Identifying Determinants of Cullin Binding Specificity Among the Three Functionally Different *Drosophila melanogaster* Roc Proteins via Domain Swapping

Patrick J. Reynolds¹, Jeffrey R. Simms², Robert J. Duronio¹,²,³*

¹ Department of Biology, University of North Carolina, Chapel Hill, North Carolina, United States of America, ² Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, North Carolina, United States of America, ³ Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina, United States of America

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**Abstract**

**Background:** 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 binds ubiquitin charged E2 and is essential for ubiquitylation. *Drosophila melanogaster* contains three highly related Roc proteins: Roc1a and Roc2, which are conserved in vertebrates, and Roc1b, which is specific to *Drosophila*. Our previous genetic data analyzing *Roc1a* and *Roc1b* mutants suggested that Roc proteins are functionally distinct, but the molecular basis for this distinction is not known.

**Methodology/Principal Findings:** Using co-immunoprecipitation studies we show that *Drosophila* Roc proteins bind specific Cullins: Roc1a binds Cul1-4, Roc1b binds Cul3, and Roc2 binds Cul5. Through domain swapping experiments, we demonstrate that Cullin binding specificity is strongly influenced by the Roc NH₂-terminal domain, which forms an intermolecular 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 that is active as an E3 ligase but cannot complement *Roc1a* mutant lethality, indicating that the identity of the RING domain can be an important determinant of CDL function. In contrast, the converse chimeric protein with a substitution of the Roc1b RING domain with that of Roc1a can rescue the male sterility of *Roc1b* mutants, but only when expressed from the endogenous *Roc1b* promoter. We also identified mutations of *Roc2* and *Cul5* and show that they cause no overt developmental phenotype, consistent with our finding that Roc2 and Cul5 proteins are exclusive binding partners, which others have observed in human cells as well.

**Conclusions:** The *Drosophila* Roc proteins are highly similar, but have diverged during evolution to bind a distinct set of Cullins and to utilize RING domains that have overlapping, but not identical, function in vivo.

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**Introduction**

Many aspects of cell and developmental biology require the regulation of protein function via ubiquitin-mediated protein destruction. Protein ubiquitylation requires the action of three families of proteins: E1 Ubiquitin Activators (Uba), E2 Ubiquitin Conjugators (Ubc), and E3 Ubiquitin Ligases (Ubl) [1,2]. 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. Vertebrates and sea urchins have two distinct E1 enzymes, and most other organisms are thought to only have a single E1 [3,4]. While there are considerably more E2’s, much of the modularity of the ubiquitin-proteasome pathway comes from the large number of different E3 ligases.

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 [5]. RING E3’s do not conjugate ubiquitin, but rather stimulate its transfer from the E2 to the substrate [6,7]. RING domains contain conserved cysteine and histidine residues that chelate zinc ions to provide a structure that interacts with an E2 [8,9]. Several RING domain structures have been solved, and they share extensive structural conservation [10,11,12,13,14]. 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 Zn²⁺ chelating residues of the RING domain (as well as the Zn²⁺ ions) are required for proper folding of the RING domain to allow binding of E2.
and function of the protein [7,9,15,16,17]. 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 [18]. 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 [18]. CDL are composed of three modules (Cullin, Roc, substrate adapter/receptor), each with a distinct role [19]. 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 serves as 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 [20,21]. For instance, the F-box family (33 members in Drosophila [22]) 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 [11,23].

The RING domain-containing Roc proteins are essential for CDL function and are also encoded by a gene family [19]. Humans and C. elegans contain two Roc proteins, Roc1 and Roc2, while there has been a radiation of the Roc1 family in Drosophilid species (T.D. Donaldson and R.J.D., unpublished). For instance, Drosophila melanogaster encodes three Roc proteins named Roc1a, Roc1b and Roc2 [24]. The level of functional redundancy among metazoan Roc proteins has remained largely unexplored. We have been addressing this issue in Drosophila melanogaster by generating and characterizing mutations in the Roc genes. We previously showed that Roc1a mutants are lethal, while Roc1b mutants are male sterile [24,25]. These different phenotypes suggest that the Rocs are not redundant in function, even though Roc1a and Roc1b are 78% identical in the RING domain. 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 [25]. 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. We analyze 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. Further, we show that not all RING domains of the individual Roc proteins are functionally interchangeable in vivo. 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.

Results

Roc proteins bind specific Cullins

We previously detected Roc-Cullin interactions in vivo by immunoprecipitating Flag-tagged Roc1a, Roc1b, and Roc2 proteins from transgenic embryo extracts and identifying interacting proteins by mass spectrometry [25]. For these experiments, each Roc protein was expressed using the ubiquitous Roc1a promoter [24]. The data indicated that Roc1a bound Cul1-4 and that Roc2 bound Cul5, but could not eliminate the possibility that certain Roc-Cullin complexes were undetectable by the IP/mass spec analysis. To more comprehensively test for the presence of Roc-Cullin complexes, we assembled a panel of anti-Cullin antibodies (see methods) and used these in IP/immunoblot analyses. Anti-FLAG immunoprecipitates of extracts made from transgenic embryos expressing FLAG-tagged Roc1a, Roc1b, or Roc2 proteins from the Roc1a promoter were probed with antibodies that specifically recognize each Cullin (Figure 1A). The data indicate that Roc1a binds Culins 1–4, but not Cul5, that Roc1b binds strongly only to Cul3, and that Roc2 binds only to Cul5. This is consistent with our previous mass spec results [25] and shows that Rocs have strong Cullin binding preferences in vivo.

The Roc Protein NH2-terminus can determine Cullin binding specificity

Available crystal structures of Roc-Cullin complexes (e.g. Roc1-Cul1 and Roc1-Cul4) reveal that the ~110 amino acid Roc proteins contain two general domains, an NH2-terminal β-strand of between 41 and 56 amino acids and a globular RING domain that chelates 3 zinc ions (Fig. 1B) [11,14]. Deletion of the NH2-terminal β-strand of human Roc1 abrogates binding to Cul1 [26], indicating that this domain is required for Cullin binding. Both the NH2-terminal β-strand and the RING domain make close contacts with the Cullin homology domain at the Cullin COOH-terminus, and thus could potentially mediate specific Cullin-Roc interaction (Fig. 1B) [11]. However, because the sequence of the RING domain is more highly conserved than the
NH$_2$-terminal β-strand, we hypothesized that the NH$_2$-terminus of Roc proteins is responsible for mediating specific Cullin interactions. For instance, the Roc1a and Roc1b RING domains are 78% identical (Fig. 1B), yet Roc1b binds strongly only to Cul3 while Roc1a binds strongly to Cullins 1–4 (Fig. 1A). To determine whether one of these two domains in Roc proteins is responsible for specific Cullin binding, we created a series of chimeric constructs that join the NH$_2$-terminus of one Roc protein to the COOH-terminal RING domain of another (RING-swap constructs, Fig. 2A). For example, AN2R contains the Roc1a NH$_2$-terminus fused to the Roc2 RING domain. All constructs have an NH$_2$-terminal V5 epitope tag and are expressed with the Roc1a promoter. If the NH$_2$-terminus is sufficient to confer Cullin binding specificity, all chimeras with the same NH$_2$-terminal Roc sequence should bind the same Cullins, regardless of the RING domain to which they are fused.

We transfected these constructs into S2 cells and determined Cullin binding by IP/immunoblot. Because we had already observed that Roc1a binds Cul1 and Cul3 but not Cul5, that Roc1b binds only Cul3, and that Roc2 binds only Cul5 (Fig. 1A), we focused our analysis on these interactions. The ANBR chimera showed the same binding preference as Roc1a: it bound both Cul1 and Cul3 (Fig. 2B). Similarly, the BNAR chimera displayed the same binding specificity as Roc1b: it bound Cul3 but not Cul1 (Fig. 2B). These data indicate that the NH$_2$-terminus of a Roc protein can direct specific Cullin binding.

Chimeras between Roc1a and Roc2 behaved slightly differently than the ANBR and BNAR proteins. For instance, AN2R failed to bind any Cullin, even though it could be stably expressed (shown for Cul1 and Cul5 in Fig. 2C). There could be several reasons why AN2R fails to bind Cullin. There may be amino acids in the Roc1a RING domain necessary for Cul1 binding that are not present in the Roc2 RING domain. We swapped several potential specificity residues in the RING domain between Roc1a and Roc2, but were unable to alter the Cullin binding (data not shown). Alternatively, since ANBR binds to Cul1, the Roc2 RING domain may contain amino acids that prevent binding to Cul1. However, we show below that the AN2R protein is not active as
an E3 ligase (for example, see Fig. 6 below), and thus this protein chimera is functionally inactive perhaps because it does not fold properly. In the reciprocal experiment, 2NAR bound strongly to Cul5, but not Cul1 as did normal Roc2 (Fig. 2C). Interestingly, we could also detect some binding of 2NAR to Cul3 (Figure 2D). These data indicate that the Roc2 NH2-terminus confers strong binding preference to Cul5, and that the Roc1a RING domain contributes somewhat to the selection of Cul1 and Cul3.

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 1. We consistently obtained similar results as in S2 cells; AN2R bound no Cullin, while 2NAR bound Cul5 but not Cul1 as did normal Roc2 (Fig. 3A). In addition, 2NAR also bound Cul3 (Fig. 3B). 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 RING domain of Roc1 plays a more important role in Cullin binding specificity than the RING domain of Roc2.

Neddylation is a small ubiquitin-like protein that is conjugated to the K720 residue of Cul-1 (and to an homologous lysine in all other Cullins as well) using Uba3 (E1), Ubc12 (E2), and Roc1 (E3) [28,29,30,31]. This modification is in turn cleaved off by the multienzyme COP9 Signosome (CSN) [32,33]. Since mutations in the pathways that conjugate and remove Neddylation are detrimental, the current model is that cycles of neddylation and de-neddylation are necessary for the proper function of CDF [34,35,36]. We observed both the unmodified and the less abundant, slower migrating de-neddylation form of Cullin in some of our experiments (e.g. Fig. 1 Cullins 1–3). Because Roc1 can influence Cullin neddylation, we examined our co-immunoprecipitation data paying particular attention to whether association with different Roc chimeras affects the steady state amount of Cullin neddylation. We could find no consistent correlation between Cullin neddylation and a particular domain of one of the Roc proteins.

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 Roc1aG1 mutation [24]. Roc1a is located on the X chromosome, and thus Roc1aG1 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 Roc1aG1/FM7 mothers and scored for the presence of rescued Roc1aG1 males. While the wild type Roc1a transgene was able to rescue the lethality of the Roc1aG1 mutation, none of 5 different ANBR transgenic insertions were able to do so (Fig. 4A). This was not a result of expression level differences, because all 5 of the ANBR proteins accumulated to a level comparable to the control Roc1a transgenic protein (Fig. 4B).

Thus, while ANBR binds Cul1 (Fig. 2) and Cul2-4 (not shown) similarly to 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, 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 Roc1aG1 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, and thus that did not contain the Roc1aG1 mutant chromosome (Fig. 4A). We do not unambiguously know the origin of these males, but since they were invariably sterile they are likely XO

![Figure 3. RING Swap constructs and Cullin binding in embryos.](image) ![Figure 4. ANBR does not rescue the lethality of Roc1a mutation.](image)
male progeny resulting from meiotic non-disjunction events in the Roc1\textsuperscript{aG1}/FM7 females. This raises the possibility that reduction of Roc1\textsuperscript{a} gene dose in females affects meiotic chromosome segregation.

**Chimeric Roc proteins can function in vivo**

Recently, Arama et al. showed that a testes-specific Cul3 isoform forms an E3 ligase with Roc1\textsubscript{b} in the testes, and Roc1\textsubscript{b} mutant males are sterile because of a failure to complete the late stages of sperm differentiation [37]. Since the BNAR construct displays the same binding specificity as Roc1\textsubscript{b}, we tested whether the BNAR chimera could rescue the male sterility caused by the Roc1\textsubscript{b}\textsuperscript{-}\textsuperscript{null} null mutation (Fig. 5)[25]. Male fertility was measured by determining the proportion of eggs that hatched into first instar larvae after mating to wild type females. Three different transgenic lines expressing V5 epitope-tagged Roc1\textsubscript{b} under the control of the Roc1\textsubscript{b} promoter were able to rescue the male sterile phenotype (Fig. 5A). Six different BNAR lines rescued the male sterility defect, five of them to the level of the control Rob1\textsubscript{b} transgenes (Fig. 5A). The BNAR chimeric proteins were expressed from the Roc1\textsubscript{b} promoter at levels comparable to normal Roc1\textsubscript{b} (Figure 5b). Because BNAR binds to Cul3, we conclude that the Roc1\textsubscript{a} RING domain can provide Roc1\textsubscript{b} function during spermatogenesis. This is consistent with our previous observations indicating the forced expression of normal Roc1\textsubscript{a} from the Roc1\textsubscript{b} promoter can partially rescue the Roc1\textsubscript{b} male fertility defect [25]. When considered together with the failure of ANBR to rescue the Roc1\textsubscript{a} mutant, these results suggest that, within the context of the male germ line-specific Cul3 E3 ligase complex, the Roc1\textsubscript{a} RING domain can productively interact with the same E2s as Roc1\textsubscript{b}, while Roc1\textsubscript{b} cannot do so with all of the E2s that function with Roc1\textsubscript{a}.

Interestingly, expression of wild type Roc1\textsubscript{b} from the Roc1\textsubscript{a} promoter was unable to rescue male sterility (Fig. 5A). This suggests that while the Roc1\textsubscript{a} promoter is active in the male body [24], it is not expressed in the male germ line in a manner appropriate to provide Roc1\textsubscript{b} function. Thus, the Roc1\textsubscript{b} regulatory sequence appears to confer expression in the testes that cannot be duplicated by the Roc1\textsubscript{a} promoter.

**Chimeric Roc proteins have E3 ligase activity in vitro**

Although ANBR binds Cul1-4, it does not rescue the lethality of Roc1\textsubscript{a} mutation. A possible explanation for this result is that the ANBR chimera protein is deficient in E3 ligase activity. To test this, we expressed all of the *Drosophila* Roc proteins and chimeras as GST fusion proteins in *E. coli* and purified them (Fig. 6A). We then tested them for E3 ligase activity using a previously described in vitro assay that detects E2- and GST-Roc-dependent polyubiquitin formation in the absence of either Cullin or a particular substrate [24,26]. The ANBR protein was fully functional in this assay (Fig. 6B). Thus, ANBR can bind Cullin and function as an E3 ligase, but it cannot provide all the function of Roc1\textsubscript{a} in vivo.

Roc1\textsubscript{a} and Roc2 displayed high E3 ligase activity, whereas Roc1\textsubscript{b} showed a weaker ability to promote poly-ubiquitylation (Fig. 6B). Comparing this activity with that of the chimeras, a pattern emerges: Roc1\textsubscript{a} and ANBR promote extensive polyubiquitylation, while that of Roc1\textsubscript{b} and BNAR is lower. Similarly, Roc2 and the 2NAR chimera are both highly active in this assay (Fig. 6B). Thus, even though the RING domain is known to mediate most of the physical interaction with the E2 [11], the Roc NH\textsubscript{2}-terminus may play a significant role in determining the efficiency of polyubiquitylation with a particular E2. As noted above, in addition to its inability to bind Cullin (Fig. 2C & 2D), the AN2R protein is non-functional as an E3 ligase in this assay (Fig. 6B).

**Roc2 and Cul5 Mutant Analysis**

Because Roc2 and Cul5 bind only to each other, we hypothesized that a Roc2-Cul5 complex would function indepen-
dently of other Roc-Cullin complexes and that mutations of each gene would cause the same phenotype. In addition, this hypothesis predicts that the Roc2 mutant phenotype would be different than the phenotype we previously determined for Roc1a and Roc1b mutants [24,25]. The Roc2 locus is complex, with two protein coding exons separated by an intron greater than 25 kb in length (Fig. 7A). Within this intron are two predicted genes (CG8234 or CG30035) that encode sugar transporters transcribed from the strand opposite Roc2 transcription (Fig. 7A). We analyzed several transposon insertion lines for expression of Roc2 and identified two mutant alleles, both of which are viable. The Roc2KG P element insertion is within the large intron, while the Roc2pBac piggyBac insertion is in a smaller intron upstream of the first protein-coding exon (Fig. 7A). RT-PCR analysis of embryonic mRNA (from mating homozygous males and virgin females) revealed that the Roc2KG allele expresses no detectable mRNA, while the Roc2pBac allele expresses a reduced amount of mRNA compared to wild type (Fig. 7C). Neither insertion reduces the expression of the CG8234 or CG30035 genes (Fig. 7C). The Roc2KG allele also produces no detectable Roc2 protein as measured by immunoblotting of embryonic protein extracts (Fig. 7E), and thus it is a strong loss of function mutation.

Roc2KG mutant animals develop normally, and we could detect no obvious phenotype, except for a slight reduction in female fecundity. The Cul5 locus is simpler than that of Roc2, and the Cul5 transcript includes 7 exons and 6 introns. We identified a P element (Cul5EY) that is inserted into the second exon of Cul5 at amino acid D346 of the predicted open reading frame (Fig. 7B). RT-PCR analysis of embryonic mRNA (from homozygous mutant males and virgin females) revealed a reduced level of Cul5 mRNA in Cul5EY (Fig. 7D), and we were unable to detect Cul5 protein by immunoblotting of protein extracts from the same mating (Fig. 7F), indicating that Cul5EY is a strong loss of function allele. As with Roc2, Cul5EY mutants develop normally and do not display any overt morphological defects. That Roc2 and Cul5 mutant animals are viable and display no obvious developmental defects is consistent with our analysis of Roc-Cullin interactions indicating that Roc2 and Cul5 bind exclusively to each other. Indeed, Roc2 and Cul5 accumulation in vivo is interdependent: Roc2 was undetectable in Cul5 mutant embryo extracts, and Cul5 was greatly reduced in Roc2 mutant embryo extracts (Fig. 7G).

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 Cul5 in the absence of Roc2, and thereby functionally substitute for Roc2. We tested this by introducing a Roc1a transgene into the Roc2KG mutant background. Even in this genotype, Roc1a bound to Cul1 but not detectably to Cul5 (Fig. 8). Moreover, as we show in Fig. 6G, 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.

**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 [26] and forms a β-strand that makes an inter-molecular β-sheet with the Cullin protein [11,14]. 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 Rob1b (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

Figure 7. Analysis of Roc2 and Cul5 mutant alleles. 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. C, 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. D, RT-PCR analysis of the Cul5 allele. –RT indicates that no reverse transcriptase was added. E, Immunoblot comparing Roc2 protein levels in wild-type (w1118) and homozygous Roc2KG embryos. F, Immunoblot comparing Cul5 protein levels in wild-type and homozygous Cul5EY embryos. In each case the embryos were derived from crosses between mutant mothers and fathers. G, Embryo extracts from Cul5 and Roc2 mutants were blotted with antibodies against the respective proteins.

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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 [24]. 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 polyubiquitination 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 ubiquitilation 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 CULD.

A similar observation was previously reported for TRAF3 and TRAF5 RING domain proteins [38]. TRAF5 can activate the NF-kB pathway when over-expressed, 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-kB 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 germ line, Roc1b forms a complex with a testes-specific Cul3 isofrom and the BTB protein KHL10 to regulate caspase activation during spermatid differentiation [37]. Consequently, Roc1b mutants are male sterile [25]. 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 [25]. 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. 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.

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 [39,40]. 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 [41]. 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 CULD. A recent paper showed that Roc1-Cul2 and Roc2-Cul5 complexes may act redundantly during meiotic cell cycle progression in C. elegans [39]. 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. Cul2- and Cul5-based E3 ligases use similar substrate adapter machineries, consisting of ElonginB, ElonginC, and a variable BC box protein [40,42,43], suggesting that Cul2 and Cul5 complexes could have overlapping substrates in some organisms. Drosophila Cul2 forms a complex with Rbx1, Elongins B and C, and VHL that supports polyubiquitin chain formation in vivo, and that is capable of ubiquitylating the HIF-1α transcription factor as occurs in mammals [44,45,46]. 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. Whether redundancy exists or not, that the Cul5-Roc2 complex has been evolutionarily conserved suggests that it plays an important role in many organisms.

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 μg/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 pGaSpeR-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. Chimera were expressed with either a Roc1a- or Roc1b-grf (genomic rescue fragment). The Roc1a-grf was previously described [24] 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 040 bp upstream from the Start codon and 350 bp downstream from the Stop codon, was also previously described [25], and here we inserted an in frame V5 tag downstream of the Start codon.

Creation of GST-fusion Proteins

Using the pCaSpeR-1 Roc constructs as template [25], 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’-CAGAGGAATTCAGGAGGATGGATGGAT-3’; Roc1b 3’Eco: 5’-CAGAGGAAATCTTATGAGGCGGTACTCTTGC-3’. Roc1b 5’Eco: 5’-TGATATGGCTGATGATCCAGAAA-3’.

GST-Roc proteins were purified using Glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. Protein concentration was determined by the method of Lowry et al. [2].

Immunoprecipitations and Western Blotting

RT-PCR Analysis

RNA was extracted from embryos using TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions. PCR was previously described [26]. Primer sequences used for RT-PCR are as follows. CG8234: 5’-CACCCATGTGCTCTTTCCG-3’ and 5’-TGACCGTTCAAAAGCCG-3’; CG30053: 5’-GAGAACATCCCGTGCCCG-3’ and 5’-GAAGGAGGTGCTATGTACC-3’. Cul5: 5’-CAACAAAGTCTTATGAGGCGCCG-3’ and 5’-TGTCGCCAGGAGATTCTC-3’. Roc2: 5’-CAGAGGACGCGTATGGCTGATGAGCAGAAACTCCATGCG-3’. Samples were run by SDS-PAGE on a 12% gel followed by Western blotting for Ubiquitin.

Ubiquitin Ligase Assay

Ligase assays were performed essentially as described [24] for 45 minutes at 37°C with the following components: 50 mM Tris-HCl pH 7.5, 5 mM MgCl2, 2 mM NaF, 0.6 mM DTT, 2 mM ATP, 10 mM Okaic Acid, 40 ng rabbit Ubc1 (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.

Binding Determinants of Rocs

For embryo lysates, overnight egg collections (0–16 hrs) were dechorionated for 3 minutes in 50% bleach and dounce homogenized with 10 volumes of NP-40 lysis buffer (50 mM Tris pH 8.3, 150 mM NaCl, 0.5% NP-40, 1 ng/ml leupeptin, 0.5 ng/ml pepstatinA, 1 mM PMSF). Lysates were centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was collected. For immunoprecipitations, 25 μl protein-A beads were washed 3× with 1 ml NP-40 lysis buffer, and then pre-incubated with antibody for 2 h before adding to 1 ml of lysis (1 mg/ml protein). Immunoprecipitations were performed overnight at 4°C.

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 [27], affinity purified rabbit anti-Cul5, guinea pig anti-Roc2 whole serum, mouse anti-FLAG (Invitrogen), mouse anti-V5 (Invitrogen), and Rabbit anti-Ab (Covance). Anti-peptide antibodies against Roc2 and Cul5 were generated using synthetic peptides (Invitrogen) coupled to KLH (Pierce). The peptide sequences are CADDENCSVDRPTDD (Roc2) and CKRDRIDFEVVPDK (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 Sulfolin Kit (Pierce).

Stocks and Genetics

The Roc1aG1 and Roc1bG1 alleles have been described previously [24,25]. The Roc2G1 (Roc1bG1), Roc2E2 (Roc1bE2) alleles were obtained from the Bloomington stock center. To test for rescue of Roc1a lethality, Roc1aG1, sn, FRT/EM7, 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 Roc1bG1/TM3, Sb and containing a transgene insertion on the second chromosome (V5-Roc1b, V5-ANBR, or V5-BNAR) were mated to Roc1bG1/TM3, Sb females. w+ (indicating the presence of the transgene), Sb+, Roc1bG1+/Roc1bG1- male progeny were then mated with w1118 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.

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Author Contributions

Conceived and designed the experiments: PJR RD. Performed the experiments: PJR JS. Analyzed the data: PJR JS RD. Wrote the paper: PJR RD. The PI: RD. Performed an experiment for part of one figure: JS. Helped write the paper and design experiments: RD.

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