protein (see Methods) and conjugated it to Keyhole Limpet Hemocyanin (KLH) to increase immunogenicity. Guinea pigs were used for the *Roc2* injections, and rabbits were used for the *Cul5* injections. High titer production bleeds were tested for their ability to recognize tagged full length *Roc2* or *Cul5* constructs induced in bacteria (Figure 4.4). Once serum of high sensitivity was identified, the serum was further tested for its ability to recognize the proteins from *Drosophila* embryo lysates (Figure 4.4). The *Cul5* antibody was then further affinity purified (see Methods) using more of the same peptide.

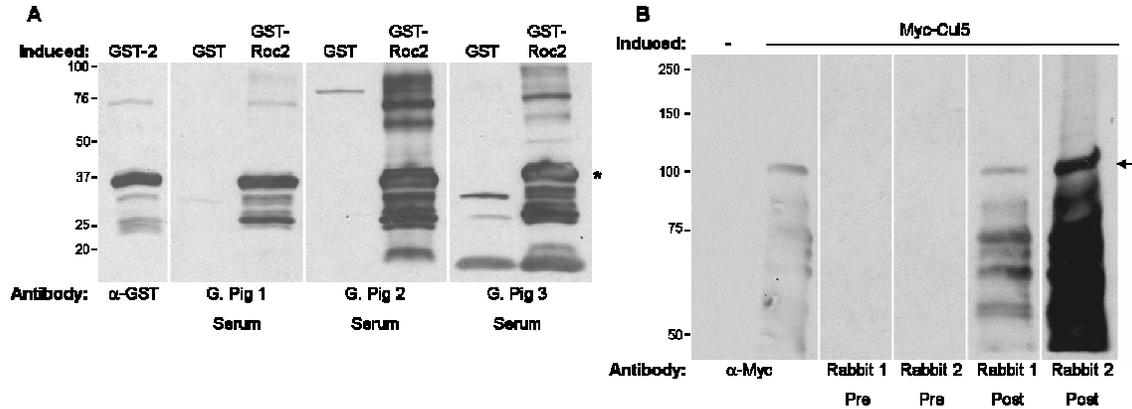


Figure 4.4: Testing of Roc2 and Cul5 Antibodies. A. Test bleeds from three guinea pigs injected with KLH-Roc2 peptide. Induced indicates the protein construct that was induced in BL21DE3 bacteria using IPTG (pGEX-1 vector). For antibody, each blot was probed with either α -GST or crude serum from one of three guinea pigs tested. Asterisk indicates GST-Roc2 protein. B. Test bleeds from two rabbits injected with KLH-Cul5 peptide. Either empty pTYB4 vector (-) or pTYB4-3xMyc-Cul5 was induced in BL21DE3 bacteria using IPTG. Each blot was probed with either α -Myc, one of the pre-immune bleeds (Pre), or one of the test bleeds (Post). Arrow indicates 3xMyc-Cul5.

We then used these antibodies to test the Roc2 and Cul5 mutants for protein expression by Western analysis. As Figure 4.5 shows, both the Roc2^{KG} and Cul5^{EY} alleles are completely deficient in their corresponding protein expression. Thus, both of these alleles are molecular nulls. Consistent with Roc2 and Cul5 forming an exclusive complex (Figure 2.1), genetically removing one protein greatly reduces expression of the other. We were therefore surprised when both mutants were viable and fairly fertile (they both display a slight reduction in female fecundity). Since both proteins are very well conserved throughout evolution, they most likely serve a functional role that is not overtly apparent in *Drosophila*.

While Roc1a does not bind Cul5 when Roc2 protein is present, it is possible that we were unable to observe a phenotype in *Roc2* mutants because Roc1a can bind the remaining low level of Cul5 in a *Roc2* mutant, and thereby functionally substitute for Roc2. We tested this by introducing a *Roc1a* transgene into the *Roc2*^{KG} null mutant background. Even in this genotype, Roc1a bound to Cul1 but not detectably to Cul5 (Figure 4.6). Moreover, as we show in Figure 4.5c, the pool of Cul5 available for binding Roc1a is greatly reduced in *Roc2* mutant animals relative to wild type. Thus, the Cul1 and Cul5 E3 ligase complexes form independently of one another and do not compete for the same pool of Roc proteins.

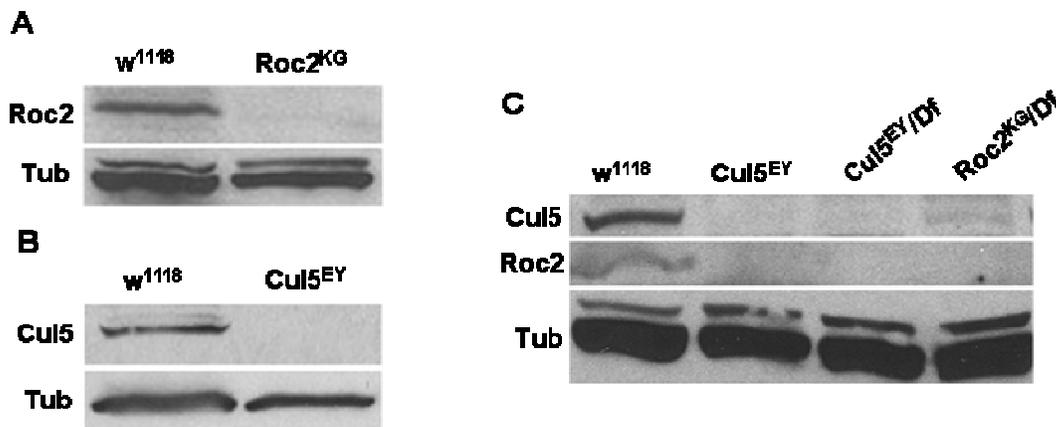


Figure 4.5: Western Analysis of Roc2 and Cul5 Mutants. A. Immunoblot comparing Roc2 protein levels in wild-type (*w¹¹¹⁸*) and homozygous *Roc2^{KG}* embryos. B. Immunoblot comparing Cul5 protein levels in wild-type and homozygous *Cul5^{EY}* embryos. In each case the embryos were derived from crosses between mutant mothers and fathers. C. Embryo extracts from *Cul5* and *Roc2* mutants were blotted with antibodies against the respective proteins.

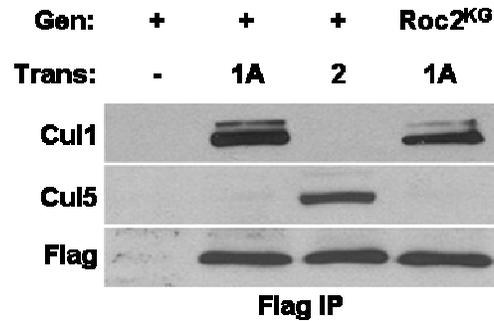


Figure 4.6: Roc2 and Cul5 are Exclusive Binding Partners. Extracts were prepared from embryos with either a wild-type (Gen=+) or *Roc2* mutant (Gen=*Roc2*^{KG}) background and the indicated transgene. The immunoprecipitated FLAG-Roc protein is indicated at the top. (-) indicates no transgene.

Discussion

Function of the Roc2-Cul5 E3 ligase

We show here that Roc2 and Cul5 only bind to each other, and that knocking out one protein greatly reduces the level of the other. The Roc2-Cul5 complex is conserved in other species including *C. elegans* and humans [8,9]. What is the function of the Roc2-Cul5 complex *in vivo*? A previous *Drosophila* study used viable P element insertions in the 5'UTR of Cul5 for over-expression experiments that suggested Cul5 is involved in cell fate specification and bouton formation in the larval CNS [26]. This study indicated that the insertion alleles were very weakly hypomorphic, and consistent with this we were not able to detect a difference in the Cul5 mRNA levels of these alleles by RT-PCR (data not shown). Here we report the identification of *Roc2* and *Cul5* transposon insertion alleles in which we cannot detect protein by immunoblot analysis of mutant embryos. These mutants develop into morphologically normal adults. Thus, Roc2-Cul5 is not required for development.

It is possible that Roc2-Cul5 is redundant with other CDL. A recent report showed that Roc1-Cul2 and Roc2-Cul5 complexes may act redundantly during meiotic cell cycle progression in *C. elegans* [9]. RNAi knockdown of Roc2 or Cul5 did not reveal an obvious phenotype, consistent with our results. However, RNAi knockdown of either Roc2 or Cul5 mRNA in a *cul-2* mutant background caused complete sterility, whereas *cul-2* mutants only display partial sterility. We occasionally observed a small, but inconsistent reduction in female fecundity in both the *Drosophila Roc2* and *Cul5* mutants, perhaps reflecting such redundancy. It should also be noted that Cul2- and Cul5-based E3 ligases use similar substrate adapter machinery, consisting of ElonginB, ElonginC, and a variable BC box protein [8,27,28]. This suggests that Cul2 and Cul5 complexes may have overlapping substrates in some organisms. Our data indicate that any potential redundancy between Cul2 and Cul5 in *Drosophila* must occur by utilizing different Roc proteins, as Roc1a is not part of a Cul5 complex, and Roc2 is not part of the Cul2 complex.

There have been several papers published on the role of a Cul5 complex in viral evasion of host immune response [10-12,29,30]. Primate lentiviruses, including HIV-1, code for a protein called Vif (Virion Infectivity Factor) that assembles with the host Cul5-ElonginB-ElonginC E3 ligase to target APOBEC3G (an antiviral cytidine deaminase) for degradation. Although a BLAST search using human APOBEC3G does not identify an obvious ortholog in *Drosophilids*, it is possible that some pathogens may use a similar Cul5 E3 ligase-hijacking system in *Drosophila*. Thus, perhaps *Drosophila* Roc2-Cul5 is involved in a stress response that is not revealed under standard laboratory growth conditions. That this specific complex has been conserved evolutionarily suggests

that it plays an important role in many organisms. The role may not be essential, however, as *Arabidopsis thaliana* have no functional homologue for Cul5 (or Cul2)[31].

Acknowledgements

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CHAPTER V

Discussion

Roc-Cullin interaction determinants

The Roc NH₂-terminus is necessary for binding to Cullin protein [1] and forms a β -strand that makes an inter-molecular β -sheet with the Cullin [2,3]. Our analysis of “RING swap” protein chimeras indicates that in some instances the Roc NH₂-terminal β -strand is the primary contributor to Roc-Cullin binding *specificity*. Both fusing the Roc2 NH₂ terminus to the Roc1a RING domain (2NAR) and fusing the Roc1a NH₂-terminus to Roc1b (ANBR) results in proteins that primarily display the Cullin binding preferences of Roc2 and Roc1a, respectively. However, our data clearly indicate that the RING domain also makes a contribution to Cullin binding preference. While a full length Roc1a construct bound Cul1-4 (Fig. 1a), a construct consisting of the Roc1a NH₂ terminus fused to the RING domain of the more distantly related Roc2 (AN2R) was unable to bind any Cullin. Substituting in the Roc1b RING domain, however, was able to restore Cullin binding, and the resulting ANBR protein displayed a Cullin binding profile identical to Roc1a. This suggests that a region of the RING domain that is more similar between Roc1a and Roc1b than Roc2 participates in Roc1a-Cullin binding preferences. Roc1a is 78% identical to Roc1b across the RING domain, while it only shares 45% identity with Roc2 in this region. Over the NH₂-terminus, Roc1a and Roc1b identity drops to 50%, and Roc1a and Roc2 identity is 42%. Thus, Roc1a and Roc1b are more similar to each other

than to Roc2 over the entire protein sequence, but their identity is much higher in the RING domain. In addition, the 2NAR protein bound detectably to Cul3 while normal Roc2 protein did not, again suggesting that the Roc1a RING domain influences Cullin selection. Taken together, we conclude from our data that the Roc NH₂-terminal β -strand makes a relatively stronger contribution to Cullin binding preference than the RING domain.

RING Chimeras

One of our RING proteins, AN2R, was expressed but completely unable to bind Cullin and lacked ligase activity. A similar observation was previously reported for the TRAF3 and TRAF5 RING domain proteins [4]. Both proteins have a high level of overall sequence conservation, especially in the RING finger and zinc finger domains. TRAF5, however, can activate the NF- κ B pathway when overexpressed, while TRAF3 cannot. This is dependent on the RING domain, as mutation of a key RING cysteine inhibits this activation. Substituting the RING domain and first zinc finger of TRAF3 with the corresponding sequence from TRAF5 creates a protein that can activate the NF- κ B pathway as well as TRAF5 alone. However, a construct that fuses portions of the RING domains of TRAF3 and TRAF5 is not capable of NF- κ B activation. The authors proposed that this chimeric protein might not be able to chelate zinc ions or fold properly. They also noted that TRAF5 has an aspartic acid in one of the zinc chelating residues where TRAF3 has a cysteine. Mutating the TRAF5 aspartic acid to cysteine inhibited TRAF5's ability to activate the NF- κ B pathway, even though both residues have the potential to coordinate metal ions. This may also explain why AN2R is unable to bind

any Cullin (and why it is inactive as a ligase). The eighth zinc coordination residue in Roc1a (and Roc1b) is an aspartic acid, while the corresponding residue in Roc2 is a cysteine. This suggests that RING proteins may have evolved to possess very specific metal-ion-interacting residues, and that all of these residues may not function identically in the context of the RING domain. However, the other Roc chimeras we constructed were able to bind cullins (ANBR, BNAR, 2NAR), and rescue of male sterility by the BNAR chimera indicates that Roc protein chimeras *can* be functional *in vivo*. While we could not test for rescue of any Roc2 mutant phenotype (because we could not detect one), the 2NAR protein was able to bind Cullins, albeit with a slightly different specificity than endogenous Roc1a or Roc2.

RING-E2 Interactions

The ANBR protein cannot rescue the lethality of *Roc1a* mutants even though it displays the same Cullin binding profile as Roc1a. Since we were able to show that ANBR can stimulate polyubiquitin chain formation using UbcH5 as the E2, why is this protein still unable to substitute for Roc1a? One possibility is that ANBR cannot bind the same E2's as Roc1a *in vivo*. We were unable, however, to find any E2's that interact with only Roc1a or Roc1b (Figure 2.7). While we were able to detect an interaction of all Rocs with Ubc5, it is possible that other factors are required *in vivo* for other Roc-E2 interactions. It has been previously shown that some RING domains will interact with E2's by *in vitro* assays that they do not interact with *in vivo*. An example of this is the Hrd1p RING domain. While the Hrd1p RING domain can use several E2's for its function in an *in vitro* ligase assay, it can only use Ubc7 (and to a lesser extent Ubc1) *in*

vivo [5]. In this case, it was determined that other portions of Hrd1p, a membrane anchoring protein Cue1p, and proper cellular localization all influence *specific* RING-E2 interactions *in vivo* [6]. Thus, it is important to confirm RING-E2 interactions *in vivo* before considering relevance for the organism. There are several other reports consistent with the idea that each RING domain only interacts with a small subset of E2's in the cell [5-10].

There is a great deal of structural conservation between the published Cbl and Rbx1 crystal structures [3,11]. In their study, Zheng *et al.* superimposed Rbx1 onto the Cbl structure [3]. The authors noted that the E2-interacting residues are conserved (or at least maintain their hydrophobicity) between the two RING proteins. It is interesting that all of the E2-interacting residues as well as all the COOH-terminal Cul1 interacting residues they describe are identical between Roc1a and Roc1b. It may be that there is a difference in overall structure between Roc1a and Roc1b (which could be imparted by non-Cul1- or E2-interacting residues) that influences E2 interactions. Or there may be unidentified factors that aid or prevent certain RING-E2 interactions *in vivo*.

Function of the Roc2-Cul5 E3 ligase

We show that Roc2 and Cul5 only bind to each other, and that knocking out one protein greatly reduces the level of the other. The Roc2-Cul5 complex is conserved in other species including *C. elegans* and humans [14,15]. What is the function of the Roc2-Cul5 complex *in vivo*? A previous *Drosophila* study used viable P element insertions in the 5'UTR of Cul5 for over-expression experiments that suggested Cul5 is involved in cell fate specification and bouton formation in the larval CNS [16]. This study indicated

that the insertion alleles were very weakly hypomorphic, and consistent with this we were not able to detect a difference in the Cul5 mRNA levels of these alleles by RT-PCR (data not shown). Here we report the identification of *Roc2* and *Cul5* transposon insertion alleles in which we cannot detect protein by immunoblot analysis of mutant embryos. These mutants develop into morphologically normal adults. Thus, Roc2-Cul5 is not required for development.

It is possible that Roc2-Cul5 is redundant with other CDL. A recent paper showed that Roc1-Cul2 and Roc2-Cul5 complexes may act redundantly during meiotic cell cycle progression in *C. elegans* [14]. Consistent with our results, RNAi knockdown of Roc2 or Cul5 did not reveal an obvious phenotype. However, RNAi knockdown of either Roc2 or Cul5 mRNA in a *cul-2* mutant background caused complete sterility, whereas *cul-2* mutants only display partial sterility. We occasionally observed a small, but inconsistent reduction in female fecundity in both the *Drosophila Roc2* and *Cul5* mutants, perhaps reflecting such redundancy. It should also be noted that Cul2- and Cul5-based E3 ligases use similar substrate adapter machinery, consisting of ElonginB, ElonginC, and a variable BC box protein [15,17,18]. This suggests that Cul2 and Cul5 complexes may have overlapping substrates in some organisms. Our data indicate that any potential redundancy between Cul2 and Cul5 in *Drosophila* must occur by utilizing different Roc proteins, as Roc1a is not part of a Cul5 complex, and Roc2 is not part of the Cul2 complex.

There have been several papers published on the role of a Cul5 complex in viral evasion of host immune response [19-23]. Primate lentiviruses, including HIV-1, code for a protein called Vif (Virion Infectivity Factor) that assembles with the host Cul5-

ElonginB-ElonginC E3 ligase to target APOBEC3G (an antiviral cytidine deaminase) for degradation. Although a BLAST search using human APOBEC3G does not identify an obvious ortholog in *Drosophilids*, it is possible that some pathogens may use a similar Cul5 E3 ligase-hijacking system in *Drosophila*. Thus, perhaps *Drosophila* Roc2-Cul5 is involved in a stress response that is not revealed under standard laboratory growth conditions. That this specific complex has been conserved evolutionarily suggests that it plays an important role in many organisms.

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