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Targeted Disruption of *Drosophila* Roc1b Reveals Functional Differences in the Roc Subunit of Cullin-dependent E3 Ubiquitin Ligases

Timothy D. Donaldson,*† Maher A. Noureddine,† Patrick J. Reynolds,† William Bradford,† and Robert J. Duronio*†‡§‖

*Lineberger Comprehensive Cancer Center, †Department of Biology, ‡Curriculum in Genetics and Molecular Biology, §Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

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Cullin-dependent ubiquitin ligases regulate a variety of cellular and developmental processes by recruiting specific proteins for ubiquitin-mediated degradation. Cullin proteins form a scaffold for two functional modules: a catalytic module comprised of a small RING domain protein Roc1/Rbx1 and a ubiquitin-conjugating enzyme (E2), and a substrate recruitment module containing one or more proteins that bind to and bring the substrate in proximity to the catalytic module. Here, we present evidence that the three *Drosophila* Roc proteins are not functionally equivalent. Mutation of *Roc1a* causes lethality that cannot be rescued by expression of *Roc1b* or *Roc2* by using the *Roc1a* promoter. *Roc1a* mutant cells hyperaccumulate Cubitus interruptus, a transcription factor that mediates Hedgehog signaling. This phenotype is not rescued by expression of *Roc2* and only partially by expression of *Roc1b*. Targeted disruption of *Roc1b* causes male sterility that is partially rescued by expression of *Roc1a* by using the *Roc1b* promoter, but not by similar expression of *Roc2*. These data indicate that Roc proteins play nonredundant roles during development. Coimmunoprecipitation followed by Western or mass spectrometric analysis indicate that the three Roc proteins preferentially bind certain Cullins, providing a possible explanation for the distinct biological activities of each *Drosophila* Roc/Rbx.

INTRODUCTION

Protein modification by ubiquitin is widely used by eukaryotic organisms to regulate many important cellular and developmental processes (Ciechanover *et al*., 2000; Ben-Neriah, 2002; Conaway *et al*., 2002; Wojcik, 2002). Three enzymatic activities, ubiquitin activation (E1), conjugation (E2), and ligation (E3), lead to the covalent attachment of ubiquitin, a 76-amino acid protein, to specific target proteins (Hershko and Ciechanover, 1998). Monoubiquitylation can influence the activity or subcellular localization of the target protein (Pickart, 2001; Raiborg *et al*., 2003). Repeated rounds of the E2-E3 reaction result in the formation of polyubiquitin chains that generally serve as a signal for destruction by the 26S proteosome, but they can also have nonproteolytic effects on protein function (Hershko and Ciechanover, 1998; Kaiser *et al*., 2000; Pickart, 2000, 2001). The E3 plays a crucial role in this pathway not only because it mediates the transfer of ubiquitin to the target protein but also because it provides substrate specificity (Jackson *et al*., 2000).

One well characterized E3 is the multisubunit SCF complex (Deshaies, 1999; Jackson and Eldridge, 2002). Consisting of four members (*S*kp1, *C*UL-1/Cdc53, an *F*-box–containing protein, and Roc1/Rbx1/Hrt1), the SCF regulates many developmental processes such as the cell cycle, signaling pathways, circadian rhythms, and apoptosis (Koepp *et al*., 1999; Maniatis, 1999; DeSalle and Pagano, 2001; Grima *et al*., 2002; Ko *et al*., 2002; Nateri *et al*., 2004). The specificity of SCF complexes is conferred by the F-box subunit, which binds to the target protein through a protein–protein interaction motif such as WD40 or leucine-rich repeats (Skowyra *et al*., 1997; Craig and Tyers, 1999; Kipreos and Pagano, 2000). Skp1 serves as an adapter by binding to the F-box domain of the F-box protein and the N-terminal portion of CUL-1 (Bai *et al*., 1996; Feldman *et al*., 1997; Zheng *et al*., 2002). CUL-1 is a scaffold that brings together the F-box/substrate complex and the E2 enzyme, which is recruited by the Roc subunit bound to the C terminus of CUL-1 (Kipreos *et al*., 1996; Patton *et al*., 1998; Kamura *et al*., 1999; Ohta *et al*., 1999; Seol *et al*., 1999; Furukawa *et al*., 2000, 2002).

The SCF complex is just one member of a family of Cullin/Roc-based E3 ubiquitin ligases. The VBC complex, which regulates the stability of transcription factors involved in the response to hypoxia and has been associated with hypervascularization of tumors and cancer progression (Kim and Kaelin, 2003), contains CUL-2, Elongins B and C, and the VHL tumor suppressor protein (Iwai *et al*., 1999; Kamura *et al*., 2001). Elongins B and C also interact with CUL-5 to form a distinct E3 ligase that functions in HIV-1 replication (Kamura *et al*., 2001; Yu *et al*., 2003). CUL-3, which is required for normal development in mice, *Drosophila*, and *Caenorhabditis elegans*, uses BTB proteins as adaptors to recruit substrates (Ou *et al*., 2002; Furukawa *et al*., 2003; Geyer *et al*., 2003; Pintard *et al*., 2003; Xu *et al*., 2003). It is thought that the DNA-damage binding protein DDB1 is one adaptor for human CUL-4A ubiquitin ligase complexes (Nag

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‖ Corresponding author. E-mail address: duronio@med.unc.edu.

et al., 2001; Groisman *et al*., 2003; Wertz *et al*., 2004), and in *C. elegans* and *Drosophila* S2 cells CUL-4 has been shown to target the DNA replication licensing factor CDT-1 for degradation (Higa *et al*., 2003; Zhong *et al*., 2003). Like the Cullin proteins, there are multiple Roc proteins in higher eukaryotes that fall within two subfamilies, Roc1 and Roc2 (Ohta *et al*., 1999). With a few exceptions, it has not been demonstrated which of the Roc family members participates in a given Cullin complex.

With regard to the SCF complex, current data indicate that the F-box subunit is the major factor in determining functional specificity (Craig and Tyers, 1999; Kipreos and Pagano, 2000). Many different F-box proteins exist, and of those that have been analyzed each seems to recruit different sets of target proteins. In yeast, the Cdc4 F-box protein regulates the stability of Sic1, Far1, and Cdc6, whereas Grr1 targets CLN1 and CLN2 (Tyers and Jorgensen, 2000). In *Drosophila*, targets of the F-box protein Slimb include Cubitus interruptus (Ci) and Armadillo (Arm), transcription factors in the Hedgehog and Wingless signaling cascades, respectively (Jiang and Struhl, 1998), the Dorsal/(nuclear factor κB) inhibitor Cactus (inhibitor of κB) (Spencer *et al*., 1999), as well as the circadian rhythm-regulating proteins Period (Per) and Timeless (Tim) (Grima *et al*., 2002; Ko *et al*., 2002). Cyclin E and Myc degradation are controlled by a different F-box protein, Archipelago (Moberg *et al*., 2001, 2004). A common theme emerging from analyses of these F-box proteins and their targets is that phosphorylation of the target seems to be a prerequisite for recognition.

Intriguingly, the *Drosophila* genome contains three members of the Roc gene family (*Roc1a*, *Roc1b*, and *Roc2*, which share 57–70% amino acid similarity), whereas the genomes of other higher eukaryotes such as worms, mice, and humans contain two (*Roc1* and *Roc2*). Previously, we have shown that clones of cells mutant for *Roc1a* fail to proliferate, implicating a role for Roc1a in cell cycle progression (Noureddine *et al*., 2002). These mutant clones also hyperaccumulate the full-length form of Ci (Noureddine *et al*., 2002), suggesting that Roc1a is part of an SCF^{Slimb} complex that targets this transcription factor for proteolytic processing (Jiang and Struhl, 1998). In this study, we sought to determine if Roc1b or Roc2 could substitute for Roc1a in the context of both the proliferation defect and the SCF^{Slimb} complex that targets Ci. Surprisingly, expression of Roc1b or Roc2 was insufficient to fully compensate for Roc1a loss, although overexpression of Roc1b could rescue the Ci hyperaccumulation phenotype. Homologous recombination-mediated gene targeting (Rong and Golic, 2000) was used to generate mutations in *Roc1b*, and we show that these mutations cause male sterility. Using similar in vivo complementation assays, we demonstrate that Roc1a, but not Roc2, is able to partially compensate for Roc1b loss. Finally, we provide evidence that each Roc protein preferentially associates with different members of the Cullin family, which may likely explain the inability of the three Roc proteins to fully compensate for each other.

MATERIALS AND METHODS

Fly Stocks and Crosses

To test for rescue of *Roc1a* lethality, *$Roc1a^{G1}$*, *FRT/FM7*, *Act-GFP* females were mated with males from stocks expressing a given Roc transgene under control of the *Roc1a* promoter and the lethal phase of green fluorescent protein (GFP)-negative males was analyzed. To examine the ability of each Roc to rescue Ci hyperaccumulation, clones of *Roc1a* mutant cells were generated using the MARCM system as described previously (Noureddine *et al*., 2002), except that the flies also carried the appropriate Roc transgene. Homozygous *Roc1b* mutant males carrying the appropriate Roc transgene were generated to analyze the rescue of the male sterility.

Rescue Constructs

The *Roc1a* genomic rescue fragment was described previously (Noureddine *et al*., 2002) and contains 980 bp of sequence upstream of the ATG initiation codon and 620 bp downstream of the translational Stop, except that a FLAG tag was inserted at the N-terminus. To create the rescue constructs in which the *Roc1a* coding region was replaced with that of *Roc1b* or *Roc2*, an NruI site was introduced into the above-mentioned construct immediately downstream of the sequences coding for the FLAG epitope, and an AatII site was introduced immediately after the Stop codon. The *Roc1b* and *Roc2* coding regions were amplified from the Origene RapidScan library with primers containing the appropriate linkers and subcloned as NruI/AatII fragments into the modified *Roc1a* rescue construct. To obtain the *Roc1b* genomic rescue transgene, the *Roc1b* genomic locus, including 840 bp upstream of the ATG codon and 330 bp downstream of the translational Stop site, was amplified from BACR13F06. To generate the constructs in which the *Roc1a* and *Roc2* open reading frames (ORFs) replaced the *Roc1b* ORF, *Roc1a* and *Roc2* were amplified from the Origene RapidScan library with a forward primer containing an *Nco*I linker and a reverse primer with an *Eag*I linker and these polymerase chain reaction (PCR) products were subcloned into the *Roc1b* genomic rescue construct. To generate *Roc1bgrf::FLAG-Roc1b*, the 840-bp *Roc1b* promoter and 330 bp downstream region were amplified in separate PCR reactions and joined in the appropriate orientation through an AgeI linker introduced during PCR. Using *Roc1agrf::FLAG-Roc1b* as a template, the *FLAG-Roc1b* ORF was amplified with primers also containing AgeI linkers and inserted in the correct orientation between the promoter and downstream region. Each of these genomic rescue constructs was cloned into pCaSpeR-4. To generate the UAS-Roc transgenes, each Roc ORF was cloned into pUAST (Brand and Perrimon, 1993). Microinjection of *Drosophila* embryos was done using standard methods, and multiple independent lines for each construct were analyzed.

Immunofluorescence

For detection of the FLAG-Roc proteins in situ, larvae from each of the *Roc1agrf::FLAG-Roc* stocks were mixed with *UAS-FLAG-dribble; en-Gal4 larvae*, dissected, fixed in 4% paraformaldehyde, and stained with mouse anti-FLAG M2 (diluted 1:250) and Cy3-conjugated goat anti-mouse (diluted 1:1000; Jackson Immunoresearch Laboratories, West Grove, PA). Discs were mounted in Fluoromount-G and photographed with a Nikon Eclipse E800 equipped with a charge-coupled device camera. For detection of Ci protein accumulation, discs were stained with rat anti-Ci 2A1 (gift of R. Holmgren, Northwestern University, Evanston, IL) and processed as described previously (Noureddine *et al*., 2002).

Western Blotting and Immunoprecipitation

To verify expression of the FLAG-Roc transgenes, 0- to 8-h embryos from each of the stocks were homogenized in sample buffer (Harlow and Lane, 1999) and boiled for 5 min. The extracts were then run on a 15% acrylamide gel and transferred to nitrocellulose in methanol-free transfer buffer (38 mM glycine, 5 mM Tris base). The blots were rinsed several times with phosphate-buffered saline plus 0.1% Tween-20 (PBT), blocked with 5% bovine serum albumin (BSA), and incubated for 1 h at room temperature with a mouse anti-FLAG M2 antibody (diluted 1:100 in PBT plus BSA; F3165, Sigma-Aldrich, St. Louis, MO). For detection, horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (diluted 1:15,000; Amersham Biosciences, Piscataway, NJ) and ECL-Plus (Amersham Biosciences) were used. For coimmunoprecipitation experiments, 4- to 8-h (for Western) or overnight (for mass spectrometry) collections of embryos were dechorionated in 50% bleach for 5 min and then lysed in NET buffer (50 mM Tris pH 7.0, 400 mM NaCl, 5 mM EDTA, 1% NP-40, 50 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1.4 μg/ml pepstatin). Samples were incubated with mouse IgG-agarose beads (A0919; Sigma-Aldrich) for 1 h at 4°C to reduce nonspecific binding, and the supernatants were then incubated with anti-FLAG-M2–conjugated agarose gel (A2220; Sigma-Aldrich) for 2 h at 4°C. After three washes with NET buffer, immunocomplexes were eluted with 66 μg/ml 3×-FLAG peptide for 30 min at 37°C and analyzed by SDS-PAGE. For Western analysis, the gel was cut in half so that the larger migrating CUL-1 bands could be blotted with traditional Western transfer buffer, whereas the smaller, FLAG-Roc bands could be blotted with methanol-free buffer. Detection of CUL-1 was performed with a rabbit anti-CUL-1 antibody (diluted 1:250; Zymed Laboratories, South San Francisco, CA) and goat anti-rabbit-HRP secondary (diluted 1:10,000; Chemicon International, Temecula, CA). Detection of the FLAG-Rocs was as described above. For mass spectrometry, the gel was fixed in 25% acetic acid/10% isopropanol for 20 min, stained overnight in 0.01% G-250 Coomassie Blue solution, and destained in 10% acetic acid. Specific bands were excised, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry by the University of North Carolina Proteomics Core Facility.

Table 1. Summary of the scheme used to target *Roc1b*

Donor line	% Mob (n)[a]	No. female germlines	No. potential HR events	No. non-HR events	No. verified	% reduction
F9	100 (86)	720	1	1	1	ND
F13	100 (93)	880	0	1	NA	NA
F14	100 (76)	400	0	0	NA	NA
F15	98.8 (83)	260	1	0	ND	NA
F16	98.9 (95)	360	0	0	NA	NA
F17	98.7 (77)	400	2	0	ND	NA
F30	95.9 (98)	360	2	0	ND	NA
F32	98.9 (88)	280	2	0	1	60.5[b]
D5	91.7 (97)	200	0	0	NA	NA
D6	100 (81)	460	1	1	ND	NA
D10	88.4 (95)	160	0	1	NA	NA
D13	73.3 (90)	360	0	0	NA	NA
D19	94.0 (67)	460	0	0	NA	NA
D24	98.3 (115)	460	3	1	1	16.0[c]
D27	98.0 (49)	120	0	0	NA	NA

NA, not applicable; ND, not done.

[a] Number of flies scored for eye color.

[b] Reduction attempted in both males and females.

[c] Reduction attempted in males only.

Construction of Roc1b Targeting Vectors

Approximately 7 kb of DNA homologous to the *Roc1b* locus was used for targeting. Recombinant PCR was used to obtain the region of homology distal to *Roc1b* (with respect to the centromere), which contains the last exon of CG1228, the gene CG1231, and the 5′ part of *Roc1b*, as well as to introduce an I-SceI cut site ~500 base pairs upstream of the Roc1b translation start site. The outside primers for these reactions were 5′-CCTCAGCGGCCGCCCTATTTCAGATGACTGCAC-3′ (which has a 5′ *Not*I linker) and 5′-CTCAACCTCTAGATCTCCTCG-3′ (which introduces two bases [underlined] into the *Roc1b* coding region, creating and *Xba*I site). The inside primers were 5′-TATTACCCTGTTATCCCTACATTATTATTAAGGAAGCTTTAC-3′ and 5′-GTAGGGATAACAGGGTAATACATTCGAGTTTGGGAAACAG (the 18 bases corresponding to the I-SceI site are underlined). The recombinant PCR product was cloned into pCR-BluntII (Invitrogen, Carlsbad, CA) and shuttled to pBluescript KS+ (pBS) as a *Not*I/*Xba*I fragment. To obtain the region of homology proximal to *Roc1b*, which includes most of *Roc1b* and the gene CG6905, and simultaneously generate the desired mutations, two separate PCR reactions were performed, one for each allele. The forward primers (both of which create an *Xba*I site, underlined) used to generate the frameshift and deletion alleles were 5′-CGAGGAGATCTAGAGGTTGAGG-3′ and 5′-AATCATCTAGACAACAAGGAGTGGGTCTAC-3′, respectively. The reverse primer for both reactions was 5′-GGAGTAGGTACCACACTGTCGCGTTATGTTATG-3′ (which has a 5′ Asp718 linker). Each PCR reaction was cloned into pCR-BluntII and subsequently inserted into pBS containing the distal region as an *Xba*I/Asp718 fragment. All PCR reactions used the BAC clone BACR13F06 as a template and were sequenced. Each targeting construct was then cloned into pTargetB (a gift of Sarah Radford and Jeff Sekelsky, University of North Carolina, Chapel Hill, NC) for injection into embryos.

Genetics of Targeting

Thirty-two potential donor constructs (18 frameshift, 14 deletion) were first crossed to flies constitutively expressing the FLP recombinase (*w; P{70FLP}10*; a gift of Kent Golic, University of Utah, Salt Lake City, UT) and the number of white-eyed progeny was divided by the total number of progeny (including those with mosaic eyes) to obtain a mobilization percentage (% Mob; Table 1) indicative of the ability of each donor line to excise. Fifteen lines with a high mobilization percentage were chosen to be sent through the targeting scheme. Virgin females carrying the donor construct were crossed to *yw; P{hsp70-FLP}, P{hsp70-I-SceI}, Sco/Cyo, S* (a gift of Kent Golic, University of Utah) males, and the progeny were heat shocked in a 37°C water bath for 1 h 3 d after egg laying. Next, w^+, *Sco* virgin female progeny were mated to *w; P{70FLP}10* males (20 females, 10 males per bottle) to screen for the presence of a w^+ element that does not mobilize in the presence of constitutive FLP expression, indicative of a potential homologous recombination (HR) event. The number of female germlines screened for each donor construct is given in Table 1. w^+ progeny were then subjected to a second round of screening by backcrossing to *w; P{70FLP}10*; this step eliminated more than half of w^+ flies obtained from the first round of screening. Flies that still contained the *w* gene after two rounds of screening were then analyzed for HR events in two ways. First, we verified that w^+ segregated with the third chromosome and then we performed inverse PCR to specifically identify class II (tandem duplication) events (Rong and Golic, 2000). Inverse PCR was performed as described on the Berkeley *Drosophila* Genome Project Web site (http://www.fruitfly.org/about/methods/inverse.pcr.html) by using *Sac*I, except that 1 fly equivalent of ligated genomic DNA was used as a template. The primers (designed to amplify a fragment only when a class II HR event occurred) were 5′-CTCTCTTGCTGTACCATG-3′ (which anneals to part of the *w* gene) and 5′-GTCAGCACACGATCATCG-3′ (which anneals to genomic sequence just outside the tandem duplication). Fragments obtained by inverse PCR were cloned into pCR-II (Invitrogen) and sequenced.

We positively identified three independent HR events. Potential HR events (Table 1) met three criteria: the w^+ is stable when crossed to *P{70FLP}10*, maps to the third chromosome, and is similar in color to the three HR events verified by inverse PCR and sequencing. We also obtained at least five nonhomologous recombination events, each from a different donor line. Two of these (D6 and D10) occurred on a chromosome that was not chromosome 3. Of the three others that were on chromosome 3, one (from F9) did not give an eye color similar to the verified targeting events, one (from D24) was unlinked from a verified targeting event (tested by meiotic recombination), and one (from F13) was sequenced and found to target a location at 89E. Males from two verified HR events (F32 and D24) were then crossed to w^{1118}*;P{v+, hsp70-I-CreI}, Sb/TM6* (a gift of Kent Golic, University of Utah) females to reduce the tandem duplication to a single copy. Progeny were heat shocked in a 36°C water bath for 30 min 3 d after egg laying. White-eyed, Sb males were collected and mated individually to *w; TM3/TM6* females. Stocks were then created from Sb^+*/TM6B* progeny and analyzed by PCR for the presence of the desired *Roc1b* mutations by using the primers 5′-CGCCGTTGTTATTTCGTAG-3′ and 5′-CTCTGTCTCACTCTCGAC-3′. The products were then digested with *Xba*I to confirm the presence of the mutation and subsequently sequenced. For reasons unknown, we observed a difference in the frequencies of reduction to generate the two mutant alleles. Reduction of the tandem duplication generated the frameshift allele at a higher frequency (60.5%) than that for the deletion allele (16%).

Characterization of Male Sterility

A single male 1–2 d posteclosion was mated to three w^{1118} virgin females and transferred daily. The number of eggs was counted immediately after transfer, and 7–10 d later the number of pupae was scored (Kusano *et al*., 2001). At least 30 males of each genotype were tested. Two aspects of male sterility were measured; the percentage of viable offspring produced (no. pupae/ no. eggs) and the number of males that were completely sterile. Pairwise comparisons of the average hatching rates of each genotype were performed using a Student's *t* test.

Reverse Transcription (RT)-PCR

RNA was isolated from tissue samples (20 testes or ~50 embryos) with TRIzol (Sigma-Aldrich) according to manufacturer's instructions. For the RT reaction, 1 μg of total RNA and 3.5 μM anchored oligo-dT_{23} were mixed in 10 μl

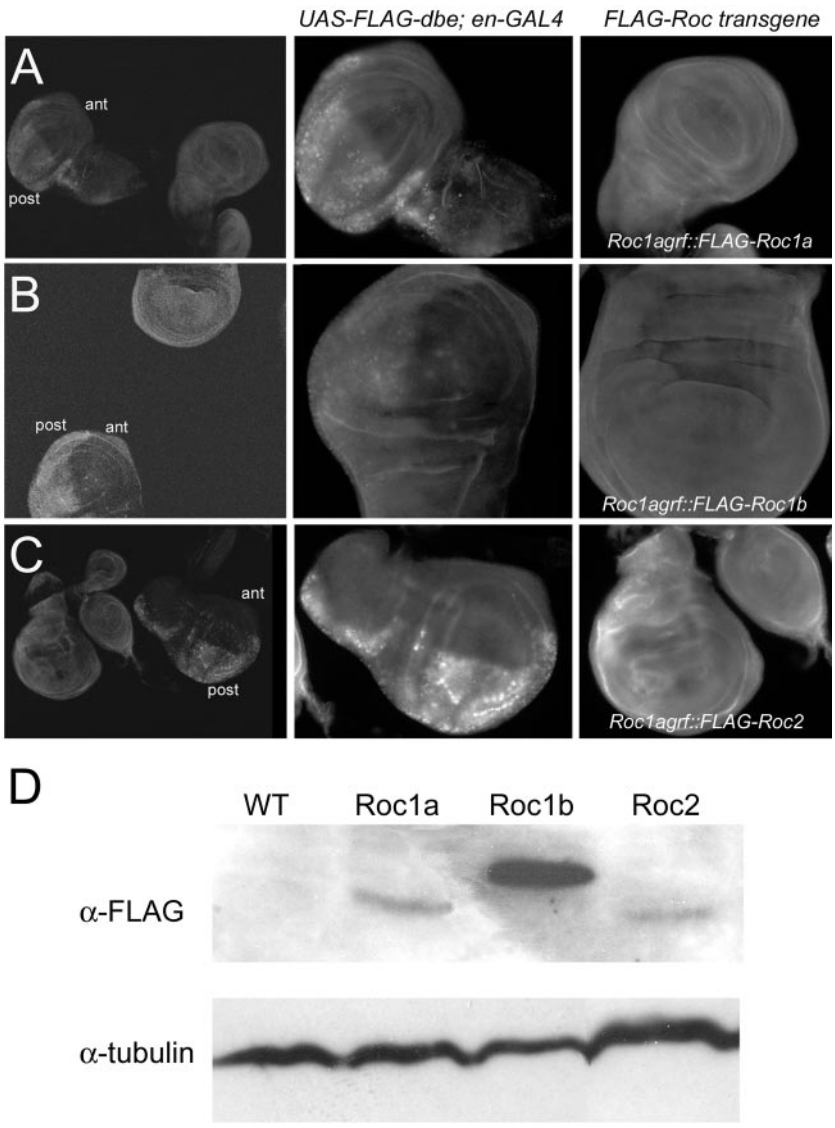


Figure 1. Expression of *Roc1agrf::FLAG-Roc* transgenes. (A–C) Expression of FLAG-Roc proteins in the wing disc. Larvae from stocks expressing either FLAG-Roc1a (A), FLAG-Roc1b (B), or FLAG-Roc2 (C) from the *Roc1a* promoter were combined with *UAS-FLAG-dribble; en-GAL4* larvae, dissected, fixed, and stained with an anti-FLAG antibody. Left, discs from both *UAS-FLAG-dribble; en-GAL4* and *Roc1agrf::FLAG-Roc* genotypes in the same frame. Middle and right, close-ups of the same control (center) and experimental (right) discs, taken with same camera settings. Because it was difficult to discern background staining from the actual signal of the low, ubiquitous expression from the *Roc1a* promoter, *UAS-FLAG-dribble; en-GAL4* discs served as both a positive and negative control for FLAG staining, because FLAG-Dribble is expressed only in the posterior compartment (post). Notice that the level of expression of each Roc protein is greater than that of the anterior compartments (ant) of the control discs where FLAG-Dribble is not expressed. (D) Western blot of embryo extracts showing expression of FLAG-Roc proteins. This level of FLAG-Roc1a expression is sufficient to rescue *Roc1a* mutant animals to adulthood. FLAG-Roc1b expression is significantly higher, but it is still unable to rescue the *Roc1a* mutation. Likewise, FLAG-Roc2 is expressed but also unable to rescue *Roc1a* mutants. Twice as much extract was loaded to the Roc2 lane. Similar results were observed from wing disc extracts (our unpublished data).

of total volume and heated to 70°C for 10 min. Next, 2 μl of 10× RT buffer (750 mM KCl, 500 mM Tris-HCl, 30 mM $MgCl_2$, 100 mM dithiothreitol, pH 8.3), 1 μl of dNTP mix (10 mM each), 1 μl of RNasin (Promega, Madison, WI), 1 μl (200 U) of M-MuLV-RT (New England Biolabs, Beverly, MA), and 5 μl of distilled H_2O were added, and the reaction was incubated at 25°C for 15 min and then at 42°C for 1 h. Three microliters of cDNA was amplified with 35 cycles by using 66°C as the annealing temperature for the *Roc1b* and *Rp49* primer sets and 58°C for the *Roc1a* and *Roc2* primer sets by using *Taq* polymerase (Roche Diagnostics, Indianapolis, IN) and analyzed on a 1% agarose gel.

RESULTS

Mutations in Roc1a Cause Lethality That Cannot Be Rescued by Roc1b or Roc2

Previously, we reported that *Roc1a* mutant animals die as late first/early second instars (Noureddine *et al*., 2002), demonstrating a unique function for Roc1a that cannot be compensated by Roc1b or Roc2. This could be the result of functional differences between the Roc proteins, or because the Roc1b/Roc2 proteins are functionally equivalent but not expressed in the same spatial or temporal pattern or at the same level as Roc1a. To directly test these possibilities, we placed FLAG-tagged versions of each Roc open reading frame under control of the *Roc1a* promoter and 3′ untranslated sequence and asked whether this was sufficient to rescue the lethality caused by the *Roc1a* mutation. Like a native *Roc1a* genomic rescue fragment (grf) (Noureddine *et al*., 2002), *Roc1agrf::FLAG-Roc1a* was able to rescue *Roc1a* mutant flies to adulthood, indicating that the presence of the FLAG epitope does not significantly affect the function of the Roc1a protein. In contrast, *Roc1a* mutant flies expressing either *Roc1agrf::FLAG-Roc1b* or *Roc1agrf::FLAG-Roc2* did not survive beyond the early second instar, precisely the same time at which *Roc1a* mutants die. Western blotting and immunostaining of wing discs revealed that each of the FLAG-Roc proteins was expressed (Figure 1). Moreover, the level of Roc1b was greater than that of Roc1a, which is sufficient for rescue (Figure 1D). These data suggest that there is at least one essential protein targeted by a Cullin-dependent E3 ligase complex containing Roc1a that is unable to be efficiently targeted by complexes containing either Roc1b or Roc2.

Roc1b, but Not Roc2, Can Partially Rescue Phenotypes of Roc1a Mutant Cells

Given that the lethality of the *Roc1a* mutation may likely be the result of the hyperaccumulation of many different SCF

targets, we asked whether Roc1b or Roc2 could substitute in the absence of Roc1a in regulating the stability of one known SCF target. We previously showed that *Roc1a* mutant clones of cells in the wing disc do not grow very large and also fail to process the Hh effector Ci from a 175-kDa transcriptional activator form to the 75-kDa repressor (Figure 2A; Noureddine *et al*., 2002). This suggests that neither Roc1b nor Roc2 normally play a major role in targeting Ci for processing, either because they are not expressed appropriately or because they are unable to assemble with the SCF complex responsible for targeting Ci. We used the MARCM system (Lee and Luo, 1999) to generate GFP-positive, *Roc1a* mutant clones in wing imaginal discs of flies carrying a specific Roc transgene and analyzed whether that transgene was able to supply a sufficient amount of Roc function to rescue either of the two *Roc1a* mutant phenotypes, namely, small clones and Ci hyperaccumulation.

As expected, a *Roc1a* genomic rescue fragment expressing either a native or FLAG-tagged version of Roc1a was able to rescue both phenotypes; the *Roc1a* mutant clones grew to a large size and did not hyperaccumulate Ci (Figure 2, B and C). When *Roc1agrf::FLAG-Roc1b* was expressed in the *Roc1a* mutant cells, we observed a partial rescue of both phenotypes. In approximately half of the discs, the clones were similar in size to *Roc1a* mutant clones, and these clones always displayed elevated levels of full-length Ci protein (Figure 3A). However, in the other half of the discs, the mutant clones grew larger than clones not expressing a transgene, but not quite as large as clones expressing *Roc1agrf::FLAG-Roc1a*. In addition, Ci hyperaccumulation was not observed in these clones (Figure 3, C–E). This partial phenotypic rescue was not caused by a reduction in Roc1b function due to inclusion of the FLAG epitope, because FLAG-Roc1b rescues the null *Roc1b* phenotype as well as untagged Roc1b, which is described below (Figure 5C and Table 2). In contrast to the effects we observed with both Roc1a and Roc1b, Roc2 was completely unable to rescue the *Roc1a* mutant phenotypes. *Roc1a* mutant clones expressing *Roc1agrf::FLAG-Roc2* were always small and always hyperaccumulated Ci (Figure 4A).

Although the *Roc1agrf::FLAG-Roc1a* transgene we used was able to rescue the phenotypes of the mutant clones as well as viability of *Roc1a* mutant animals, we suspected that using the endogenous *Roc1a* promoter to express the Roc transgenes may provide at best only moderate levels of gene expression. This could potentially explain the inability of either Roc1b of Roc2 to fully rescue the *Roc1a* mutant clones, if these proteins shared some redundancy. Therefore, we tested whether higher levels of Roc1b or Roc2 expression could provide additional rescue of *Roc1a* mutant clones. For this we used the UAS-Gal4 system (Brand and Perrimon, 1993) to express each of the *Roc* genes, which when used in the MARCM system expresses at high levels and only in the cells that become mutant for *Roc1a*. Just as with *Roc1agrf*, expression of *UAS-Roc1a* was able to fully rescue both the small clone size and Ci hyperaccumulation phenotypes associated with the *Roc1a* mutation (Figure 2D). Expression of *UAS-Roc1b* was also able to rescue the Ci hyperaccumulation phenotype similarly to *UAS-Roc1a* (Figure 3B). However, even though the *UAS-Roc1b*–rescued clones were larger than *Roc1a* mutant clones, they were not quite as large as those obtained by rescue with either a *Roc1a* genomic fragment or *UAS-Roc1a* (Figure 3B). In addition, unlike the rescue observed with *Roc1agrf::FLAG-Roc1b*, the effect of *UAS-Roc1b* expression was fully penetrant; every disc analyzed showed some degree of rescue. In sharp contrast, expression of *UAS-Roc2* was unable to rescue either of the *Roc1a* mutant phenotypes; we always observed small clones that always hyperaccumulated Ci (Figure 4B).

We observed one additional phenotype in *Roc1a* mutant clones expressing *Roc1agrf::FLAG-Roc1b* and (to a lesser extent) *UAS-Roc1b*. Occasionally, these clones were associated with small punctate regions of GFP expression (Figure 3, F and G). We attribute this to apoptosis of the *Roc1a* mutant cells, which as they died left behind fragments of cell membranes still expressing the CD8-tagged, membrane-anchored GFP. This was not observed in any other *Roc1a* mutant clones, perhaps because the clones were either rescued (as those expressing exogenous Roc1a) or were never able to get large enough before dying to notice the fragments of membranes (as those expressing Roc2 or no transgene).

Mutations in Roc1b Cause Male Sterility

To further our analysis of potential redundancies between Roc proteins, we generated mutations in *Roc1b* by using homologous recombination-mediated gene targeting (Rong and Golic, 2000). We obtained two mutant alleles of *Roc1b*. One allele is a deletion of the *Roc1b* coding region ($Roc1b^{dc3}$) and is both a protein and genetic null. In the other allele, a frameshift/premature stop codon was introduced after the fifth codon of the *Roc1b* open reading frame ($Roc1b^{F28M}$; see *Materials and Methods*).

Flies homozygous for either of the *Roc1b* mutant alleles are viable, indicating that Roc1b is not essential for *Drosophila* development. However, these mutations caused male-specific sterility. To examine this male sterility in detail, we calculated the percentage of viable progeny produced from 30 individual males and compared those values among wild-type and various mutant genotypes (see *Materials and Methods*; Figure 5A). Homozygous null $Roc1b^{dc3}$ males were completely sterile; none of the eggs laid by the females of the cross hatched. In contrast, ~2% of the eggs laid by females mated with homozygous $Roc1b^{F28M}$ males hatched. Additionally, about half of these males were completely sterile, whereas the fertility of the other half ranged from 0.3 to 12%. Although the frameshift allele would be expected to be a protein null, this partial sterility suggests that this allele is actually a hypomorph, perhaps due to reinitiation of translation at a downstream methionine. All males transheterozygous for both alleles ($Roc1b^{dc3}/Roc1b^{F28M}$), or for the frameshift allele over a deficiency that uncovers *Roc1b* ($Roc1b^{F28M}/Df(3L)emc^{E12}$), were also completely sterile, thus confirming the hypomorphic nature of the $Roc1b^{F28M}$ allele. The sterility induced by both *Roc1b* mutant alleles was rescued by a transgene containing the *Roc1b* genomic locus (Figure 5C), although the fertility of the rescued males was still somewhat lower than wild type (~70% compared with 95%).

Squashed preparations of live testes from $Roc1b^{dc3}$ homozygous males revealed the presence of sperm bundles that complete the individualization process normally (our unpublished data). However, these sperm cells were completely immotile. This is the most common of the male sterile phenotypes and suggests that the mitotic and meiotic cell divisions are normal and that there is a defect in some aspect of sperm differentiation or maturation. Consistent with the hypomorphic nature of the $Roc1b^{F28M}$ allele, homozygotes produce some live, motile sperm, but in quantities much less than heterozygous or wild-type males.

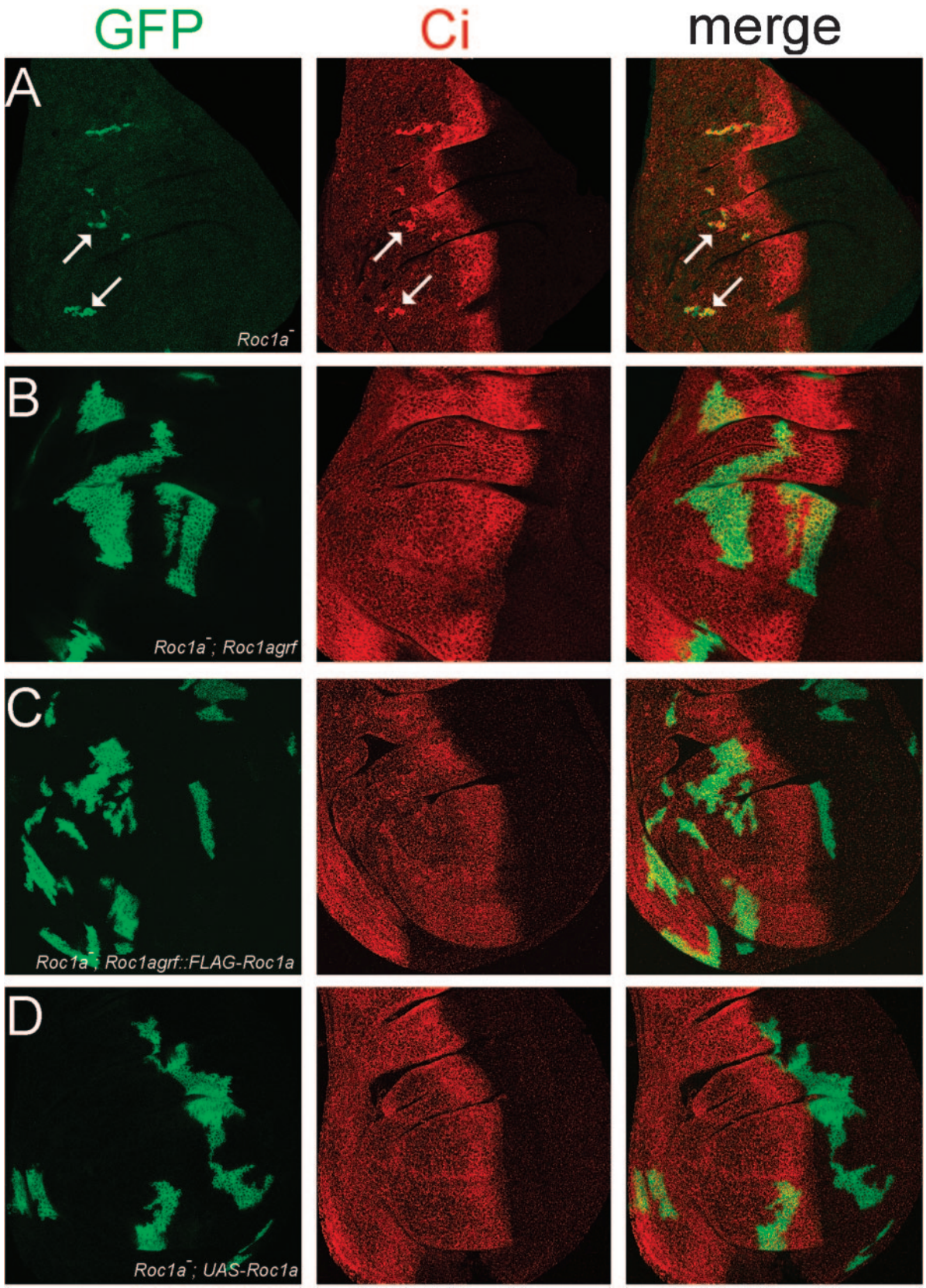


Figure 2. Expression of Roc1a can fully rescue *Roc1a* mutant phenotypes. (A) *Roc1a* mutant clones (positively marked with GFP, arrows) have a proliferation defect and accumulate full-length Ci. (B–D) Expression of native Roc1a (B) or FLAG-Roc1a (C) with the *Roc1a* promoter or UAS-Roc1a (D) can rescue both the proliferation and Ci hyperaccumulation defects of the *Roc1a* mutation.

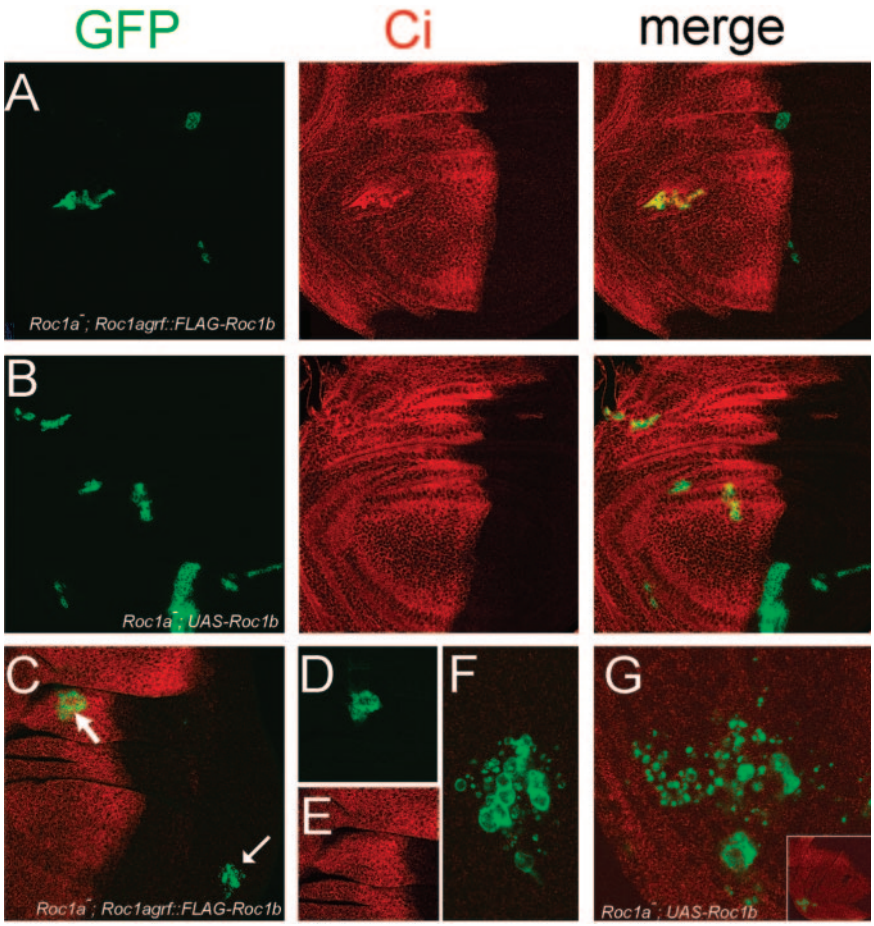


Figure 3. Roc1b can partially substitute for Roc1a. (A) *Roc1a* mutant clones expressing *Roc1agrf::FLAG-Roc1b*. The clones are small and hyperaccumulate unprocessed Ci in about half of the wing discs. (B) *Roc1a* mutant clones expressing *UAS-Roc1b*. A partial rescue of the proliferation and Ci hyperaccumulation defects is observed. (C–F) A wing disc containing *Roc1a* mutant clones, depicting an example where *Roc1agrf::FLAG-Roc1b* expression rescues the *Roc1a* phenotypes (i.e., a normal level of Ci is observed). Single channels of the clone in the merged image (C, arrowhead) are shown in D and E. (F) Enlarged image of *Roc1a* mutant clone (C, arrow) showing the cell death phenotype. (G) Cell death phenotype of *Roc1a* mutant clones expressing *UAS-Roc1b*. Notice the area of the clone is larger than that in (F), consistent with the fact that UAS expression of *Roc1b* provides a higher degree of rescue than expression with the *Roc1a* promoter. Inset shows a lower magnification view of the wing pouch, indicating the position of the clone.

Roc1a but Not Roc2 Can Partially Rescue the Male Sterile Phenotype of Roc1b Mutations

The male sterile phenotype of *Roc1b* mutants indicates a unique role for Roc1b in spermatogenesis. The other two Roc proteins may not be able to compensate for loss of Roc1b because they may not be expressed in the correct time or place in the testes. To test this, we first performed RT-PCR of testes from wild-type and $Roc1b^{dc3}$ mutant males. Both *Roc1a*- and *Roc1b*-specific primers were able to amplify bands of the correct size from wild-type testes, and as expected no *Roc1b* mRNA was detected in the $Roc1b^{dc3}$ null mutant line (Figure 5B). *Roc2* mRNA is detected in embryos, but not in the testes (Figure 5B). This was somewhat unexpected because *Roc2* is expressed in all stages of *Drosophila* development and in many mammalian tissues, including the testis (Duan *et al*., 1999; Noureddine *et al*., 2002). This suggests that all necessary Cullin-mediated ubiquitylation reactions in the testes can be carried out using either Roc1a or Roc1b.

Next, we placed *Roc1a* and *Roc2* ORFs under the control of the *Roc1b* promoter and 3′ untranslated region, and asked whether these transgenes could rescue the male sterility of the Roc1b mutants. Expression of *Roc1a* was able to rescue the male sterile phenotype of the Roc1b mutation to some degree (Figure 5C). For example, $Roc1b^{F28M}$ homozygous males expressing *Roc1bgrf::Roc1a* had hatching rates ranging from 2 to 85%, and none were completely sterile (compared with approximately half of $Roc1b^{F28M}$ homozygous males). However, the rescue of both *Roc1b* alleles with *Roc1bgrf::Roc1a* differed significantly ($p < 0.02$) from that observed with *Roc1bgrf*, indicating that Roc1a is not fully able to compensate for loss of Roc1b (Table 2).

In contrast, Roc2 was completely unable to rescue the *Roc1b* male sterile phenotype. $Roc1b^{dc3}$ males expressing *Roc1bgrf::Roc2* were completely sterile, and the hatching

Table 2. *Roc1a* does not fully rescue the male sterility of the *Roc1b* mutations

	1a #7	1a #8	1b #1	1b #5	1b #8	1b #10
1a #7		N	Y	Y	Y	Y
1a #8	N		Y	Y	Y	Y
1b #1	Y	N		N	N	Y
1b #5	Y	Y	N		N	N
1b #8	Y	Y	N	N		N
1b #10	Y	Y	N	N	N	
FLAG1b #23	Y	Y	N	N	N	N
FLAG1b #27	Y	Y	N	N	N	N
FLAG1b #40	Y	Y	N	N	N	N

Pairwise comparisons of the ability of each *Roc1bgrf* (transgenic lines #1, 5, 8, and 10), *Roc1bgrf::FLAG-Roc1b* (transgenic lines #23, 27, and 40) and *Roc1bgrf::Roc1a* (transgenic lines #7 and 8) transgene to rescue the $Roc1b^{-}$ male sterile phenotype. Y indicates a significant difference ($p < 0.02$) in the ability of the indicated transgenes to rescue the $Roc1b^{F28M}$ hypomorphic (unshaded) or $Roc1b^{dc3}$ null (shaded) mutations (see *Materials and Methods*). N indicates no significant difference. Note that for the most part, 1) the transgenic lines of a given construct are not significantly different from each other, and 2) the *Roc1a* transgenic lines are significantly different from the *Roc1b* lines.

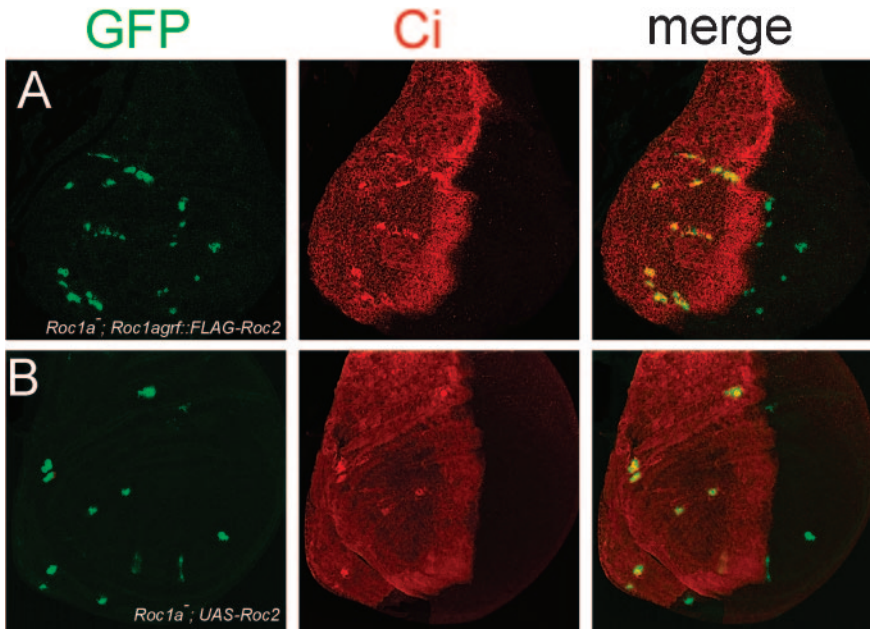


Figure 4. Roc2 cannot substitute for Roc1a. tk;2Expression *Roc1agrf::FLAG-Roc2* (A) or *UAS-Roc2* (B) in *Roc1a* mutant clones does not rescue either the proliferation or Ci hyperaccumulation defects caused by the *Roc1a* mutation.

rates from crosses of $Roc1b^{F28M}$ males with or without the transgene were not significantly different, even at $p < 0.001$ (Figure 5C). Using RT-PCR, we verified that mRNA from each transgenic line was expressed (Figure 5, D–F), suggesting that the lack of complete rescue is due to biological differences of the Roc proteins.

The Drosophila Roc Proteins Preferentially Bind Different Members of the Cullin Family

One possible explanation for the inability of a given Roc protein to rescue the phenotype of a different Roc mutant is that each Roc protein may form a unique set of E3 ubiquitin ligase complexes by preferentially interacting with different Cullin family members. To test this, we performed coimmunoprecipitation experiments with our *Roc1agrf::FLAG-Roc* transgenes. Lysates from control, nontransgenic (w^{1118}) embryos or embryos expressing each of the FLAG-Roc transgenes were incubated with anti-FLAG-agarose and immunocomplexes were analyzed by Western blotting or mass spectrometry. Western analysis with a CUL-1 antibody showed that CUL-1 efficiently coprecipitates with FLAG-Roc1a (Figure 6A). Relatively little, but still above-background, amounts of CUL-1 was present in immunocomplexes from FLAG-Roc1b or FLAG-Roc2 lysates (Figure 6A). This result shows that whereas Roc1a, Roc1b, and Roc2 are each able to bind to CUL-1 when expressed from the *Roc1a* promoter, Roc1a does so much more efficiently. We also analyzed immunocomplexes from each of the FLAG-Roc transgenic lysates by mass spectrometry. Proteins from a Coomassie-stained polyacrylamide gel that migrated with the predicted molecular weight of the Cullins and that were present in one or more of the transgenic lines but absent from wild-type, nontransgenic lysate (Figure 6B) were excised and identified by tandem mass spectrometry. Using this approach, we identified CUL-1 and CUL-2 in Roc1a immunocomplexes, CUL-3 in Roc1b immunocomplexes, and CUL-5 in Roc2 immunocomplexes (Figure 6C). Because weaker Cullin–Roc interactions may not permit the precipitation of enough Cullin protein to be visible on a Coomassie-stained gel, this technique does not rule out any particular Cullin–Roc interactions. However, the data do suggest that there is a preference for the formation of certain Cullin–Roc complexes.

DISCUSSION

Drosophila Roc Proteins Have Distinct Functions

Our results indicate that there are significant differences in the biological roles of the three *Drosophila* Roc proteins and that these differences are not simply the result of distinct expression patterns during development. In all of our experimental paradigms, Roc1a and Roc1b could partially, but not completely, substitute for one another, whereas Roc2 showed no ability to substitute for either Roc1 paralogue. Results of coimmunoprecipitation experiments suggest that these differences are due to preferential interactions between Roc and Cullin family members. For example, CUL-1 seems to interact most strongly with Roc1a, suggesting that a majority of SCF (i.e., CUL-1) targets require Roc1a. However, we cannot rule out that Roc1b or Roc2 function within the context of an SCF complex, as both showed weak interactions with CUL-1. Indeed, Roc1b seems to be capable of participating in SCF-mediated ubiquitylation, because it was able to rescue the aberrant accumulation of Ci, a bona fide SCF target, when overexpressed.

Because the *Drosophila* Roc proteins share between 40 and 60% overall sequence identity, it is somewhat surprising that we did not observe a higher degree of complementation in our rescue assays. Most of the conservation is within the C-terminal 67 residues, which contains the catalytic RING domain. Roc1a and Roc1b share 76% identity and 88% similarity in this domain, whereas Roc1a and Roc2 are 45% identical and 59% similar. In the N-terminal regions, the sequence identity/similarity is lower (38%/50% between Roc1a and Roc1b; 41%/57% between Roc1a and Roc2). It was found previously that deletion of the Rbx1 (Roc1) N-terminus prevents interaction with CUL-1 in 293T cells (Furukawa *et al*., 2002). The crystal structure of the SCF complex (Zheng *et al*., 2002) shows that the association between Rbx1 and the C-terminal portion of the CUL-1 protein (termed the Cullin homology domain or CHD) consists of two parts.

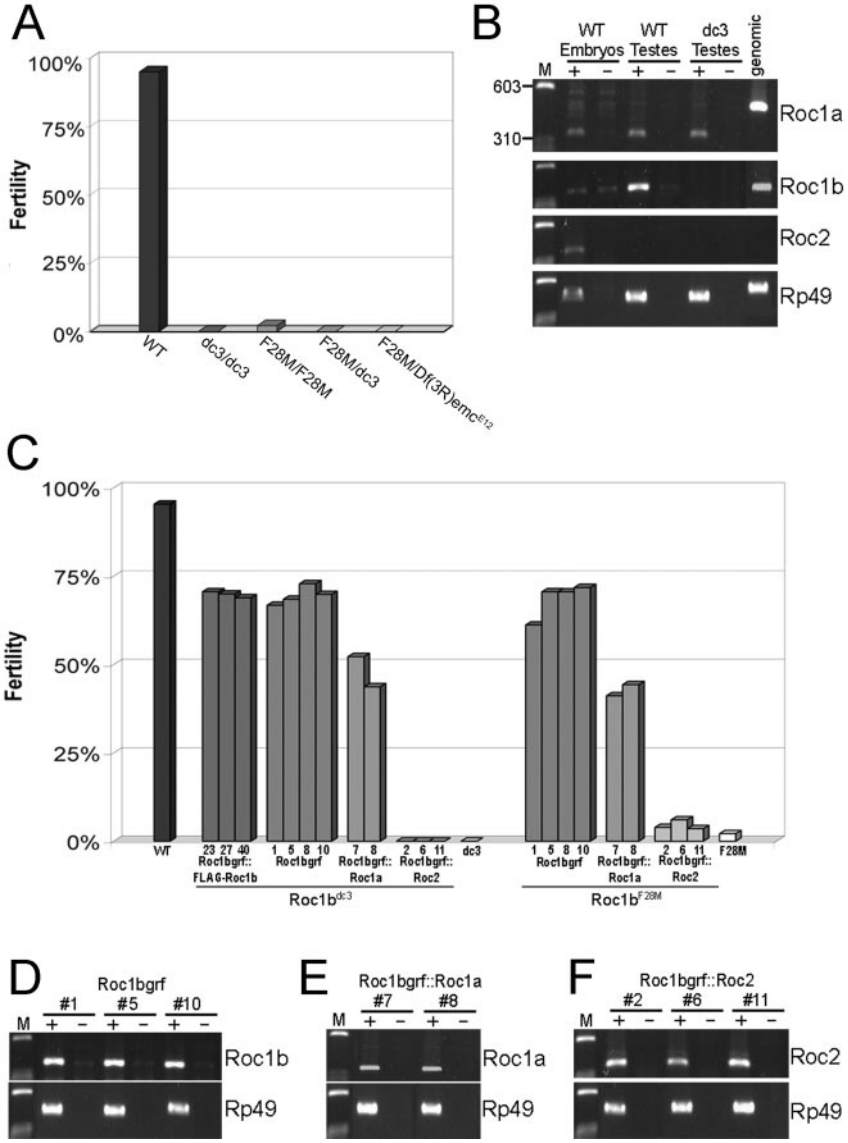


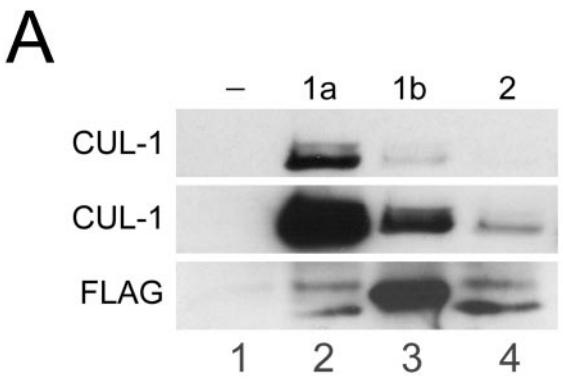
Figure 5. The *Roc1b* mutation causes male sterility. (A) Graph showing fertility of wild-type and various *Roc1b* mutant males. "Fertility" is the percentage of viable progeny produced by 30 males mated individually to three virgin females. (B) RT-PCR performed on indicated tissues using primer sets designated on the right. Genomic DNA was used as a control for each primer set. + and − indicate reactions with or without reverse transcriptase. The *Roc1a* and *Rp49* primer sets span small introns and thus produce slightly larger PCR fragments. The *Roc2* primer set spans a 26-kb intron. WT embryos were also used as a control to test primer sets that do not amplify messages in the testes (i.e., *Roc2*). This also reaffirms our previous observation that each *Roc* is expressed in the embryo (Noureddine *et al*., 2002). *Roc1b* does not contain an intron and the primers amplify a band in the no-RT control, indicating the presence of some contaminating DNA. Both *Roc1a* and *Roc1b* are expressed in the testis, but *Roc2* is not. Note the absence of *Roc1b* message in the $Roc1b^{dc3}$ mutant. M indicates Marker and is the same in other panels. (C) Expression of FLAG-Roc1b, Roc1b, and Roc1a, but not Roc2, can rescue the male sterile phenotype. *Roc* transgenes expressed with the *Roc1b* promoter were crossed into the $Roc1b^{dc3}$ (null, left) or $Roc1b^{F28M}$ (hypomorph, right) backgrounds and the percentage of viable progeny from individual males was determined. Three transgenic lines expressing FLAG-Roc1b (#23, #27, and #40) and four lines expressing native Roc1b (#1, #5, #8, and #10) were found to rescue the mutations. Two transgenic lines expressing Roc1a (#7 and #8) also rescue the mutations, but to a significantly lower degree (see Table 2). None of three Roc2 transgenic lines (#2, #6, and #11) showed any significant rescue. (D–F) RT-PCR of testes from $Roc1b^{dc3}$ mutant males expressing a *Roc1bgrf* transgene. Three of the four individual Roc1b lines (D), both Roc1a lines (E), and all three Roc2 lines (F) used in the rescue assay were tested, and each indicates a similar level of expression.

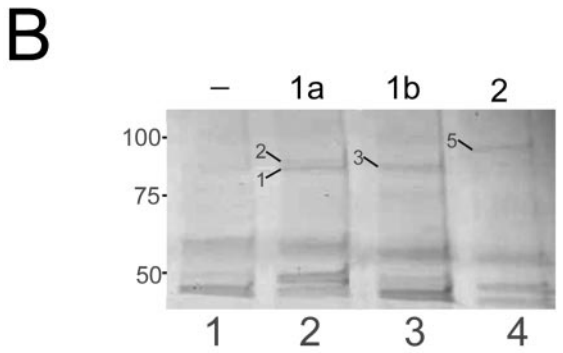
First, the RING domain of Rbx1 packs into a V-shaped groove formed by the α/B and WH-B domains of CUL-1. Second, the Rbx1 N terminus threads into CUL-1 and makes a five-stranded intermolecular β-sheet (four strands provided by CUL-1 and one by Rbx1). This intermolecular β-sheet seems to provide the primary mechanism of Rbx1 recruitment. Together, these data implicate the N terminus of the *Drosophila* Roc proteins as the region responsible for mediating the differential binding to Cullins.

Additional factors may also contribute to the differences in the biological roles of Roc proteins. Specific interactions between E2s and RING domains have been observed, suggesting that the identity of the Roc protein can influence which E2 gets recruited to the complex. Roc1-containing immunoprecipitates from Cullin-transfected cells possess ubiquitin ligase activity with both UbcH5 and Ubc3/Cdc34; however, similar complexes containing Roc2 are only active with UbcH5 (Furukawa *et al*., 2002). The RING proteins RAD5 and RAD18 form a macromolecular complex with the E2s RAD6 and Ubc13-MMS2 that affects postreplicative DNA damage. RAD5 is able to directly interact with Ubc13-MMS2, but not with RAD6, and the converse is true for RAD18 (Ulrich and Jentsch, 2000). Protein mapping studies have identified specific residues important for the interaction between Ubc13-MMS2 and the RING domain of RAD5 (Ulrich, 2003). Finally, although the RING domain protein BARD1 interacts with UbcH5, the BARD1 RING domain is not required for this and the interaction is instead mediated by the RING domain protein BRCA1 (Brzovic *et al*., 2003). Thus, one possible model is that Roc1a, Roc1b, and Roc2 recruit a unique (set of) E2(s) that each act on a different set of targets, thereby providing an additional level of functional specificity in vivo. Other issues such as protein half-life or subcellular localization may also be important contributing factors to the biological differences among the three *Drosophila* Roc proteins.

The Roc1b Mutant Phenotype

We were able to use the two step method of homologous recombination mediated gene targeting to obtain two mutant alleles of *Roc1b*. Our results confirm the validity of the technique for use in generating specific mutations in a gene of interest. Furthermore, our results are consistent with pre-





C

Band	Protein Name	# Peptides Identified
1	CUL-1 (lin-19, *D. melanogaster* CG1877)	13
2	CUL-2 (*D. melanogaster* CG1512)	8
3	CUL-3 (gft, *D. melanogaster* CG11861)	21
5	CUL-5 (*D. melanogaster* CG1401)	11

Figure 6. The three *Drosophila* Roc proteins preferentially bind different Cullins. (A) Lysates from embryos expressing no transgene (−; lane 1) or one of the Roc1agrf::FLAG-Roc proteins (1a, 1b, or 2; lanes 2–4) were incubated with anti-FLAG agarose and immunocomplexes were analyzed by Western blot with anti-CUL-1. Top, short exposure showing that Roc1a efficiently immunoprecipitates both neddylated and unmodified CUL-1. Middle, longer exposure of the same blot showing that Roc1b and Roc2 can also immunopurify CUL-1, although much less efficiently than Roc1a. Bottom, blot probed with anti-FLAG to demonstrate immunoprecipitation of the FLAG-Roc proteins. The slower migrating bands in lanes 2 and 4 correspond to unidentified proteins, possibly read-through translation products. (B and C) Immunocomplexes from the embryo lysates of the same genotypes as in A were analyzed by mass spectrometry. (B) Coomassie-stained gel showing that each Roc protein immunoprecipitates a unique set of proteins in the size range expected for the Cullins. Bands indicated with arrows were excised and analyzed by mass spectrometry (the corresponding Cullin protein is identified by the number). (C) Protein identification of the bands excised from the gel in B. CUL-1 and CUL-2 were each identified FLAG–Roc1a immunocomplexes, CUL-3 was identified in FLAG–Roc1b immunocomplexes, and CUL-5 was identified in FLAG–Roc2 immunocomplexes. The number of peptides matching a theoretical digest of the corresponding protein is indicated.

vious work (Rong *et al*., 2002) stating that a minimum distance of 400 base pairs between the I-SceI cut site and the mutation to be introduced is sufficient to prevent the mutation from being lost due to gap enlargement. Our mutation was ~520 base pairs away from the I-SceI site and all three targeting events that we analyzed molecularly retained the mutation. Our results also demonstrate that a simple deletion of the start codon or the introduction of a premature Stop codon early in the reading frame may not be sufficient to generate a null mutation due to the possibility of translation initiation at downstream AUG codons. Although we did not definitively determine what causes the *Roc1b*^{F28M} mutation to be hypomorphic, we suspect that this is the result of a low frequency of translation reinitiation that leads to a reduction in the total Roc1b protein levels and/or the production of an N-terminally truncated protein with reduced activity.

We do not know the precise cause of the sterility of *Roc1b* mutant males. Given the role that SCF complexes play in regulation of the cell cycle, it is attractive to speculate that the *Roc1b* mutation may disrupt some aspect of the mitotic or meiotic divisions of the germ cells. However, this seems unlikely given the fact that the null mutation is completely sterile. Aberrant mitotic or meiotic divisions generally only reduce fertility and result in other cytological defects not seen in the *Roc1b* mutant. Furthermore, the only discernable phenotype of the *Roc1b* mutation is the production of elongated individualized, but nonmotile sperm, suggesting that the mitotic and meiotic divisions are normal.

The fact that the *Roc1b* mutation results in a male sterile phenotype is somewhat surprising given the observation that *Roc1a* is also expressed in adult (Figure 5B) and larval (Noureddine and Duronio, unpublished data) testes. However, Roc1a and Roc1b may be expressed in different cell types in the testes (e.g., soma vs. germline). This idea is supported by the observation that Roc1a partially rescues the male sterile phenotype when expressed with the Roc1b promoter. However, the difference in expression pattern may not be the only difference between the two proteins, because the phenotypic rescue observed with Roc1a was less than that with Roc1b.

Ubiquitylation is believed to play a variety of roles in spermatogenesis. In addition to the proposed role in the regulation of the meiotic cell cycle, ubiquitylation may affect aspects such as synaptonemal complex formation or the chromatin remodeling and reorganization that occurs during nuclear condensation and elongation (Roest *et al*., 1996; Grootegoed *et al*., 1998; Baarends *et al*., 2003). Furthermore, many components of the ubiquitin system show high and/or unique expression patterns in the testes. For example, the UbcD1 gene of *Drosophila*, which is involved in maintaining telomere structure, encodes three transcripts, one of which is expressed solely in the male germline (Cenci *et al*., 1997). Mutations in this gene disrupt male meiosis, leading to infertility (Cenci *et al*., 1997). Another *Drosophila* E2, Ubc7, also encodes a male specific transcript and plays a role in spermatogenesis, although mutations are pleiotropic and affect other processes such as courtship behavior and neural development/function (Orgad *et al*., 2000).

At the molecular level, the *Roc1b* mutant male sterile phenotype is likely the result of a specific target protein of a Cullin-Roc1b E3 ligase that fails to be ubiquitylated. Presumably, this ubiquitylation occurs in the context of an SCF-like complex for several reasons. First, Roc1a has been shown to associate with SCF components (Bocca *et al*., 2001) and is able to partially rescue the *Roc1b* mutation. Second, we observed enhancement of the *Roc1b*^{F28M} induced sterility by

halving the gene dose of either CUL-1 or CUL-3 (Donaldson and Duronio, unpublished results), suggesting that both of these Cullins have specific targets within the testes. This is consistent with the observation from the coimmunoprecipitation experiments that Roc1b and CUL-3 interact strongly and that Roc1b can interact with CUL-1 in vivo. Interestingly, mutations in the Slimb homologue β-TRCP1 result in male infertility in the mouse (Guardavaccaro *et al*., 2003). However, unlike the *Roc1b* mutation, the lack of β-TRCP1 disrupts the meiotic divisions, and mutant testes contain spermatocytes that arrest in metaphase I, reducing the number of postmeiotic spermatids (Guardavaccaro *et al*., 2003). This, however, does not exclude the possibility that Roc1b and Slimb might be part of the same complex, because Slimb may also have additional functions later in spermatogenesis. Along these lines, mutations in both *Slimb* and *SkpA* (the *Drosophila Skp1* homologue) have been associated with centrosome overduplication in other tissues (Wojcik *et al*., 2000; Murphy, 2003) and mutations in *centrosomin* result in defects in cytokinesis, karyokinesis, and growth of the axoneme during spermatogenesis (Megraw *et al*., 1999). Thus, it is possible that an SCF^{Slimb} complex with Roc1a has a function early in sperm development, and an SCF^{Slimb} complex with Roc1b regulates later stages.

The Roc Subunit of Cullin-dependent E3 Ligases

In this study, we have used the powerful genetic techniques of the fruit fly to assess how the RING domain subunit contributes to the function of Cullin-dependent ubiquitin ligases. We have found that the *Drosophila* Roc proteins have nonredundant roles during development and that these differences may be mediated by the formation of specific Cullin–Roc ligase complexes. Our results are consistent with studies of mammalian Roc proteins showing that although both Rbx1 and mammalian Roc2 can associate with all Cullin proteins, these interactions, as well as the associated ligase activities of the different complexes, seem to show certain preferences (Furukawa *et al*., 2002). Because each Cullin family member may use a distinct mechanism to target nonoverlapping sets of proteins for ubiquitylation (Kamura *et al*., 2001; Nag *et al*., 2001; Groisman *et al*., 2003; Yu *et al*., 2003; van den Heuvel, 2004; Wertz *et al*., 2004), preferential Cullin binding provides a sufficient, if not the only, explanation for the functional differences among the three *Drosophila* Roc proteins. Further experiments are needed identify which complexes exist in vivo and to determine exactly what mediates these specific Cullin–Roc interactions.

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