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

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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 proteasome, but they can also have nonproteolytic effects on protein function (Hershko and Ciechanover, 1998; Kaiser et al., 2000; Pickart, 2000). 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 (Deshaines, 1999; Jackson and Eldridge, 2002). Consisting of four members (Skp1, CUL-1/Cdc53, an F-box–containing protein, and Roc1/Rbx1/Hrt1), the SCF regulates many developmental processes such as the cell cycle, signalizing pathways, circadian rhythms, and apoptosis (Koepp et al., 1999; Maniatis, 1999; DeSalle and Pagano, 2001; Grima et al., 2002; Ko et al., 2002; Natari 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 Culin-/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; Wu 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 DBD1 is one adaptor for human CUL-4A ubiquitin ligase complexes (Nag...
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 stabilizes the stability of Sic1, Far1, and Cdc6, whereas Gr1 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 demonstrate full phosphorylation of their target to be a prerequisite for recognition.

To test for rescue of Roc1a lethality, Roc1a−/−, FRT/TM7, 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.

**MATERIALS AND METHODS**

**Fly Stocks and Crosses**

To test for rescue of Roc1a lethality, Roc1a−/−, FRT/TM7, 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 sequence coding for the FLAG epitope, and an AatII site was introduced immediately after the Stop codon. The intronless 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 a NruI linker and a reverse primer with an EagI linker and these polymerase chain reaction (PCR) products were subcloned into the Roc1b genomic rescue construct. To generate Roc1aFlag::FLAG-Roc1b, the 840-bp Roc1a Flag ORF and 330 bp downstream region were deleted and the appropriate orientation through an EagI linker introduced downstream of the PCR. Using Roc1aFlag::FLAG-Roc1b as a template, the FLAG-Roc1b ORF was amplified with primers also containing EagI and NruI linkers and inserted into the correct orientation between the promoter and downstream region. Each of these genomic rescue constructs was cloned into pCaSpeR4. 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 Roc1aFlag::FLAG-Roc1b stocks were mixed with UAS-FLAG-dribble+mU4 larval, 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, the brains from each genotype were 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 m-MOPS and free transfer buffer (30 mM MOPS, 1 mM Tris base). The blots were rinsed several times with phosphate-buffered saline plus 0.1% Tween-20 (PB), blocked with 5% bovine serum albumin (BSA), and incubated for 1 h at room temperature with a mouse anti-FLAG M2 antibody (diluted 1:300; Sigma), followed by an HRP-conjugated donkey anti-mouse secondary antibody (diluted 1:3000; Jackson ImmunoResearch Laboratories, West Grove, PA). Discs were transferred to Fluoromount-G and photographed with a Nikon Eclipse E800 equipped with a charge-coupled device camera. For detection of Ci protein accumulation, the brains from each genotype were processed as described previously (Noureddine et al., 2002).

**Immunocomplex Mass Spectrometry**

To verify the presence of the FLAG-Roc transgene, 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 m-MOPS and free transfer buffer (30 mM MOPS, 1 mM Tris base). The blots were rinsed several times with phosphate-buffered saline plus 0.1% Tween-20 (PB), blocked with 5% bovine serum albumin (BSA), and incubated for 1 h at room temperature with a mouse anti-FLAG M2 antibody (diluted 1:300; Sigma), followed by an HRP-conjugated donkey anti-mouse secondary antibody (diluted 1:3000; Amersham Biosciences, Piscataway, NJ) and ECL-Plus (Amersham Biosciences) were used. For immunoprecipitation 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 polyethylene glycol, 0.1 μg/ml leupeptin, 14 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 μl/mg 3%–5% 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 UAS bands could be blotted with traditional Western transfer buffer, whereas the smaller, FLAG-Roc bands could be blotted with methanol-free buffer. Detection of UAS-1 was performed with a rabbit anti-CU1 antibody (diluted 1:250; Zymed Laboratories, South San Francisco, CA) and goat anti-rabbit-HRP secondary (diluted 1:1000; 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.
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 to vector pairs bases upstream of the Roc1b translation start site. The outside primers for these reactions were 5′-CTCTAGACCTCGAC-3′ (which has a 5′ NotI linker) and 5′-CTCACAACCCTGCTGGC-3′ (which introduces two bases [underlined] into the Roc1b coding region, creating and existing site). The inside primers were 5′-TATTACGCTTATCCTATTATATTAAAGAGGCCTTAC-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 NotI/Xbal 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 XbaI site, underlined) designed to generate the frameshift and deletion alleles were 5′-CGGAGGATCTAGCTGGGACG-3′ and 5′-AATCATCTAG ACAACAAGGATGCGTCTAC-3′, respectively. The reverse primer for both reactions was 5′-CGATTAGGTTACACATTCTGCCATAT-3′ (which has a 5′ Asp718 linker). Each PCR reaction was cloned into pCR-BluntII and subsequently inserted into pBS containing the distal I/Asp718 fragment. All PCR reactions used the BAC clone I/Asp718 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{70FLP10}, 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 w; P{hsp70-I-CreI} hsp70-I-CreI, Sco hemizygous, 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{hsp70-L-SceI}, Sco hemizygous, S 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 P{hsp70-L-SceI} this step eliminated more than half of the w* lines 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 SacI, except that 1 by 5′ 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′-CTCCTCTGCTGAGCTGATC-3′ (which anneals to part of the w gene) and 5′-GTCACAACCGATCACGATCC-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{hsp70-L-SceI}, 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 99E. Males from two verified HR events (F32 and D24) were then crossed to w; hsp70-I-CreI, hsp70-I-CreI, Sco/TM6B (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 d after egg laying. White-eyed, Sc males were collected and mated individually to w; Sco/TM6B females. Stocks were then created from Sc/TM6B progeny and analyzed by PCR for the presence of the desired Roc1b mutations by using the primers 5′-CCGCGGT- GTATTTCCGAT-3′ and 5′-CTCCTCTGCTGATC-3′. The products were then digested with XhoI 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* 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-dT23 were mixed in 10 μL
of total volume and heated to 70°C for 10 min. Next, 2/\mu l of RNasin (Promega, Madison, WI), 1/\mu l of dNTP mix (10 mM each), 1/\mu l of M-MuLV-RT (New England Biolabs, Beverly, MA), and 5/\mu l of distilled H2O 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 mutants 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 or 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-Ga4 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, expres-

sion 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 del/M) 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 Drosohila 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 6-3 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 6-3/Roc1b F28M), or for the frameshift allele alone 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 6-3 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.
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 Roc1bdc3 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 Roc1bdc3 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, Roc1bF28M homozygous males expressing Roc1agrf::Roc1a had hatching rates ranging from 2 to 85%, and none were completely sterile (compared with approximately half of Roc1bF28M homozygous males). However, the rescue of both Roc1b alleles with Roc1agrf::Roc1a differed significantly (p < 0.02) from that observed with Roc1agrf, 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. Roc1bF28M males expressing Roc1agrf::Roc2 were completely sterile, and the hatching rates varied significantly (p < 0.02) from that observed with Roc1agrf, indicating that Roc1a is not fully able to compensate for loss of Roc1b (Table 2).

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

<table>
<thead>
<tr>
<th>Roc1b mutant</th>
<th>Roc1agrf::FLAG-Roc1b</th>
<th>UAS-Roc1b</th>
<th>Roc1agrf::FLAG-Roc1b + UAS-Roc1b</th>
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<tbody>
<tr>
<td>roc1bF28M</td>
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<tr>
<td>roc1bdc3</td>
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Pairwise comparisons of the ability of each Roc1agrf (transgenic lines #1, 5, 8, and 10), Roc1agrf::FLAG-Roc1b (transgenic lines #23, 27, and 40) and Roc1agrf::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 male sterile phenotype. 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.
rates from crosses of Roc1bF28M 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 (w1118) 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 parologue. 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.
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 interaction as 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-
Figure 6. The three Drosophila Roc proteins preferentially bind different Cullins. (A) Lysates from embryos expressing no transgene (−; lane 1) or one of the Roc1α/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 Roc1α efficiently immunoprecipitates both neddylated and unmodified CUL-1. Middle, longer exposure of the same blot showing that Roc1b and Roc2 can also immunoprecipitatively bind CUL-1, although much less efficiently than Roc1α. 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 embryonic 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–Roc1α 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.
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 communoprecipitation 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 centroamin result in defects in cytokinesis, karyokinesis, and growth of the axoneme during spermatogenesis (Megraw et al., 1999). Thus, it is possible that an SCFSlimb complex with Roc1a has a function early in sperm development, and an SCFSlimb 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 theRING 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., 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 to identify which complexes exist in vivo and to determine exactly what mediates these specific Cullin–Roc interactions.

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