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Drosophila Roc1a Encodes a RING-H2 Protein with a Unique Function in Processing the Hh Signal Transducer Ci by the SCF E3 Ubiquitin Ligase

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Summary

Substrate specificity of SCF E3 ubiquitin ligases is thought to be determined by the F box protein subunit. Another component of SCF complexes is provided by members of the Roc1/Rbx1/Hrt1 gene family, which encode RING-H2 proteins. *Drosophila* contains three members of this gene family. We show that Roc1a mutant cells fail to proliferate. Further, while the F box protein Slimb is required for Cubitus interruptus (Ci) and Armadillo/β-catenin (Arm) proteolysis, Roc1a mutant cells hyperaccumulate Ci but not Arm. This suggests that Slimb and Roc1a function in the same SCF complex to target Ci but that a different RING-H2 protein acts with Slimb to target Arm. Consequently, the identity of the Roc subunit may contribute to the selection of substrates by metazoan SCF complexes.

Introduction

Proper control of growth and cell cycle progression during development requires the regulation of gene expression at many different levels. One level of regulation is the rapid and irreversible elimination of proteins via ubiquitin-mediated proteolysis. This process involves the polymerization of ubiquitin onto selected proteins, allowing them to be recognized as a substrate by the proteosome. Ubiquitin conjugation is a highly regulated process involving three different enzymatic activities, termed E1, E2, and E3 (reviewed in Hershko and Ciechanover [1998]). Substrate recognition by the ubiquitination machinery is usually the limiting step in proteolysis and appears to be provided primarily by the E3. Several varieties of E3 exist, and they play important roles in many cellular processes, including signal transduction, transcription, and cell cycle control. The SCF complex is a multisubunit E3 that regulates progress through the G1-S transition by mediating the ubiquitination of G1 cyclins (e.g., cyclin E and Cln2p) and G1 cyclin/cdk inhibitors (e.g., p27 and Sic1p). Many recent studies have contributed to an understanding of the overall architecture of the SCF complex and the biochemical role played by each component. SCF is composed of four core subunits: Skp1, Cul1 (Cdc53), an F box-containing protein, and Roc1 (Rbx1/Hrt1) (reviewed in Deshaies [1999]). The specific recognition and ubiquitination of phosphorylated substrate proteins, such as Cln1p, Cln2p, and Sic1p in yeast and IkB and Smad3 in mammals, can be reconstituted in vitro in the presence of E1, E2, and purified, recombinant SCF components (Fukuchi et al., 2001; Furukawa et al., 2000; Kamura et al., 1999b; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1997, 1999; Tan et al., 1999; Verma et al., 1997). Cullins are large molecules that may act as a scaffold to bring the E2 and the other SCF components into close proximity. The C-terminal region of Cul1 binds to Roc1 and the E2, and the N-terminal region of Cul1 binds to Skp1, which recruits an F box containing protein by binding to the F box domain (Michel and Xiong, 1998; Ohta et al., 1999; Patton et al., 1998; Schulman et al., 2000; Seol et al., 1999; Skowyra et al., 1999). F box proteins also typically contain either WD40 repeats or a leucine-rich region that interacts directly with phosphorylated substrate proteins (reviewed in Kipreos and Pagano [2000]).

SCF components are evolutionary conserved among eukaryotes and are found in organisms from yeast to humans. Studies in *S. cerevisiae* have provided a great deal of insight into SCF function, including much biochemical and genetic evidence supporting the idea that individual SCF complexes and the substrates they recognize in vivo are defined by the F box subunit (the so called "F box hypothesis") (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997). For instance, SCF^{Cdc4} targets Sic1p, Far1p (both CKIs), and Cdc6 (a replication factor) for ubiquitination; SCF^{Grr1} targets Cln1p and Cln2p (G1 cyclins), and SCF^{Met30} targets Swe1p (a CDK inhibitory kinase) and Met4p (a transcription factor) (Blondel et al., 2000a; Deshaies et al., 1995; Drury et al., 1997; Feldman et al., 1997; Kaiser et al., 1998, 2000; Patton et al., 1998, 2000; Seol et al., 1999; Sia et al., 1998; Skowyra et al., 1997, 1999). Each of these complexes contains Skp1p, Cdc53p, and Rbx1p/Hrt1p in common, and they are therefore functionally distinguished in terms of substrate recognition by the Cdc4p, Grr1p, and Met30p F box proteins. Moreover, these analyses have indicated that individual SCF complexes are capable of recognizing more than one substrate.

In animal systems, in vivo evidence supporting the F box hypothesis and the role played by each SCF subunit is less extensive. Mutations in genes encoding SCF components have been identified and characterized through studies of developmentally important signal transduction cascades. An excellent example is provided by the *Drosophila slimb* gene. *slimb* encodes an F box/WD40-repeat protein involved in the Wingless (Wg) and Hedgehog (Hh) signaling pathways, which are important for growth and axis formation during limb development. Cytoplasmic Armadillo (Arm) protein is normally unstable and accumulates only in response to Wg signaling, whereby it translocates to the nucleus and participates in the activation of Wg-responsive genes (reviewed in Peifer and Polakis [2000]). Similarly, in the absence of Hh signal, full-length Cubitus interruptus Ci (Ci^{155}) is processed by the proteosome to generate a smaller form (Ci^{75}) that functions as a transcriptional repressor of Hh target genes. Cells receiving Hh signal block the processing of Ci, allowing Ci^{155} to accumulate and activate transcription (Aza-Blanc et al., 1997; Chen et al., 1999; Hepker et al., 1997; Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998). In *slimb* mutant cells, both Arm and Ci^{155} accumulate inappropriately in the

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absence of signal, resulting in phenotypes that resemble ectopic Wg and Hh activation, including the limb duplication phenotypes for which the gene was named (supernumerary *limb*s) (Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000; Theodosiou et al., 1998). Slimb is also required for the degradation of other proteins, including the Dorsal/NFkB inhibitor Cactus/IkB in early embryos, and an unidentified substrate regulating centrosome duplication in larval neuroblasts (Spencer et al., 1999; Wojcik et al., 2000). The idea that F box proteins in *Drosophila* contribute to SCF substrate specificity is supported by the observation that Arm and Ci are not stabilized by mutations of *archipelago* (*ago*), which encodes an F box protein required for destruction of cyclin E (Moberg et al., 2001).

A common feature of many E3 ubiquitin ligases is the presence of a RING finger, a highly conserved domain that forms a defined tertiary structure by chelating two zinc ions (Borden et al., 1996; Lovering et al., 1993). RING fingers are found either as a single domain of a multidomain E3 (c-Cbl) or as a distinct subunit of a multiprotein complex, such as SCF and APC, an E3 ligase that mediates destruction of proteins during mitosis, and VCB, an E3 ligase that regulates responses to hypoxia via ubiquitination of the transcription factor Hif1α (Aso et al., 2000; Kamura et al., 1999b; Ohta et al., 1999; Skowyra et al., 1999; Tan et al., 1999; Yu et al., 1998; Zachariae et al., 1998; Zheng et al., 2000). It is becoming increasingly clear that RING fingers function as allosteric activators of E2 enzymes and play an essential role in the ubiquitination process, although their precise mechanism of action is unknown (reviewed in Freemont [2000]; Jackson et al. [2000]). Purified RING finger proteins, including Roc1 and APC11, can by themselves stimulate the ubiquitin transferase activity of E2 in vitro when ubiquitin is activated by E1, either in the presence or absence of a particular substrate (Furukawa et al., 2002; Gmachl et al., 2000; Leverson et al., 2000; Lorick et al., 1999). *S. cerevisiae* cells lacking Roc1 accumulate Sic1p and Cln2p and, consequently, arrest in G1 phase with multiple buds (Blondel et al., 2000b; Kamura et al., 1999b; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999). This phenotype is completely rescued by homologous *Drosophila* and human Roc1 genes but not by the related APC11, suggesting evolutionary conservation in Roc1 function that is distinct from the function of the RING domain in the APC complex (Bocca et al., 2001; Kamura et al., 1999a; Ohta et al., 1999; Seol et al., 1999). However, no genetic analysis of Roc1 function in a multicellular organism has yet been reported. Here we demonstrate that *Roc1a* plays a nonredundant role during *Drosophila* development that provides insight into the in vivo function of metazoan SCF complexes.

Results

D. melanogaster Contains Three Members of the Roc/Rbx/Hrt Gene Family that Stimulate the Formation of Polyubiquitin Conjugates by E2 In Vitro

The Roc/Rbx/Hrt and Apc11 genes encode a related subfamily of RING-H2 finger proteins required for SCF and APC E3 ubiquitin ligase activity, respectively. In this group of small proteins, the RING-H2 domain comprises nearly the entire molecule (Deshaies, 1999). Whereas yeast contains a single *Roc* gene, there are two classes of metazoan *Roc* genes, designated *ROC1* and *ROC2*. In human and nematode, each class is thus far represented by a single gene. In contrast, we identified three highly conserved *Roc*-like genes in *Drosophila*. One of these genes (*Roc2*) encodes a 113-residue protein that is 64% identical to human ROC2, while the other two (designated *Roc1a* and *Roc1b*) encode proteins that are clearly more similar to human ROC1, demonstrating 85% and 59% identity to human ROC1, respectively (Figure 1A). Roc1a and human ROC1 appear to be orthologous, as their identity rises to 100% in the RING domain (residues 42 to 97). In addition, other groups have previously demonstrated that both *Roc1a* and human ROC1 complement *S. cerevisiae Rbx1/Hrt1* deletion strains (Bocca et al., 2001; Kamura et al., 1999b; Ohta et al., 1999; Seol et al., 1999).

To gauge the potential of these *Drosophila* Roc proteins to function as ubiquitin ligases, we expressed and purified all three proteins from *E. coli* as GST fusions (Figure 1B) and tested whether they could stimulate ubiquitination in vitro. GST-Roc1a stimulated the formation of high molecular weight [^{32}P]ubiquitin conjugates in the presence of E1 and the E2 UbcH5 (Figure 1C). This activity was not observed with E1 or UbcH5 alone or when E1 and E2 were combined in the absence of GST-Roc1a (Figure 1C). GST-Roc1b and GST-Roc2 could also induce the formation of ubiquitin conjugates, although slightly less efficiently than GST-Roc1a (Figure 1D). A modest level of ubiquitin conjugation was also detected using UbcH2 or UbcY4 and each of the three GST-Roc proteins (data not shown). Thus, each *Drosophila* Roc protein behaves in vitro as a functional RING-H2 protein to stimulate E1- and E2-dependent ubiquitin conjugation.

Roc1a Provides an Essential Function for *Drosophila* Development

The high degree of amino acid identity between human ROC1 and Roc1a led us to initially focus on the *Roc1a* gene. A homozygous viable P element insertion [EP(X)1216a], located $\sim$6.3 kb downstream of *Roc1a* (Figure 2A), was used as the starting point for local mutagenic tranposition events (see Experimental Procedures). Using this methodology, a chromosome containing a small deletion was recovered that removes both *Roc1a* and the genes on either side of *Roc1a*. When homozygous, this deletion causes complete lethality that is rescued by a transgene containing a wild-type *Roc1a* gene (Figure 2A) but not by a transgene containing a *Roc1a* allele with an inactivating N59R/C68R double missense mutation (based on an *Rbx1* allele [Skowyra et al., 1999]). Moreover, loss of the genes on either side of *Roc1a* does not contribute to the lethality of this deletion, because, when each gene is individually mutated, homozygous flies are fully viable and fertile and display no obvious morphological defects (see Experimental Procedures). These data indicate that *Roc1a* function is essential for *Drosophila* development.

The lethality caused by *Roc1a* deletion indicates that neither *Roc1b* nor *Roc2* can compensate for loss of *Roc1a*. This was somewhat surprising, since Roc1b is

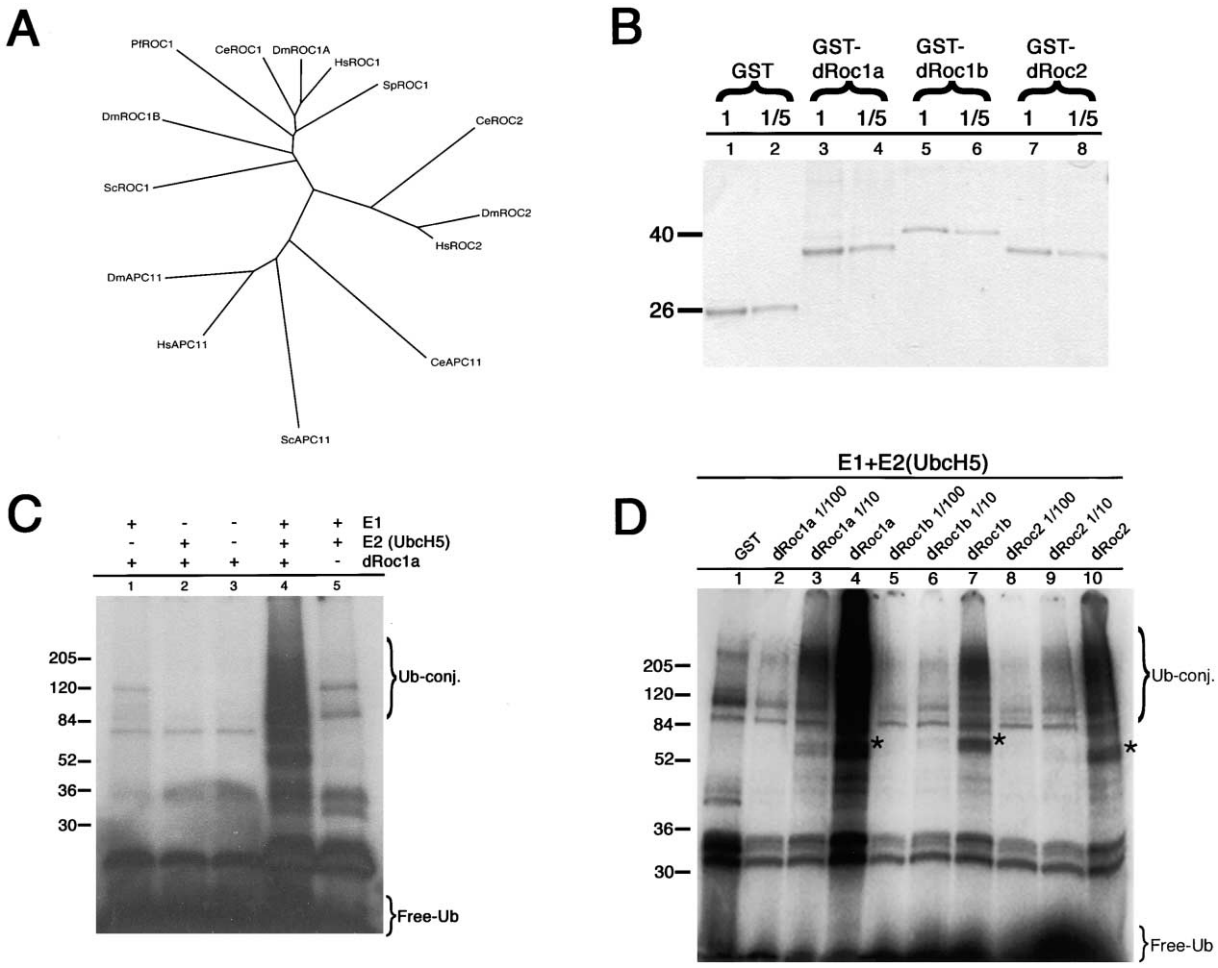

Figure 1. The Family of *Drosophila* RING-H2 Proteins Stimulate Polyubiquitin Formation In Vitro

(A) An unrooted guide tree using Clustal W was generated with the predicted primary amino acid sequence of Roc-like genes from human, nematode, fly, yeast, and trypanosomes. Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Pf, *Plasmodium falciparum*.

(B) Coomassie-stained GST-Roc fusion proteins expressed in bacteria and purified using glutathione agarose beads. Lanes 1 and 2, GST; lanes 3 and 4, GST-Roc1a; lanes 5 and 6, GST-Roc1b; Lanes 7 and 8, GST-Roc2. Odd- and even-numbered lanes represent approximately 50 ng and 10 ng of protein, respectively.

(C) Ubiquitination reaction mixes containing [^{32}P]ubiquitin and the indicated components were subjected to SDS-PAGE and autoradiography.

(D) Dose-dependent stimulation of ubiquitin ligase activity by Roc1a, Roc1b, and Roc2. Ten nanograms of GST (lane 1) or 0.1 ng (lanes 2, 5, and 8), 1 ng (lanes 3, 6, and 9), or 10 ng (lanes 4, 7, and 10) of the indicated RING finger protein were added to a ubiquitin ligase reaction containing 40 ng rabbit E1 and 60 ng E2 (UbcH5). Asterisks denote [^{32}P]GST-Roc proteins.

46% percent identical to Roc1a (78% in the RING-H2 domain). If the *Roc* genes were expressed at mutually exclusive times or in different tissues during development, then each could provide a distinct function for the animal, even though they might perform similar or even identical biochemical functions within the cell. The expression of *Roc1a*, *Roc1b*, and *Roc2* during different stages of development was examined by RT-PCR (Figure 2B). Each of the three *Roc* genes is expressed during all stages of development. *Roc1a* mRNA is the most ubiquitous and is expressed in embryonic, larval, and adult tissues. *Roc1b* and *Roc2* mRNAs were also detected throughout development, but their expression is more variable than *Roc1a*. For instance, *Roc2* mRNA is most abundant in pupae, and *Roc1b* mRNA is barely detectable in adult females but readily detectable in adult males (Figure 2B). These data indicate that each Roc gene has a different developmental profile of expression. However, these profiles substantially overlap. Moreover, *Roc1a* and *Roc1b* are both expressed uniformly throughout wing imaginal discs (data not shown). Consequently, developmental stage-specific gene expression is not a sufficient explanation for the lethality caused by mutation of *Roc1a*.

Roc1a Is Required for Cell Proliferation

Animals homozygous for the *Roc1a* deletion die between first and early second instar larval stages. Both RT-PCR and in situ hybridization analysis of 0–2 hr embryos indicate that *Roc1a* is expressed maternally (data not shown), and this may provide enough function to support early development. Homozygous mutant larvae

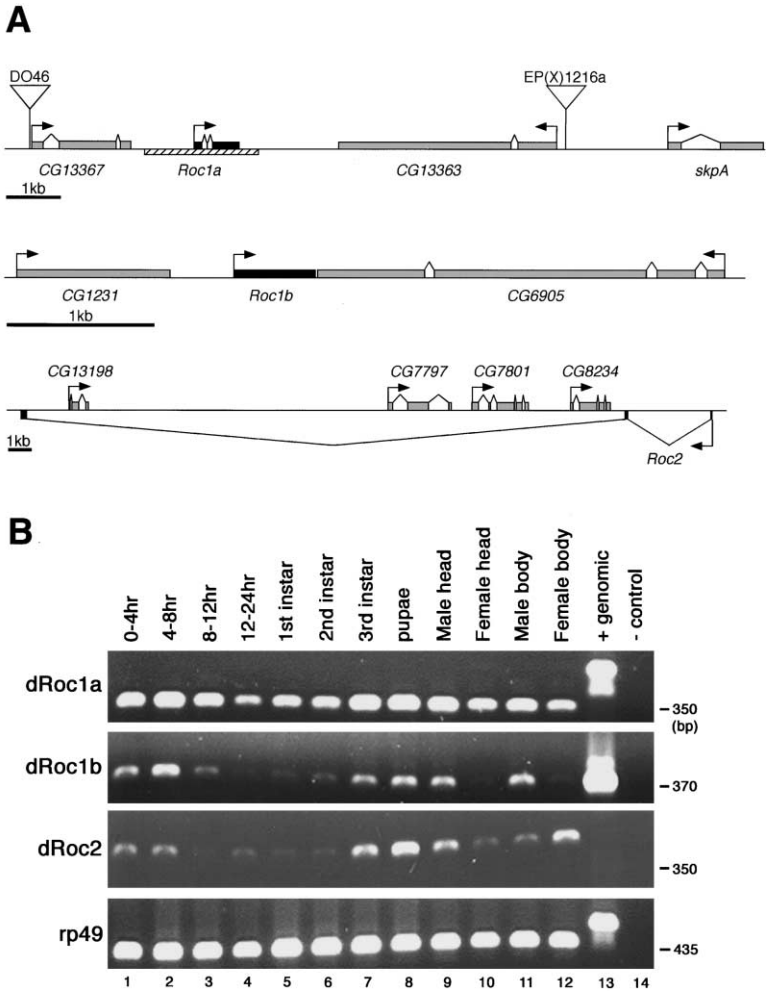

Figure 2. Expression Profile of *Drosophila Roc* Genes during Development

(A) Schematic of each *Drosophila Roc* locus. Non-*Roc* exons, gray boxes; *Roc* exons, black boxes. Arrows indicate the direction of transcription. P element insertions are indicated by a triangle. Deletion of the region between EP(X)1216a and DO46 generated the $Roc1a^{G1}$ allele used throughout. The hatched box indicates the genomic region used to construct the rescuing *Roc1a* transgene.

(B) The accumulation of *Roc1a*, *Roc1b*, *Roc2* and the ubiquitously expressed *rp49* transcript were monitored by RT-PCR of first strand cDNAs derived from different tissues and developmental stages (lanes 1–12). Lane 13, adult genomic; lane 14, no template. Note that the absence of the larger, intron-containing *Roc1a* and *rp49* amplicons in lanes 1–12 indicate that the cDNA samples are free of contaminating genomic DNA.

displayed no apparent defects in cuticle structure, although imaginal disc growth appeared impaired. We were unable to recover eggs lacking maternal Roc1a using techniques to generate clones of *Roc1a* mutant cells in the female germline. One possibility for this result is that *Roc1a* mutant cells are unable to proliferate. To examine this more carefully and to determine whether loss of *Roc1a* causes any specific cellular phenotypes, clones of *Roc1a* mutant cells were generated using hsp70-FLP/FRT-mediated mitotic recombination and analyzed in imaginal tissue. Mutant cells were detected by positive marking with a plasma membrane-localized GFP using the MARCM system (Lee and Luo, 1999). Compared to the large clones generated using a wild-type control chromosome (Figure 3A), *Roc1a* mutant clones were invariably small (Figure 3B). Wild-type clones varied greatly in size but were typically composed of 10–100 cells, with several large clones containing more than 100 cells (Figure 3C). In contrast, the *Roc1a* mutant clones rarely exceeded 6–10 cells in size, and the majority of clones were composed of only one cell (Figure 3C). Small *Roc1a* mutant clones occurred in all regions of wing, leg, haltere, and eye/antennal discs. These data suggest that *Roc1a* is required for imaginal cell proliferation.

Because mutations of yeast *ROC1* cause G1 arrest, we used FACS analysis of *Roc1a* mutant cells to determine whether they accumulate in a particular phase of the cell cycle. Imaginal discs containing wild-type or enlarged (see Experimental Procedures) *Roc1a* mutant cell clones were dissected from late third instar larvae and dissociated with trypsin, and the cells were subjected to FACS analysis to determine DNA content. In control discs containing clones of GFP^+ wild-type cells, both the GFP^+ and GFP^- cell populations were roughly equally split between 2C and 4C DNA content (Figure 3D). A similar distribution of 2C and 4C cells was seen with discs containing GFP^+ *Roc1a* mutant clones (Figure 3D). Since the FACS profile of *Roc1a* mutant cells is indistinguishable from wild-type, we conclude that cells lacking *Roc1a* dot not arrest at a particular phase of the cell cycle, perhaps because of phenotypic pleiotropy with respect to cell cycle targets (see Discussion).

Mutant cells with a growth disadvantage relative to wild-type neighbors are often actively eliminated during imaginal disc development via apoptosis and thus can be absent from or underrepresented in adult structures (Neufeld et al., 1998). The procedure for positively marking mitotic clones provides a simple method for determining whether any *Roc1a* mutant cells can survive to

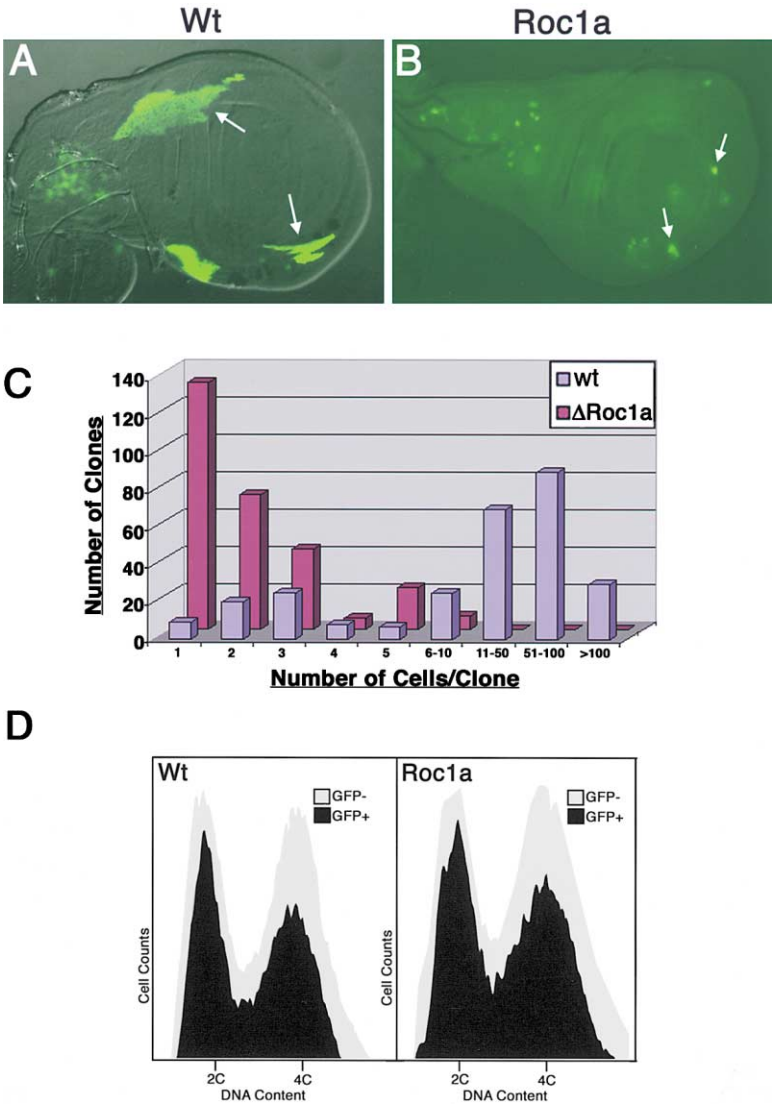

Figure 3. *Roc1a* Is Required for Cell Proliferation

GFP$^+$ wild-type or *Roc1a* mutant mitotic clones were generated as described in Experimental Procedures and analyzed in third instar larval wing imaginal discs.

(A) Clones of GFP$^+$ wild-type cells (e.g., arrows).

(B) Clones of GFP$^+$ *Roc1a*-null mutant cells. Note that the size of the clones (e.g., arrows) is very small relative to that of wild-type.

(C) Quantification of wild-type and *Roc1a* mutant clone size. Wing, leg, and haltere imaginal discs were harvested from third instar larvae carrying positively marked wild-type or *Roc1a* mutant clones. The survey includes a total of 285 wild-type or mutant clones selected randomly from all sections of a given imaginal disc (20 larvae/genotype).

(D) FACS analysis of wild-type and *Roc1a* mutant cells isolated from trypsin dissociated imaginal discs.

adulthood. When larvae harboring *Roc1a* mutant clones were allowed to complete development, GFP$^+$ mutant cells were identified in many adult tissues, including wing, leg, thorax, abdomen, and head (shown for wing in Figure 4). *Roc1a* mutant clones in the adult wing were small in size and concentrated at the anterior margin, while wild-type control clones were large and spread throughout the entire wing blade (Figures 4A and 4B). Induction of mitotic recombination in *Roc1a*/+ animals carrying an *hsp70-Roc1a* cDNA caused a distribution of GFP$^+$ clones across the wing blade that was indistinguishable from that of wild-type (Figures 4C–4E). To test whether the lack of *Roc1a* mutant cells in the wing blade was due to cell elimination via apoptosis during development, we expressed the baculovirus caspase inhibitor P35 in clones of *Roc1a* mutant cells (Hay et al., 1994). This caused the appearance of *Roc1a* mutant clones throughout the entire wing (Figure 4F). However, unlike wild-type or *hsp70-Roc1a* rescued clones, these *Roc1a* mutant clones were invariably small and similar in size to those found in wing imaginal discs. These results indicate that *Roc1a* mutant cells have a proliferation defect and are therefore eliminated from much of the wing via apoptosis. During development the cells along the margin cease proliferating well before those in the rest of the wing blade (Johnston and Edgar, 1998). This may explain the preferential retention of *Roc1a* mutant cells at the wing margin, even when apoptosis is not prevented, since these cells may be subjected to less competition than those near cells that proliferate throughout imaginal development.

Roc1a Mutant Cells Inappropriately Accumulate Full-Length Ci^{155} Protein

The *Drososphila slimb* gene encodes an F box/WD40 protein that can interact with other SCF components in vitro, including Roc1a (Bocca et al., 2001). *slimb* is required for the conversion of phosphorylated Ci^{155} transcriptional activator to a 75 kDa form that acts as a transcriptional repressor in the absence of Hh signaling (Jiang and Struhl, 1998; Methot and Basler, 2001; Price and Kalderon, 1999). During wing development, Ci is

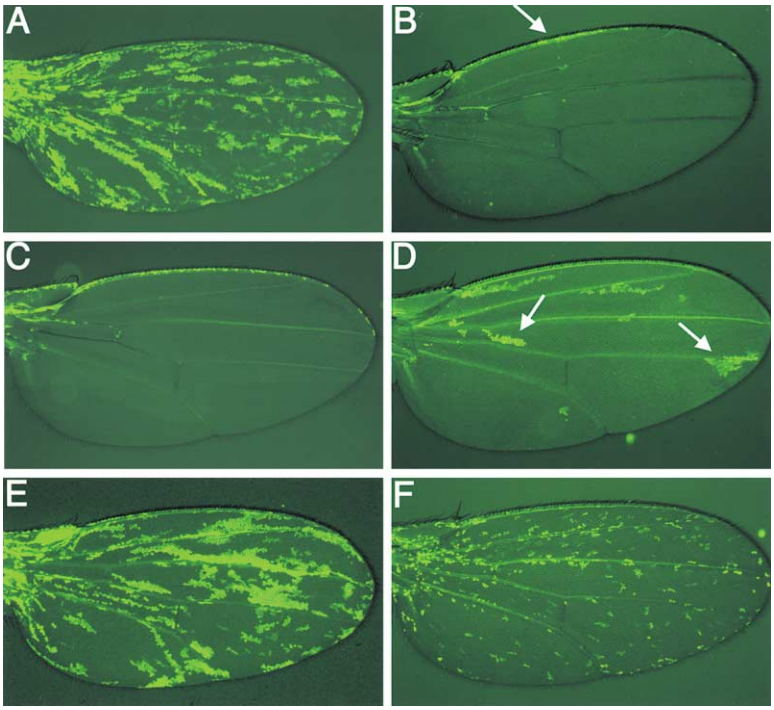

Figure 4. *Roc1a* Mutant Cells Contribute to Adult Tissues

Wild-type and *Roc1a* mutant cell clones were induced during larval stages, the animals were allowed to complete development, and adult wing blades were mounted for fluorescent microscopy.

(A) Wild-type clones induced with a single heat shock treatment at mid second instar are spread throughout the wing blade, margin, and hinge.

(B) Small *Roc1a*-null mutant clones induced with a single heat shock treatment at mid second instar stage appear in the hinge region and anterior margin (arrow).

(C) *Roc1a* clones generated by one heat shock treatment every 8 hr beginning at second instar and ending at late third larval instar stages.

(D) *Roc1a* clones induced as in C but containing one copy of an *hsp70-Roc1a* cDNA transgene. Note the appearance of larger clones located in the middle sectors of the wing blade (e.g., arrows).

(E) *Roc1a* mutant cell clones containing the *hsp70-Roc1a* cDNA transgene were induced at mid second instar stage and subjected to a heat shock once every 8 hr until mid pupal stage.

(F) *Roc1a*-null mutant clones expressing UAS-P35 to block apoptosis were induced with a single heat shock treatment at mid second instar stage.

expressed in all cells of the anterior compartment but not in cells of the posterior compartment (Figure 5A) (Aza-Blanc et al., 1997). The diffusible ligand Hh is expressed in posterior compartment cells and induces the hyper-accumulation of Ci^{155} along the A/P boundary in anterior cells that receive the Hh signal (Ruiz i Altaba, 1999; Strigini and Cohen, 1997; Tabata and Kornberg, 1994). This accumulation results from the inhibition of Ci^{155} proteolysis and can be detected in situ using a monoclonal antibody (2A1) raised against the C terminus of Ci (Figure 5A) (Aza-Blanc et al., 1997; Motzny and Holmgren, 1995; Wang and Holmgren, 1999). *slimb* mutant clones in the anterior compartment display cell-autonomous accumulation of Ci^{155}, even in cells that receive little, if any, Hh signal (Figures 5D–5F) (Jiang and Struhl, 1998). Similarly, clones of *Roc1a* mutant cells anywhere in the anterior compartment have elevated levels of Ci protein relative to those of neighboring wild-type cells (Figures 5G–5I). This was also apparent in clones close to the A/P boundary, suggesting that Ci can hyperaccumulate, even in *Roc1a* mutant cells receiving Hh signal (Figures 5G–5I). In contrast, *Roc1a* mutant cell clones located in the posterior compartment did not accumulate Ci protein (Figure 5H). This suggests that mutation of *Roc1a* does not induce *ci* transcription, because Ci hyperaccumulation occurs only in cells that normally express the *ci* gene (i.e., those in the anterior compartment). We conclude from these data that mutation of *Roc1a* stabilizes Ci protein via a posttranscriptional mechanism.

The typically small size of the *Roc1a* mutant clones made our cytological observations a bit difficult. This was exacerbated because some of the cells in the clones were somewhat irregular in shape, perhaps because they were entering apoptosis. These problems were reduced by analyzing clones expressing P35 to block apoptosis. In these cases it was clear that all cells displaying hyperaccumulation of Ci were GFP^+, indicating that this mutant phenotype is cell autonomous (Figures 5J–5O). Moreover, the hyperaccumulated Ci appears to be cytoplasmic, much as is the endogenous Ci^{155} at the anterior-posterior boundary (Figures 5J–5O). We also examined Ci accumulation in *Roc1a* homozygous mutant animals carrying the *hsp70-Roc1a* transgene and that were rescued to third instar stage by applying a heat pulse every 8 hr during larval development. Wing discs harvested 24 or 48 hr after the last heat shock were stained with 2A1 antibody and displayed hyperaccumulation of Ci in all cells of the anterior compartment but nowhere else (data not shown).

To determine whether it was full-length Ci^{155} protein that was inappropriately accumulating in *Roc1a* mutant cells, clones were analyzed with a different antibody (AbN), which recognizes an epitope in the N-terminal part of Ci, upstream of the processing site (Aza-Blanc et al., 1997). In wild-type discs, this antibody uniformly stains all cells in the anterior compartment that express Ci but does not detect the additional accumulation of Ci^{155} at the A/P boundary (Figures 6A–6C). *Roc1a* mutant cells in enlarged clones located in the anterior compartment but not in the posterior compartment also stained more intensely with the N-terminal anti-Ci antibody than neighboring $Roc1a^+$ cells (Figures 6D–6F). The staining was cytoplasmic and cell autonomous (Figures 6G–6I). This

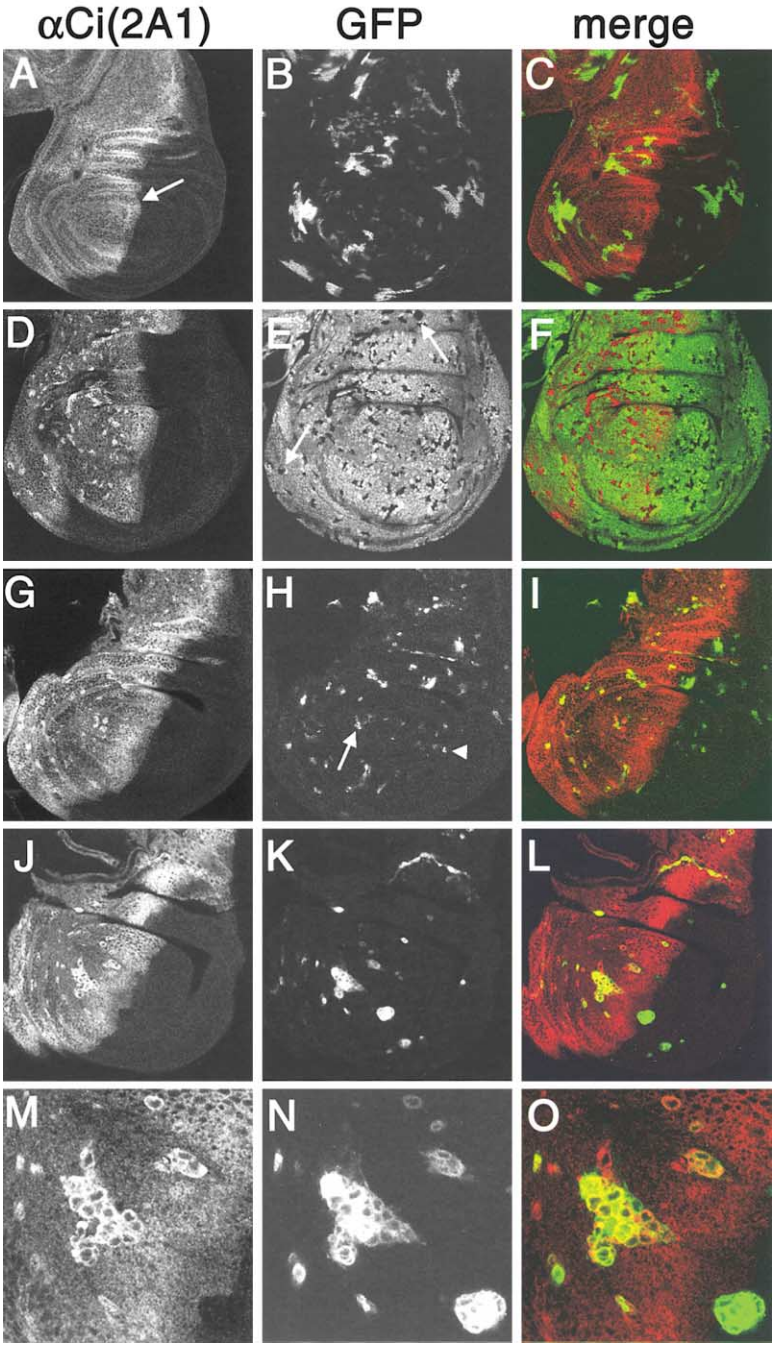

Figure 5. Ectopic Accumulation of Ci Protein in *Roc1a* Mutant Cells

Third instar wing imaginal discs harboring mitotic clones were stained with COOH-terminal-specific Ci antibody 2A1. Discs are oriented anterior to the left and dorsal at the top. Left panels show Ci, middle panels show GFP, and right panels show a merged image (red, Ci; green, GFP).

(A–C) Wild-type clones are randomly distributed throughout the wing disc and show no abnormal accumulation of Ci protein.

(D–F) Clones of *slmb*^{P1493} mutant cells. Unlike all other panels, *slimb* mutant cells were identified by the lack of GFP staining.

(G–I) *Roc1a* mutant cells in the anterior compartment of the wing disc accumulate more Ci protein (arrow) than neighboring phenotypically wild-type cells. Clones in the posterior compartment show no sign of ectopic Ci protein (arrowhead).

(J–L) *Roc1a* mutant clones expressing UAS-P35.

(M–O) Higher magnification view of the pouch clones shown in (J)–(L). The examination of multiple confocal planes in stained tissue preparations confirmed that all cells with elevated Ci are also GFP positive.

result strongly suggests that full-length, unprocessed Ci^{155} hyperaccumulates in *Roc1a* mutant cells that express *ci*. Taken together, these data indicate that *Roc1a*, like *slimb*, is required for proteolytic processing of Ci.

Stabilization of Ci in *Roc1a* Mutant Cells Results in Ectopic Activation of *dpp* Expression

Ci^{155} is a transcription factor necessary for the activation of genes in response to Hh signaling (Hepker et al., 1997; Methot and Basler, 2001). Among this group of genes is *decapentaplegic* (*dpp*), which encodes a signaling molecule of the TGFβ class that plays an important role in limb development. Wild-type *dpp* expression occurs along the A/P boundary in cells that normally accumulate Ci^{155}, and this pattern of expression is reproduced by a *dpp-lacZ* enhancer trap line (Figures 6J–6L) (Blackman et al., 1991). Clones were generated in animals carrying *dpp-lacZ* to test whether the accumulation of Ci^{155} in *Roc1a* mutant cells causes ectopic Hh signaling. While *Roc1a* mutant clones are randomly distributed throughout the disc, only those clones located in the anterior part of the wing disc most distal from the A/P

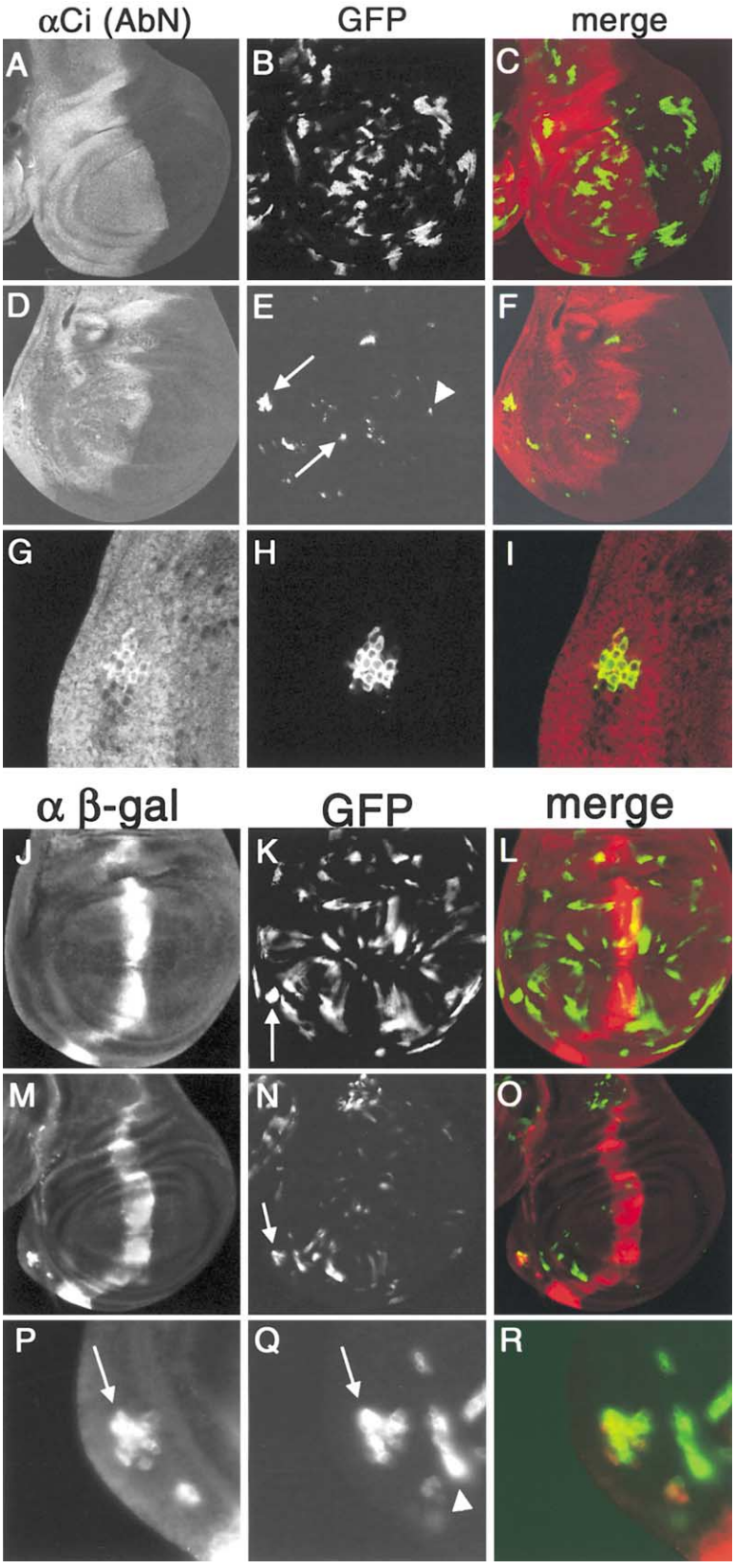

Figure 6. *Roc1a* Mutant Clones Accumulate Full-Length Ci that Activates *dpp* Expression

Wing imaginal discs were prepared and analyzed as in Figure 5. Panels (A), (D), and (G) show Ci protein detected with NH2-terminal-specific antibody AbN, and panels (J), (M), and (P) show β-gal protein (i.e., *dpp* expression). All middle panels show GFP, and all right panels show a merged image (red, Ci or β-gal; green, GFP).

(A–C) Wild-type clones with abnormal accumulation of Ci. Unlike 2A1, staining with AbN is uniform across the anterior compartment.

(D–I) Enlarged *Roc1a*-null mutant clones in the anterior compartment of the wing disc ectopically accumulate Ci (arrows). Clones located in the posterior compartment show no Ci accumulation (arrowhead).

(G–I) Higher magnification view of anterior clone shown in panels (D)–(F) (upper arrow in [E]).

(J–L) Wild-type clones distributed randomly throughout the disc, including the anterior-most region (arrow), display no ectopic *dpp-lacZ* expression.

(M–O) Enlarged *Roc1a*-null mutant clones containing the *dpp-lacZ* reporter transgene. Only clones located in the anterior most section of the wing pouch contain cells that ectopically activate *dpp-lacZ* expression (arrow).

(P–R) Higher magnification view of (M)–(O) showing *dpp-lacZ* expression in the anterior most GFP^+ *Roc1a* mutant clone ([P] and [Q], arrow) but not within a GFP^+ *Roc1a* mutant clone located slightly more posteriorly ([Q], arrowhead).

boundary ectopically activate the *dpp-lacZ* reporter (Figures 6M–6R). We failed to detect β-gal expression in mutant clones located anywhere else in the wing imaginal disc, including those located in the anterior compartment near the A/P boundary that normally hyperaccumulate Ci^{155}. Thus, while ectopic *dpp* expression and activation of Hh signaling can occur by mutation of *Roc1a*, the sensitivity of the mutant cells to inappropriate accumulation of the Ci^{155} transcriptional activator is modulated along the A/P axis, probably by other developmental inputs.

Mutation of *Roc1a*, Unlike *slimb*, Does Not Cause Ectopic Accumulation of Armadillo

The presence of metazoan gene families that encode each of the components of SCF, including the Roc RING-H2 subunit, provides the potential for the formation of many functionally redundant complexes. Alternatively, individual SCF components could assemble into functionally distinct complexes containing only a single type of Roc protein. For instance, if Slimb could assemble only into an SCF complex containing Roc1a, then all proteins that hyperaccumulate in *slimb* mutant cells should also hyperaccumulate in *Roc1a* mutant cells. To test this possibility we generated *Roc1a* mutant clones and stained wing imaginal discs with antibodies that recognize Arm. As previously reported, clones of *slimb* mutant cells ectopically accumulate cytoplasmic/nuclear Arm in a cell-autonomous manner (Figures 7A–7F) (Jiang and Struhl, 1998). In contrast, *Roc1a*-null mutant cells do not accumulate Arm (Figures 7G–7I). Neither clones within the wing pouch near the D/V axis, where Wg signaling occurs (Figures 7J–7L), nor clones within the wing hinge accumulate Arm inappropriately (Figures 7M–7O). Thus, not all targets of Slimb are affected by mutation of *Roc1a*. Consequently, since RING-H2 proteins are an essential component of SCF complexes, these data suggest that the F box protein Slimb can assemble into more than one complex, each containing a different member of the Roc protein family.

Discussion

Drosophila contains multiple genes encoding each of the known components of the multisubunit SCF E3 ubiquitin ligase. Here we show that the *Drosophila Roc1a*, *Roc1b*, and *Roc2* genes encode RING-H2 proteins that stimulate E1- and E2-dependent ubiquitination in vitro and that perform nonredundant roles in vivo. *Roc1a* mutant cells fail to proliferate normally, and, consequently, loss of *Roc1a* function is lethal. The mechanistic basis for this proliferation defect is not known. *S. cerevisiae* Rbx1/Hrt1 mutants arrest in G1 phase because of a failure to proteolytically destroy Sic1p, an inhibitor of the Clb5,6/CDC28p kinases that are required for S phase (Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999). In contrast, *Drosophila* cells that lack *Roc1a* function do not appear to arrest at a specific point in the cell cycle, as determined by FACS analysis of mutant imaginal cells. This suggests that the block to cell proliferation is not a consequence of the failure to degrade a single regulator that plays a key role in one cell cycle transition. There are likely to be many substrates of Roc1a-containing SCF complexes, and phenotypic pleiotropy could mask a role for Roc1a in a specific cell cycle transition. We did not detect inappropriate accumulation of cyclin E, dE2F, or the p21/p27-like cdk inhibitor dacapo in *Roc1a* mutant imaginal cells (data not shown). While such negative results are difficult to interpret, these proteins are all known to be substrates for SCF-mediated ubiquitination and degradation in other systems (Carrano et al., 1999; Ganoth et al., 2001; Koepp et al., 2001; Marti et al., 1999; Nakayama et al., 2000; Spruck et al., 2001; Strohmaier et al., 2001; Sutterluty et al., 1999; Tsvetkov et al., 1999).

Both *Roc1a* and *slimb* mutant cells inappropriately accumulate Ci^{155} protein. Since Roc1a and Slimb proteins are capable of interacting with each other and with other components of *Drosophila* SCF (e.g., Cul1) in vitro (Bocca et al., 2001), the simplest interpretation of this result is that Roc1a and Slimb are part of a common SCF complex that targets Ci^{155} for ubiquitination and subsequent proteolysis. However, there are notable differences between the *Roc1a* and *slimb* mutant phenotypes. First, *Roc1a*-null mutant clones do not cause limb duplications, as do clones of cells homozygous for a hypomorphic allele of *slimb* (Jiang and Struhl, 1998). While differences in the spectrum of proteins affected by loss of each of these genes could very well explain this phenotypic difference, another simple explanation is that *Roc1a*-null mutant cells cannot proliferate. Indeed, null mutant clones of *slimb* are unable to proliferate extensively and do not cause large wing duplications (Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000; Theodosiou et al., 1998). Second, whereas *slimb* mutant cells inappropriately activate *dpp* expression no matter where they arise in the anterior compartment of the wing disc, *Roc1a* mutant cells do not. Only when *Roc1a* mutant cells are found far from the A/P axis is *dpp* expression ectopically activated. One possible explanation for this result is that less Ci^{155} protein accumulates in *Roc1a* mutant cells than in *slimb* mutant cells. Although such differences were not readily apparent in our antibody staining, this method does not provide very good quantitative measurements. The cells most distal from the A/P boundry are known to be more sensitive to misregulation of the Hh signaling pathway than cells closer to the A/P boundary (Capdevila et al., 1994). Moreover, regulatory events in addition to stabilization of Ci^{155}, such as nuclear import, are also required for Ci^{155} to transduce the Hh signal (Hepker et al., 1999; Methot and Basler, 1999, 2001; Wang and Holmgren, 1999, 2000). Consequently, the elevated amount of Ci^{155} protein in *Roc1a* mutant cells may not be sufficient, or the protein may not be appropriately activated, to stimulate *dpp* expression close to the compartment boundary. Why would less Ci^{155} accumulate in *Roc1a* mutant cells relative to *slimb* mutant cells if Ci^{155} were degraded by an SCF complex containing Slimb and Roc1a? There may be some redundancy among the different Roc proteins, such that some Ci^{155} is processed in the absence of *Roc1a* but that none is processed in the absence of *slimb*. Finally, *slimb* and *Roc1a* may affect the accumulation of Ci^{155} by completely independent mechanisms that affect other aspects of Ci regulation (Lefers et al., 2001; Wang et al., 2000).

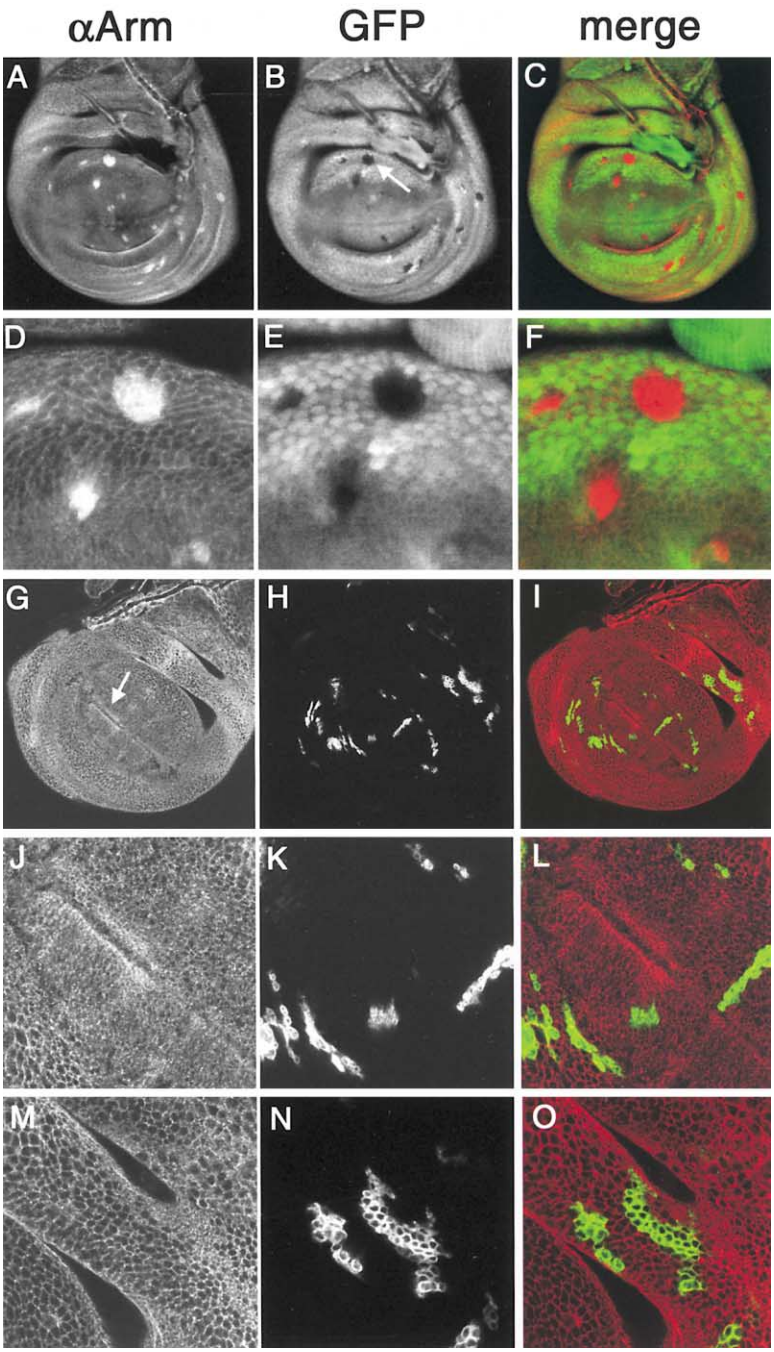

Figure 7. *Roc1a* Mutant Cells Do Not Inappropriately Accumulate Armadillo Protein

(A–F) Clones of *slmb*^{P1493} mutant cells (negatively marked) were generated in third instar wing imaginal discs and stained with anti-Arm monoclonal antibody. Left panels show Arm protein, middle panels show GFP, and right panels show a merged image (red, Arm; green, GFP).

(A–C) Arm protein normally localizes to adherens junctions in epithelial cells that do not receive Wg signal, causing the cortex of all cells to be visualized with anti-Arm antibodies (Peifer and Wieschaus, 1990). *slmb*^{P1493} mutant cells accumulate cytosolic and nuclear Arm protein inappropriately ([B], arrow).

(D–F) Higher magnification view of clones in panels (A)–(C).

(G–I) *Roc1a*-null mutant clones. The normal accumulation of cytosolic Arm along the D/V boundary in response to Wg signaling is shown ([G], arrow). Higher magnification view of *Roc1a* mutant cells in the wing pouch (J–L) and hinge region (M–O).

Perhaps the most revealing difference between the *slimb* and *Roc1a* mutant phenotypes is the differential effect on Arm destruction. While *slimb*-null mutant clones clearly accumulate cytoplasmic/nuclear Arm, we could detect no difference in the abundance of Arm protein between *Roc1a*$^{+}$ and *Roc1a* mutant cells. Thus, mutation of *Roc1a* and *slimb* affect the steady-state levels of different sets of proteins. Similarly, while we did not observe any changes in cyclin E levels in *Roc1a* mutant cells, mutations of *Drosophila ago*, which, like *slimb*, encodes an F box/WD-repeat protein, cause inappropriate accumulation of cyclin E and hyperplasia (Moberg et al., 2001). If *slimb* and *Roc1a* function in vivo only in the context of an SCF complex, then SCF complexes containing both Slimb and Roc1a are not absolutely required for targeting Arm for ubiquitin-mediated proteolysis. The different effect that mutation of *Roc1a* has on Arm and Ci accumulation has important implications for our understanding of how SCF functions in animal cells. Two quite different models could explain the data. In the first model, the Roc genes encode proteins with fully redundant biochemical properties, and an SCF complex containing any one of them is capable of targeting Arm for ubiquitination. In this case, the loss

of Roc1a would be compensated by another Roc protein. Indeed, two groups have reported in Wormbase that RNAi inactivation of the apparent *C. elegans* Roc2 ortholog (R10A10.2) causes no phenotype, suggesting that Roc1 (ZK287.5) is sufficient to provide all SCF function in this organism (Stein et al., 2001). However, biochemical redundancy among the *Drosophila* Roc proteins can only explain the Ci phenotype if Ci is more sensitive to reductions of SCF activity than Arm. In this situation, the absence of *Roc1a* would reduce the total cellular complement of SCF activity (i.e., the sum of all Roc1a-, Roc1b-, and Roc2-containing complexes) below a critical threshold required for Ci, but not Arm, proteolysis. In the second model, the Roc genes encode proteins with independent biochemical properties, such that an SCF complex containing Slimb and Roc1a would be capable of targeting Ci for ubiquitination but incapable of targeting Arm. Since Slimb is required for Arm proteolysis, it would perform this role as part of an SCF complex containing a RING finger protein other than Roc1a (e.g., Roc1b or Roc2). Consequently, this model suggests that the F box protein Slimb does not by itself dictate the selection of a substrate by a particular SCF. Both models provide a possible explanation for the uniqueness of *Roc1a* function during development.

To begin testing these models genetically, we have attempted to rescue the *Roc1a* mutant by UAS-Roc1b overexpression with tubulin-GAL4 and by expression of the *Roc1b* coding sequence from the endogenous *Roc1a* promoter. In neither case did we observe rescue of the *Roc1a* lethality, as occurs with the *Roc1a* transgene itself, suggesting that the Roc1a and Roc1b proteins are not functionally interchangeable. These data are more consistent with the model in which distinct Roc proteins confer certain biochemical properties to individual SCF complexes that affect the overall efficiency of ubiquitination of specific substrates in vivo. The identity of the Roc subunit could lead to differences in regulatory posttranslational modifications of SCF (e.g., cullin neddylation), recruitment of different E2s with different specificities to SCF, or SCF subcellular localization. Any of these properties could potentially affect how efficiently Ci and Arm or any other substrate is ubiquitinated in the context of a specific cellular Roc-SCF^{slimb} complex.

Experimental Procedures

Transgene Constructs and RT-PCR

Genomic DNA containing *Roc1a* (including 980 bp upstream of the ATG) was PCR amplified from Canton S flies and cloned into pCaSpeR-4 to generate the rescuing transgene. Developmental gene expression profiles for *Roc1a*, *Roc1b*, and *Roc2* were performed using the Rapid-Scan Gene Expression Panel (OriGene Technologies). At least one cDNA encoding each Roc gene has been identified by the BDGP (e.g., *Roc1a*: GH12110, *Roc1b*: LD25631, and *Roc2*: RE61847).

Ubiquitin Ligase Assay

GST-Roc fusion proteins were prepared from *E. coli* (Frangioni et al., 1992). [^{32}P]ubiquitin labeling and ligase assays were as described (Ohta et al., 1999).

Roc1a Deletion Alleles

Two X-linked P element insertions in line EP(X)1216 were separated by meiotic recombination, and the element near *Roc1a* was renamed EP(X)1216a (located at bp 31,812 of cosmid 115C2; GenBank AL031581). Homozygous viable EP(X)1216a females were crossed to Sb, Δ2,3/TM6 males to induce P element transposition. Male progeny were crossed to $C(1)DXy^1w^1f^1/Dp(1;Y)y^2$ sc females, and individual w^- males or w^+ males with a change of eye color relative to EP(X)1216a were isolated. The $Dp(1;Y)y^2$ sc Y chromosome carries a duplication of the *Roc1a* region from the X, allowing the recovery of lethal transposition or excision events. After brooding, both w^- and w^+ males were screened by PCR using several primers that hybridize to the *Roc1a* region or the P element inverted repeat. From the w^- excisions, deletions of CG13363 (Figure 2A) that do not extend into *Roc1a* were identified. These deletions are completely viable and fertile as homozygotes, indicating that CG13363 is nonessential. From the w^+ events, one chromosome was recovered that contained both EP(X)1216a and an additional P insertion (DO46 located at bp 22,454 of cosmid 115C2) near the start of transcription of CG13367 (Figure 2A). The DO46 insertion event caused local chromosome aberrations that disrupt CG13367. The sequence of this allele has been deposited in Genbank (AY082967). Flies homozygous for this double insertion chromosome were completely viable and fertile, indicating that CG13367 is also nonessential. The intervening sequence between closely spaced P elements is deleted with high frequency in the presence of transposase (Cooley et al., 1990). Lethal *Roc1a* deletion mutations were isolated by subjecting the DO46 chromosome to the same transposition scheme outlined above. Of 100 w^-, X chromosome lethal lines recovered from this scheme, 45 were completely rescued by a *Roc1a* transgene. These same lines complemented a lethal deletion allele of *skpA* (kindly provided by Terence Murphy). All phenotypic analyses were carried out using $Roc1a^{G1}$, which deletes all sequence between the insertion points of DO46 and EP(X)1216a (Figure 2A).

Mitotic Clonal Analysis

Roc1a mutant mitotic clones were generated using the MARCM method (Lee and Luo, 1999), and *slimb* mutant clones were negatively marked using GFP. Clones were induced with hsp70-FLP by placing culture vials containing second instar larvae in a 37°C water bath for 1 hr. MARCM animals carrying a UAS-P35 transgene were used to block apoptosis in marked clones. To generate "enlarged" *Roc1a* mutant clones, MARCM animals containing a third-chromosome *hsp70-Roc1a* transgene were subjected to single heat shocks at second and third instar larval stages, and discs were harvested 24 hr later. In these animals heat shock will simultaneously induce mutant clone formation and provide wild-type *Roc1a* expression to support some cell proliferation. These enlarged *Roc1a* clones hyperaccumulate Ci, indicating that any heat shock-induced Roc1a protein declines below a functional threshold within 24 hr (Figure 6). For flow cytometry, these animals were heat-shocked three times per day for 2 days from mid second until early early instar. Wing, leg, and haltere imaginal disc were dissected 24 hr later, dissociated with trypsin in the presence of Hoechst 33342, and then immediately analyzed using a MoFlo high-speed molecular flow cytometer (Neufeld et al., 1998).

Immunostaining of Imaginal Discs

Dissected imaginal tissues were fixed in 4% formaldehyde for 45 min. Primary antibodies were rabbit anti-Ci (AbN, 1:5000; gift of Tom Kornberg) or rat anti-Ci (2A1, 1:2; gift of Bob Holmgren), mouse anti-Arm (N27A1, 1:50; gift of Mark Peifer), and rabbit anti-β-gal (1:500; Upstate Biotechnology). Fluorescent secondary antibodies were goat anti-mouse Cy3 (1:1000), goat anti-rat Cy3 (1:1000), and goat anti-rabbit rhodamine (1:1000) (Jackson Research Laboratories). Discs were mounted in Fluoromount-G and analyzed with a Zeiss 410 confocal microscope.

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References

Aso, T., Yamazaki, K., Aigaki, T., and Kitajima, S. (2000). Drosophila von Hippel-Lindau tumor suppressor complex possesses E3 ubiquitin ligase activity. Biochem. Biophys. Res. Commun. *276*, 355–361.

Aza-Blanc, P., Ramirez-Weber, F.A., Laget, M.P., Schwartz, C., and Kornberg, T.B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell *89*, 1043–1053.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263–274.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., and Gelbart, W.M. (1991). An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. Development *111*, 657–666.

Blondel, M., Galan, J.M., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies, R.J., and Peter, M. (2000a). Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. EMBO J. *19*, 6085–6097.

Blondel, M., Galan, J.M., and Peter, M. (2000b). Isolation and characterization of HRT1 using a genetic screen for mutants unable to degrade Gic2p in Saccharomyces cerevisiae. Genetics *155*, 1033–1044.

Bocca, S.N., Muzzopappa, M., Silberstein, S., and Wappner, P. (2001). Occurrence of a putative SCF ubiquitin ligase complex in Drosophila. Biochem. Biophys. Res. Commun. *286*, 357–364.

Borden, K.L., Lally, J.M., Martin, S.R., O'Reilly, N.J., Solomon, E., and Freemont, P.S. (1996). In vivo and in vitro characterization of the B1 and B2 zinc-binding domains from the acute promyelocytic leukemia protooncoprotein PML. Proc. Natl. Acad. Sci. USA *93*, 1601–1606.

Capdevila, J., Estrada, M.P., Sanchez-Herrero, E., and Guerrero, I. (1994). The Drosophila segment polarity gene patched interacts with decapentaplegic in wing development. EMBO J. *13*, 71–82.

Carrano, A.C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. *1*, 193–199.

Chen, C.H., von Kessler, D.P., Park, W., Wang, B., Ma, Y., and Beachy, P.A. (1999). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. Cell *98*, 305–316.

Cooley, L., Thompson, D., and Spradling, A.C. (1990). Constructing deletions with defined endpoints in Drosophila. Proc. Natl. Acad. Sci. USA *87*, 3170–3173.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. *15*, 435–467.

Deshaies, R.J., Chau, V., and Kirschner, M. (1995). Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. EMBO J. *14*, 303–312.

Drury, L.S., Perkins, G., and Diffley, J.F. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. EMBO J. *16*, 5966–5976.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221–230.

Frangioni, J.V., Beahm, P.H., Shifrin, V., Jost, C.A., and Neel, B.G. (1992). The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. Cell *68*, 545–560.

Freemont, P.S. (2000). RING for destruction? Curr. Biol. *10*, R84–R87.

Fukuchi, M., Imamura, T., Chiba, T., Ebisawa, T., Kawabata, M., Tanaka, K., and Miyazono, K. (2001). Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol. Biol. Cell *12*, 1431–1443.

Furukawa, M., Ohta, T., and Yue Xiong, Y. (2002). Activation of UBC5 ubiquitin conjugating (E2) enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all cullins. J. Biol. Chem., in press.

Furukawa, M., Zhang, Y., McCarville, J., Ohta, T., and Xiong, Y. (2000). The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. Mol. Cell. Biol. *20*, 8185–8197.

Ganoth, D., Bornstein, G., Ko, T.K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001). The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nat. Cell Biol. *3*, 321–324.

Gmachl, M., Gieffers, C., Podtelejnikov, A.V., Mann, M., and Peters, J.M. (2000). The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *97*, 8973–8978.

Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. Development *120*, 2121–2129.

Hepker, J., Blackman, R.K., and Holmgren, R. (1999). Cubitus interruptus is necessary but not sufficient for direct activation of a wing-specific decapentaplegic enhancer. Development *126*, 3669–3677.

Hepker, J., Wang, Q.T., Motzny, C.K., Holmgren, R., and Orenic, T.V. (1997). Drosophila cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. Development *124*, 549–558.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. *10*, 429–439.

Jiang, J., and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F- box/WD40-repeat protein Slimb. Nature *391*, 493–496.

Johnston, L.A., and Edgar, B.A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. Nature *394*, 82–84.

Kaiser, P., Flick, K., Wittenberg, C., and Reed, S.I. (2000). Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. Cell *102*, 303–314.

Kaiser, P., Sia, R.A., Bardes, E.G., Lew, D.J., and Reed, S.I. (1998). Cdc34 and the F-box protein Met30 are required for degradation of the Cdk-inhibitory kinase Swe1. Genes Dev. *12*, 2587–2597.

Kamura, T., Conrad, M.N., Yan, Q., Conaway, R.C., and Conaway, J.W. (1999a). The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. Genes Dev. *13*, 2928–2933.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C., et al. (1999b). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657–661.

Kipreos, E.T., and Pagano, M. (2000). The F-box protein family. Genome Biol. *1*, REVIEWS3002.

Koepp, D.M., Schaefer, L.K., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. Science *294*, 173–177.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.

Lefers, M.A., Wang, Q.T., and Holmgren, R.A. (2001). Genetic dissection of the Drosophila Cubitus interruptus signaling complex. Dev. Biol. *236*, 411–420.

Leverson, J.D., Joazeiro, C.A., Page, A.M., Huang, H., Hieter, P., and Hunter, T. (2000). The APC11 RING-H2 finger mediates E2-dependent ubiquitination. Mol. Biol. Cell *11*, 2315–2325.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S., and Weissman, A.M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc. Natl. Acad. Sci. USA *96*, 11364–11369.

Lovering, R., Hanson, I.M., Borden, K.L., Martin, S., O'Reilly, N.J., Evan, G.I., Rahman, D., Pappin, D.J., Trowsdale, J., and Freemont, P.S. (1993). Identification and preliminary characterization of a protein motif related to the zinc finger. Proc. Natl. Acad. Sci. USA *90*, 2112–2116.

Marti, A., Wirbelauer, C., Scheffner, M., and Krek, W. (1999). Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat. Cell Biol. *1*, 14–19.

Methot, N., and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell *96*, 819–831.

Methot, N., and Basler, K. (2001). An absolute requirement for Cubitus interruptus in Hedgehog signaling. Development *128*, 733–742.

Michel, J.J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth Differ. *9*, 435–449.

Miletich, I., and Limbourg-Bouchon, B. (2000). Drosophila null slimb clones transiently deregulate Hedgehog- independent transcription of wingless in all limb discs, and induce decapentaplegic transcription linked to imaginal disc regeneration. Mech. Dev. *93*, 15–26.

Moberg, K.H., Bell, D.W., Wahrer, D.C., Haber, D.A., and Hariharan, I.K. (2001). Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines. Nature *413*, 311–316.

Motzny, C.K., and Holmgren, R. (1995). The Drosophila cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. Mech. Dev. *52*, 137–150.

Nakayama, K., Nagahama, H., Minamishima, Y.A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., et al. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J. *19*, 2069–2081.

Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. (1998). Coordination of growth and cell division in the Drosophila wing. Cell *93*, 1183–1193.

Ohlmeyer, J.T., and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. Nature *396*, 749–753.

Ohta, T., Michel, J.J., Schottelius, A.J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol. Cell *3*, 535–541.

Patton, E.E., Peyraud, C., Rouillon, A., Surdin-Kerjan, Y., Tyers, M., and Thomas, D. (2000). SCF(Met30)-mediated control of the transcriptional activator Met4 is required for the G(1)-S transition. EMBO J. *19*, 1613–1624.

Patton, E.E., Willems, A.R., Sa, D., Kuras, L., Thomas, D., Craig, K.L., and Tyers, M. (1998). Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev. *12*, 692–705.

Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science *287*, 1606–1609.

Peifer, M., and Wieschaus, E. (1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. Cell *63*, 1167–1176.

Price, M.A., and Kalderon, D. (1999). Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A. Development *126*, 4331–4339.

Ruiz i Altaba, A. (1999). Gli proteins and Hedgehog signaling: development and cancer. Trends Genet. *15*, 418–25.

Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M., and Pavletich, N.P. (2000). Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. Nature *408*, 381–386.

Seol, J.H., Feldman, R.M., Zachariae, W., Shevchenko, A., Correll, C.C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., et al. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. *13*, 1614–1626.

Sia, R.A.L., Bardes, E.S.G., and Lew, D.J. (1998). Control of Swe1p degradation by the morphogenesis checkpoint. EMBO J. *17*, 6678–6688.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209–219.

Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N., Conaway, R.C., Conaway, J.W., Elledge, S.J., and Harper, J.W. (1999). Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. Science *284*, 662–665.

Spencer, E., Jiang, J., and Chen, Z.J. (1999). Signal-induced ubiquitination of IkappaBalpha by the F-box protein Slimb/beta-TrCP. Genes Dev. *13*, 284–294.

Spruck, C., Strohmaier, H., Watson, M., Smith, A.P., Ryan, A., Krek, T.W., and Reed, S.I. (2001). A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol. Cell *7*, 639–650.

Stein, L., Sternberg, P., Durbin, R., Thierry-Mieg, J., and Spieth, J. (2001). WormBase: network access to the genome and biology of Caenorhabditis elegans. Nucleic Acids Res. *29*, 82–86.

Strigini, M., and Cohen, S.M. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the Drosophila wing. Development *124*, 4697–4705.

Strohmaier, H., Spruck, C.H., Kaiser, P., Won, K.A., Sangfelt, O., and Reed, S.I. (2001). Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature *413*, 316–322.

Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999). p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. *1*, 207–214.

Tabata, T., and Kornberg, T.B. (1994). Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. Cell *76*, 89–102.

Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z.Q. (1999). Recruitment of a ROC1–CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I kappa B alpha. Mol. Cell *3*, 527–533.

Theodosiou, N.A., Zhang, S., Wang, W.Y., and Xu, T. (1998). slimb coordinates wg and dpp expression in the dorsal-ventral and anterior-posterior axes during limb development. Development *125*, 3411–3416.

Tsvetkov, L.M., Yeh, K.H., Lee, S.J., Sun, H., and Zhang, H. (1999). p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr. Biol. *9*, 661–664.

Verma, R., Feldman, R.M., and Deshaies, R.J. (1997). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Mol. Biol. Cell *8*, 1427–1437.

Wang, G., Amanai, K., Wang, B., and Jiang, J. (2000). Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of cubitus interruptus. Genes Dev. *14*, 2893–2905.

Wang, Q.T., and Holmgren, R.A. (1999). The subcellular localization and activity of Drosophila cubitus interruptus are regulated at multiple levels. Development *126*, 5097–5106.

Wang, Q.T., and Holmgren, R.A. (2000). Nuclear import of cubitus interruptus is regulated by hedgehog via a mechanism distinct from Ci stabilization and Ci activation. Development *127*, 3131–3139.

Wojcik, E.J., Glover, D.M., and Hays, T.S. (2000). The SCF ubiquitin

ligase protein slimb regulates centrosome duplication in Drosophila. Curr. Biol. *10*, 1131–1134.

Yu, H., Peters, J.M., King, R.W., Page, A.M., Hieter, P., and Kirschner, M.W. (1998). Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science *279*, 1219–1222.

Zachariae, W., Shevchenko, A., Andrews, P.D., Ciosk, R., Galova, M., Stark, M.J., Mann, M., and Nasmyth, K. (1998). Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. Science *279*, 1216–1219.

Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533–539.

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