following reaction: 2 ascorbate + H_2O_2 → 2 monodehydroascorbate + 2 H_2O.

9. The transgenic plants were grown and exposed to EL as described (*8*) with the following modifications: The photoperiod was 8 hours at 200 ± 45 μmol of photons m^{-2} s^{-1}, night temperature was 16° ± 1°C, EL exposure was 2700 ± 300 μmol of photons m^{-2} s^{-1}. Six- to eight-week-old plants were used in these experiments. EL was generated with a HMV 1200 lamp (Pani, Vienna). Chlorophyll a fluorescence parameters were determined with a portable modulating fluorimeter FMS1 and the manufacturer's software (Hansatech, Kings Lynn, UK).
10. The *APX2* promoter was cloned by polymerase chain reaction (PCR) amplification from the bacteriophage λ clone of *APX2* [M. Santos *et al.*, *Planta* **198**, 64 (1996)] with the following primers: 5′-CCAAGAAGAGGAAAACCGGTACCAAGGTAATCTCAATACTTG-3′ (top strand; coordinates are −1438 to −1397, counted back from the first base of the *APX2* start codon) and 5′-CCGGGTAACTCTTCTTCACCATGGTTTTCAAATTCGCTTCCTTC-3′ (bottom strand, coordinates are +22 to −22). The underlined residues G and GG were changes introduced from the wild-type sequence (C and AA, respectively). These sites introduced a Kpn I and a Nco I site at the 5′ and 3′ ends of the promoter fragment, respectively. The PCR fragment was cut with these enzymes and cloned into pJIT163 [F. Guerineau, A. Lucy, P. Mullineaux, *Plant Mol. Biol.* **18**, 815 (1990)], substituting the cauliflower mosaic virus 35S promoter with the *APX2* promoter. The promoter region was completely sequenced and was confirmed to be a faithful copy of the gene sequence, apart from the introduced changes. The modified firefly luciferase coding sequence cassette (LUC+, Promega, Madison, WI) was inserted as a Nco I–Eco RI fragment, thus creating an *APX2-LUC-CaMV* polyA chimeric gene fusion. This chimeric gene fusion was inserted as a Kpn I–Bgl II fragment into the Kpn I–Bam HI sites of the binary Ti plasmid pBIN19 [M. Bevan, *Nucleic Acids Res.* **12**, 8711 (1984)]. *Arabidopsis thaliana* ecotype Columbia was transformed with an *Agrobacterium*-based procedure [M. Valvekens, M. Van Montagu, M. van Lusebettens, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536 (1988)]. All of the data in this report were derived from T_4 generation homozygous progeny for one transgenic line. Thus, we have been careful to cross-check all luciferase data by Northern blotting or by RNA slot blotting to examine the response of the native *APX2* gene. RNA isolations, Northern, and slot blot hybridizations were made as described (*8*). We have also performed key experiments on the wild-type plant, which confirm the observed phenomenon.
11. Luciferase activity was imaged with a Berthold luminograph LB 980 charge-coupled device (CCD) camera (Berthold Instruments, Milton Keynes, UK) and processed with the associated software according to the manufacturer's instructions, using an aperture setting of 1.8. Rosettes or detached leaves were misted with 1 mM D-(-)-luciferin (Sigma) until they were uniformly wetted, and then they were incubated for 10 min under LL conditions before imaging. Luciferase activity was also assayed in vitro with a luciferase assay kit (Promega) according to the manufacturer's instructions and measured on a luminometer, model 1250 (BioOrbit, Helsinki). Luciferase activity was expressed as relative light units (RLUs) per gram of fresh weight.
12. Detached leaves were vacuum infiltrated for 3 min in the presence of the various compounds or enzymes dissolved in H_2O or appropriate buffers, respectively. The leaves were then floated on a solution of the same compound or enzyme for up to 2 hours in petri dishes, with the cut end of the petiole submerged below the surface of the liquid, and incubated in LL conditions. After this period, the leaves were exposed to EL. In pilot experiments, the following treatments were done, and the minimum concentration that gave the maximum effect for test parameters was determined: (i) H_2O_2 (0.5 to 100 mM). Ten millimolar H_2O_2-treated leaves gave the highest luciferase activity under LL. (ii) Catalase (bovine) (Sigma) (10 to 200 units per milliliter for 2 hours). Maximum inhibition of EL-induced luciferase activity was achieved with 200 units per milliliter. (iii) Superoxide dismutase (bovine) (Sigma) (10 to 200 units per milliliter). No effect was observed at the highest concentration after 40 min in EL. (iv) DCMU and DBMIB (1 to 20 μM). After 1 hour of incubation in LL, 10 μM DCMU-treated leaves achieved a target q_p value of ≤0.2, and 12 μM DBMIB-treated leaves achieved the highest induction of *APX2-LUC* in LL and a 30% reduction of q_p. Two millimolar stock solutions of DCMU in ethanol and DBMIB in dimethyl sulfoxide were used. In all cases, the q_p value was calculated with measurements made at 300 μmol of photons m^{-2} s^{-1} of actinic light. For the F_v/F_m parameter, leaves were adapted to the dark for a minimum of 30 min before taking the measurement. In combined DCMU and H_2O_2 treatments, leaves were first treated with DCMU and then with H_2O_2. H_2O_2 amounts were determined as described before (*8*).
13. For the construction of *APX1-LUC* transgenic plants, the *APX1* promoter was recovered from pGUS1 (a gift from D. Inzé, University of Gent, Gent, Belgium). pGUS1 harbors a 1.55-kb portion of the *Arabidopsis APX1* immediately upstream of the translation initiation codon [G. H. Kubo, H. Saji, K. Tanaka, N. Kondo, *FEBS Lett.* **315**, 313 (1992)]. This portion was recovered as a Hind III–Nco I fragment and inserted into the same sites of pNonedescript [A. Edwards *et al.*, *Plant Physiol.* **112**, 89 (1996)]. This insert could then be recovered as a Kpn I–Nco I fragment and was inserted into the corresponding sites of *APX2-LUC* (*10*), thus replacing the *APX2* promoter with the *APX1* promoter. The rest of the manipulation steps and transformation were as in (*10*). More than 10 independent *APX1-LUC* transgenic lines were recovered, and all displayed high luciferase activity in LL.
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Rbx1, a Component of the VHL Tumor Suppressor Complex and SCF Ubiquitin Ligase

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The von Hippel–Lindau (VHL) tumor suppressor gene is mutated in most human kidney cancers. The VHL protein is part of a complex that includes Elongin B, Elongin C, and Cullin-2, proteins associated with transcriptional elongation and ubiquitination. Here it is shown that the endogenous VHL complex in rat liver also includes Rbx1, an evolutionarily conserved protein that contains a RING-H2 fingerlike motif and that interacts with Cullins. The yeast homolog of Rbx1 is a subunit and potent activator of the Cdc53-containing SCF^{Cdc4} ubiquitin ligase required for ubiquitination of the cyclin-dependent kinase inhibitor Sic1 and for the G_1 to S cell cycle transition. These findings provide a further link between VHL and the cellular ubiquitination machinery.

The VHL tumor suppressor gene on chromosome 3p25.5 is mutated in most sporadic clear cell renal carcinomas and in VHL disease, an autosomal dominant familial cancer syndrome that predisposes affected individuals to a variety of tumors (*1*). The VHL protein is expressed in most tissues and cell types and appears to perform multiple functions, including repression of hypoxia-inducible genes (*2*), regulation of p27 protein stability (*3*), and regulation of fibronectin matrix assembly (*4*). VHL is found in a multiprotein complex with Elongin B, which is ubiquitin-like, and Elongin C and CUL2, which share sequence similarity with the Skp1 and Cdc53 components of the SCF ubiquitin ligase (*5*, *6*). The Elongin BC complex interacts with a short BC-box motif in VHL and bridges its interaction with CUL2 (*6*). A large fraction of VHL mutations alter the BC-box and disrupt the VHL complex (*1*, *4*, *6*).

To investigate VHL function, we purified the endogenous VHL complex from rat liver (Fig. 1) (*7*). Greater than 90% of the detectable VHL protein copurified with CUL2, Elongins B and C, and a polypeptide of ~16 kD (Fig. 1, B and C). The identities of the VHL, CUL2, and Elongin B and C polypeptides were confirmed by immunoblotting, peptide sequencing, or both. Ion trap mass

spectrometry (*8*) of the ~16-kD protein revealed that it was a previously undescribed RING-H2 fingerlike protein, which we designate RING-box protein Rbx1 (Fig. 1D). Rbx1 is highly homologous to *Drosophila melanogaster* open reading frame (ORF) 115C2.11, *Caenorhabditis elegans* ORF ZK287.5, and *Saccharomyces cerevisiae* ORF YOL133w. In addition, Rbx1 is similar in sequence to *S. cerevisiae* anaphase-promoting complex subunit APC11 (*9*).

We next studied Rbx1 interactions with VHL, CUL2, and the Elongin BC complex. Sf21 insect cells were coinfected with various combinations of baculoviruses encoding MYC-Rbx1, FLAG-VHL, hemagglutinin A (HA)–CUL2, HPC4–Elongin B, and herpes simplex virus (HSV)–Elongin C, and complexes were immunoprecipitated from infected cell lysates with antibodies to the epitope (*10*, *11*). Rbx1 assembled into a complex with VHL, CUL2, and Elongins B and C when all five proteins were coexpressed (Fig. 2A, lanes 5 and 9). In addition, Rbx1 assembled with VHL and Elongins B and C independent of coexpressed CUL2 (lanes 6 and 10) and with CUL2 and Elongins B and C independent of coexpressed VHL (lane 11). Suggesting that Rbx1 interacts independently with VHL, CUL2, and the Elongin BC complex, Rbx1-VHL, Rbx1-CUL2, and Rbx1–Elongin BC complexes could be isolated from lysates of cells not expressing other VHL complex components (Fig. 2B). Consistent with these results, complexes containing Rbx1, Elongin BC, and VHL or Rbx1 and Elongin BC could be reconstituted in vitro with bacterially expressed proteins (Fig. 2C) (*12*).

CUL2 is a member of the Cullin protein family that includes CUL1 and its *S. cerevisiae* homolog Cdc53 (*13*). CUL1/Cdc53 proteins are components of SCF (Skp1–Cdc53–F-box protein) complexes, the E3 ubiquitin ligases that target diverse proteins for ubiquitin-mediated proteolysis (*14–18*). In SCF complexes, CUL1 is linked to one of a number of F-box proteins through an adapter protein, Skp1. F-box proteins interact with ubiquitination substrates through COOH-terminal protein-protein interaction domains and with Skp1 through the F-box motif (*14*, *18*). The finding that Rbx1 interacts with CUL2 led us to test whether it might also interact with CUL1. As shown in Fig. 2D, Rbx1 bound to CUL1 in insect cells, and this interaction was independent of Skp1. Rbx1 did not associate with Skp1 directly.

SCF complexes are best understood in *S. cerevisiae*, where they have been implicated in multiple phosphorylation-dependent proteolysis pathways, including destruction of the cyclin-dependent kinase inhibitor Sic1 by SCF^{Cdc4}, a process required for the G_1 to S transition (*14–16*, *18*, *19*). We therefore examined whether Rbx1 is involved in SCF function in *S. cerevisiae*. An *S. cerevisiae* strain lacking the *RBX1* gene was constructed by replacing the complete coding sequence (ORF YOL133w) with the *HIS3* gene (*20*, *21*). Sporulation and tetrad dissection showed 2:0 segregation for viability, indicating that *RBX1* is an essential gene (*21*). Inviable spores produced microcolonies of 10 to 20 cells, many of which were abnormally elongated or contained multiple, abnormally shaped buds. *Saccharomyces cerevisiae* strains containing mutations in genes encoding the SCF components Cdc53, Skp1, Cdc4, and Cdc34 exhibit a similar morphology (*14*).

The viability defect of the *rbx1* deletion strain was rescued by expression of either MYC-tagged mammalian Rbx1 (mRbx1) or a mutant mRbx1 (M4), in which putative RING finger cysteines at positions 53 and 56 were replaced with serines, but not by expression of a mutant mRbx1 (M3), in which putative ring finger cysteines 42 and 45 were replaced by serines. When expressed in either the *rbx1* deletion strain or in a wild-type background, MYC-tagged mRbx1 interacted with endogenous Cdc53 (Fig. 3A). Interaction of wild-type and mutant mRbx1 proteins with Cdc53 correlated with their abilities to rescue the deletion-phenotype: Substantially more Cdc53 was

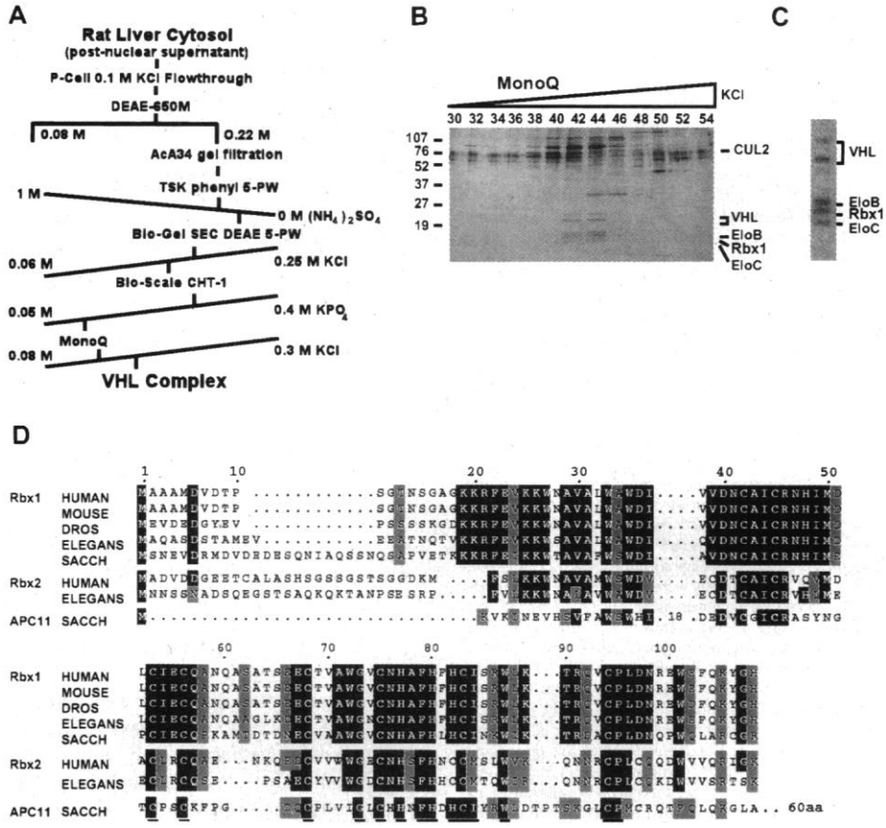

Fig. 1. Copurification of the VHL complex with Rbx1. (**A**) Purification of the VHL complex. P-cell, phosphocellulose P11. (**B**) Cochromatography of Rbx1 with the VHL complex. Samples of column fractions from the MonoQ column were subjected to 12% SDS-PAGE, and proteins were detected by silver staining. VHL, von Hippel–Lindau protein; CUL2, CUL2 protein; EloB, Elongin B; EloC, Elongin C. (**C**) SDS-polyacrylamide (5 to 20%) gel of sample used for peptide sequencing. (**D**) Alignment of predicted Rbx1 protein sequences from human, mouse, *D. melanogaster* (DROS), *C. elegans* (ELEGANS), and *S. cerevisiae* (SACCH) with Rbx2 from human and *C. elegans* and APC11 from *S. cerevisiae*. The alignment was generated with the MACAW program (*30*). Black shading indicates sequence identity, gray shading sequence similarity. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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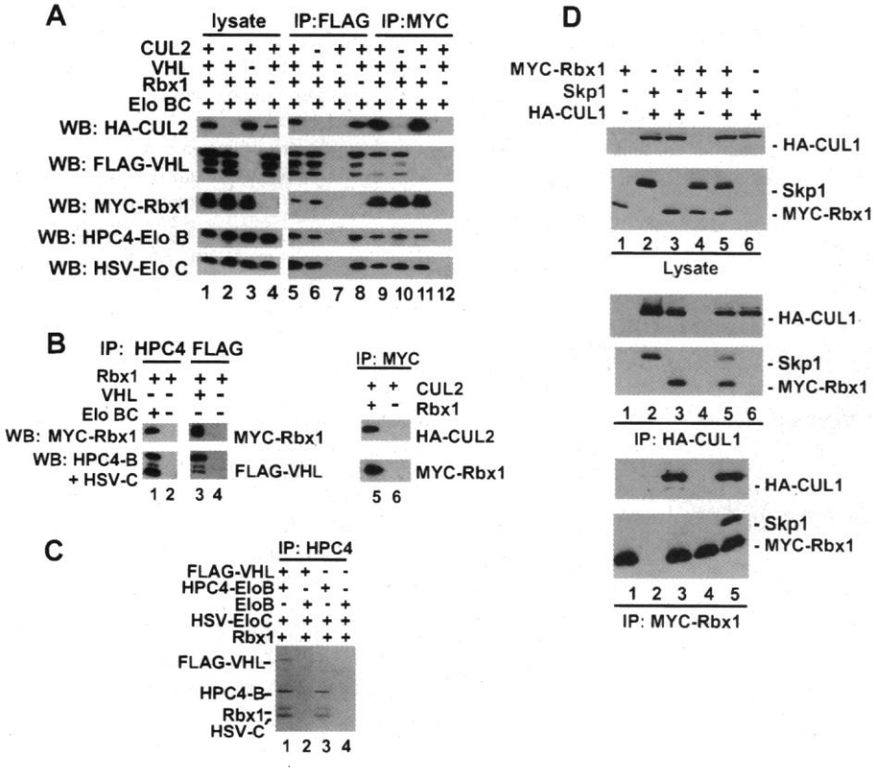

Fig. 2. Reconstitution of Rbx1-containing complexes. (**A**) Rbx1 forms complexes with VHL and CUL2 in the presence of Elongin BC. Lysates from Sf21 cells expressing the indicated viruses were immunoprecipitated with anti-FLAG or anti-MYC. Immunoprecipitated proteins in (A) to (D) were detected by immunoblotting. (**B**) Interaction of Rbx1 with Elongin BC, VHL, and CUL2. Lysates from Sf21 cells expressing the indicated viruses were immunoprecipitated with anti-HPC4, anti-FLAG, or anti-MYC. (**C**) In vitro binding of recombinant Rbx1, VHL, and Elongin BC. Proteins expressed in and purified from *E. coli* were mixed together in the combinations indicated, renatured by dilution and dialysis, and immunoprecipitated with anti-HPC4. (**D**) Skp1-independent association of mRbx1 with CUL1. Lysates from Hi5 cells expressing the indicated viruses were immunoprecipitated with anti-HA or anti-MYC.

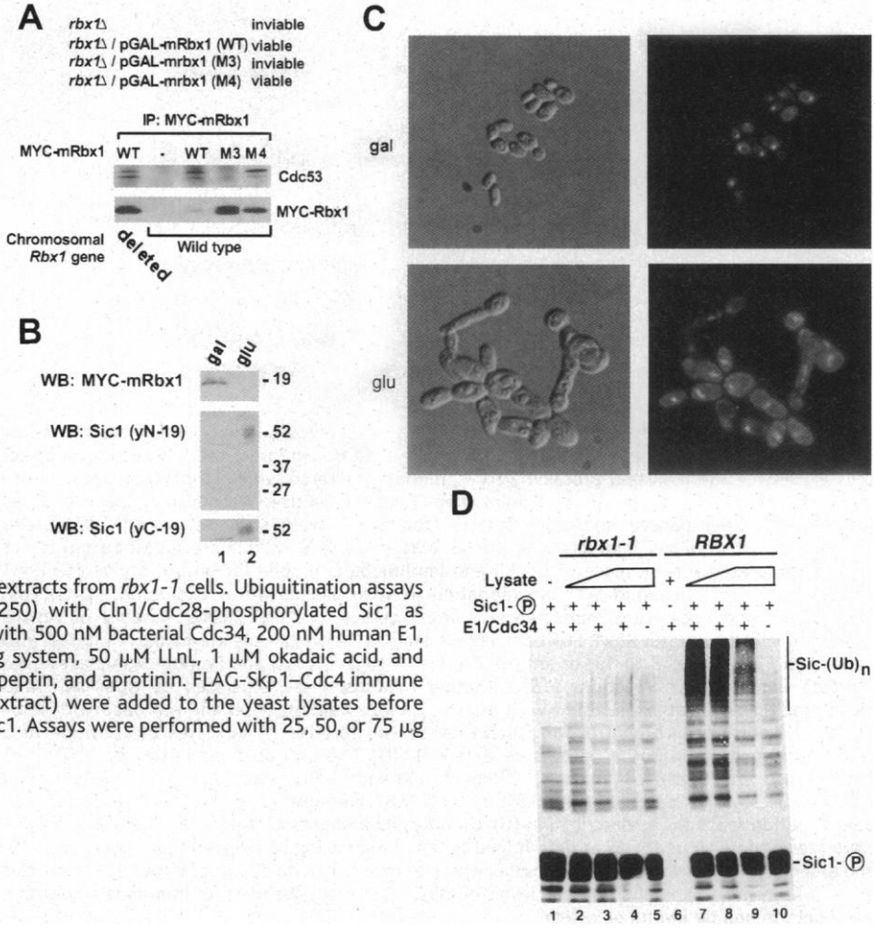

Fig. 3. Rbx1 function in yeast. (**A**) Binding of Rbx1 to endogenous yeast Cdc53 correlates with function. (Upper panel) Phenotypes of *rbx1*Δ cells expressing wild-type or mutant mRbx1. (Lower panel) Lysates from cells expressing wild-type and mutant mammalian MYC-Rbx1 proteins in the *rbx1* deletion strain (deleted) or in the parental strain MCY453 (wild type) were immunoprecipitated with anti-MYC and immunoblotted with anti-MYC or anti-Cdc53. (**B**) Sic1 accumulates in Rbx1-depleted cells. *rbx1*Δ*/pGAL-mrbx1*(M4) cells were grown to an absorbance at 600 nm of 1 in galactose-containing medium and then shifted into glucose medium. Cells were harvested after 8 hours of growth in glucose, and cell lysates were analyzed by immunoblotting with anti-MYC and two different anti-Sic1 antibodies. (**C**) Morphological changes associated with Rbx1-depletion. *rbx1*Δ/pGAL-mrbx1(M4) cells were grown in galactose (gal) or for 8 hours after glucose shift (glu) before fixation and staining. Nuclear morphology was visualized by DAPI (4′,6′-diamidino-2-phenylindole) staining. (Left) Differential interference contrast; (right) DAPI. (**D**) Sic1 ubiquitination is defective in extracts from *rbx1-1* cells. Ubiquitination assays were performed in fractionated yeast lysates (F250) with Cln1/Cdc28-phosphorylated Sic1 as substrate (*15*, *31*). Reactions were supplemented with 500 nM bacterial Cdc34, 200 nM human E1, 20 μM ubiquitin, 5 mM ATP, an ATP-regenerating system, 50 μM LLnL, 1 μM okadaic acid, and protease inhibitors (5 μg/ml each of pepstatin, leupeptin, and aprotinin. FLAG-Skp1–Cdc4 immune complexes (100 ng of Cdc4 per 25 μg of yeast extract) were added to the yeast lysates before addition of substrate to increase activity toward Sic1. Assays were performed with 25, 50, or 75 μg of yeast proteins.

coimmunoprecipitated with M4 than with M3 (Fig. 3A) (*22*). Consistent with these in vivo interactions, *S. cerevisiae* Rbx1 associated with Cdc53 (Fig. 4B) and assembled into SCF^{Cdc4} complexes (Fig. 4A) when coexpressed in insect cells. Efficient assembly into SCF complexes required the presence of Cdc53 (Fig. 4A, lane 4). Rbx1 also associated with Cdc4 in the absence of Cdc53 and Skp1 (Fig. 4A, lane 5), an interaction that was reduced in the presence of Skp1 (Fig. 4A, lane 4). This interaction may be analogous to that between Rbx1 and VHL (Fig. 2B).

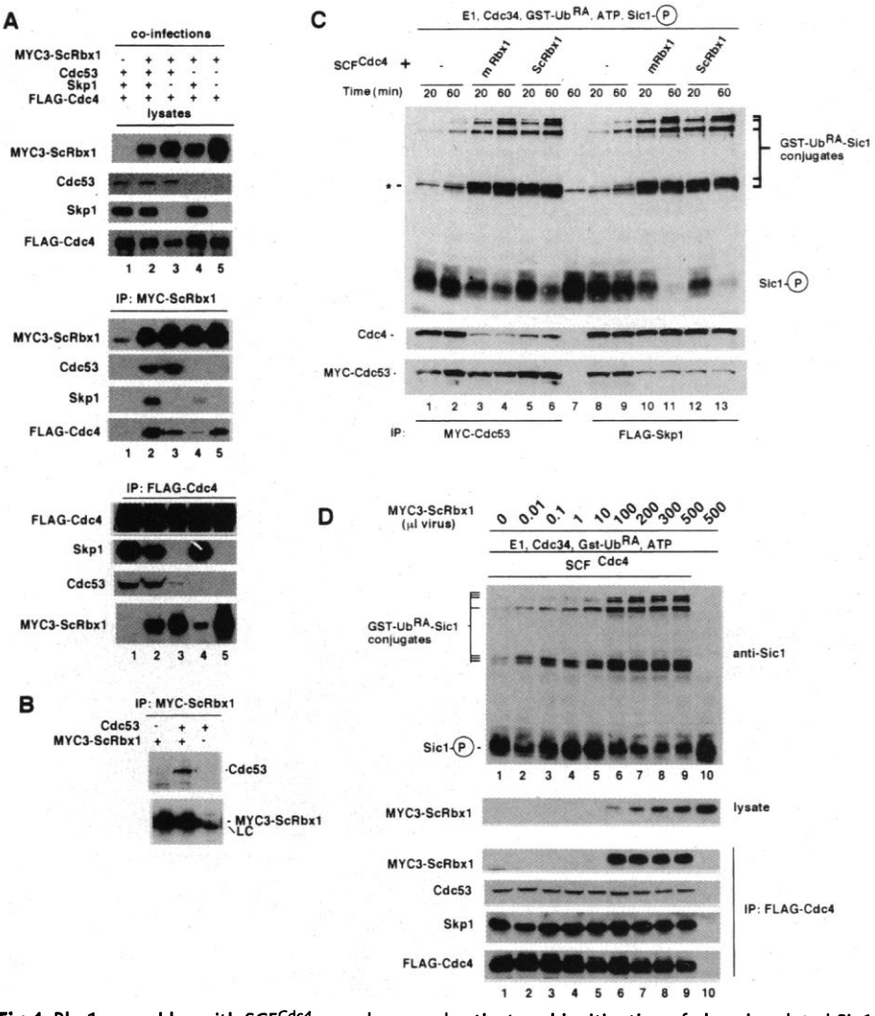

Fig 4. Rbx1 assembles with SCF^{Cdc4} complexes and activates ubiquitination of phosphorylated Sic1 in vitro. (**A**) Rbx1 associates with SCF^{Cdc4} complexes and Cdc4 in insect cells. Lysates from insect cells expressing the indicated proteins were immunoprecipitated with anti-MYC to immunoprecipitate MYC-Rbx1 or anti-FLAG to immunoprecipitate FLAG-Cdc4. Washed immune complexes (middle and lower panels) and crude lysates (top panel) were separated by SDS-PAGE and immunoblotted. (**B**) Rbx1 interacts with Cdc53. MYC-Rbx1 was immunoprecipitated from insect cells in the presence or absence of Cdc53 and immunoblots probed for both proteins. (**C**) Rbx1 stimulates SCF^{Cdc4}-mediated Sic1 ubiquitination in vitro. Baculoviruses expressing FLAG-Skp1, MYC-Cdc53, and Cdc4 were coinfected into insect cells in the presence or absence of viruses expressing mouse or yeast Rbx1. SCF complexes from 4×10^6 cells were immunoprecipitated with either anti-FLAG (lanes 8 to 13) or anti-MYC (lanes 1 to 6) (10 μl) and immune complexes used for Sic1 ubiquitination reactions (*15*). Reaction mixtures were separated by SDS-PAGE and Sic1-ubiquitin conjugates visualized with anti-Sic1. Blots were stripped and reprobed with anti-Cdc53 and anti-Cdc4 to control for protein levels. The asterisk (*) indicates the position of a cross-reacting band in some batches of anti-Sic1. (**D**) The indicated quantities of MYC-Rbx1 baculovirus were coinfected into 2×10^6 insect cells with 100 μl each of Skp1, FLAG-Cdc4, and Cdc53 viruses ($\sim 10^8$ particles per milliliter). Anti-FLAG immune complexes were used for Sic1 ubiquitination reactions as described in (C). Comparable amounts of Skp1, Cdc53, and FLAG-Cdc4 were present in each reaction as determined by immunoblotting of stripped blots (lower panels), and assembly of Rbx1 with the SCF complex was near maximal, with 100 μl of virus ($\sim 10^8$ particles per milliliter) (lane 6). In addition, the levels of MYC-Rbx1 in lysates used for immunoprecipitation were determined by immunoblotting.

To address the role of Rbx1 in Sic1 ubiquitination, we introduced MYC-tagged wild-type or M4 mRbx1 into the *rbx1* deletion strain on a high copy number plasmid under control of the GAL1,10 promoter. When cells carrying the plasmid were shifted from galactose to glucose medium, Rbx1 protein was depleted, and the fraction of cells exhibiting the elongated bud morphology increased substantially (Fig 3, B and C). Cells expressing M4 stopped growing within a few hours of the glucose shift, whereas cells expressing wild-type Rbx1 continued to grow slowly, presumably owing to the presence of residual Rbx1. Consistent with the hypothesis that M4 cells arrest because they cannot ubiquitinate and destroy Sic1, the M4 cells accumulated Sic1 protein when shifted into glucose (Fig 3B).

A common property of temperature-sensitive mutations in the SCF components Skp1, Cdc53, Cdc4, and Cdc34 is that Sic1 overexpression is lethal at permissive temperatures (*14*, *19*). Sic1 overexpression from the GAL1,10 promoter was lethal in cells expressing an *RBX1* temperature-sensitive mutant, *rbx1-1* (*23*), but not in cells expressing wild-type *RBX1*. Even on glucose, *rbx1-1* cells grew more slowly in the presence of the SIC1 plasmid than did cells containing wild-type *RBX1*, suggesting that low levels of SIC1 expression are toxic in combination with the *rbx1-1* mutation. Moreover, extracts from *RBX1* cells displayed Sic1 ubiquitination activity when supplemented with Cdc34, E1, and an adenosine triphosphate (ATP)–regenerating system, whereas extracts from *rbx1-1* were deficient in Sic1 ubiquitination (Fig. 3D). These data indicate that Rbx1 is involved in SCF^{Cdc4} function and in Sic1 ubiquitination.

To test directly the role of Rbx1 in SCF^{Cdc4} function, we used an in vitro Sic1 ubiquitination assay dependent on Sic1 phosphorylation and the E2 Cdc34 (*15*, *24*). SCF^{Cdc4} components were coexpressed in insect cells in the presence or absence of mammalian or yeast Rbx1, and complexes were purified by immunoprecipitation of MYC-tagged Cdc53 (MYC-Cdc53) or FLAG-tagged Skp1 (FLAG-Skp1) subunits. Complexes were supplemented with phosphorylated Sic1, Cdc34, E1 ubiquitin-activating enzyme, ATP, and glutathione S-transferase (GST)–Ub^{RA} before analysis of Sic1 conjugates by immunoblotting. GST-Ub^{RA} forms polyubiquitinated products poorly, so Sic1 conjugates are integrated into a ladder of bands differing by ~35 kD, the size of GST-Ub^{RA}. Low but detectable amounts of Sic1-GST-Ub^{RA} conjugates were produced by the SCF^{Cdc4} complex after a 60-min reaction (Fig. 4C, lanes 2 and 9). In the presence of Rbx1, accumulation of Sic1-GST-Ub^{RA} conjugates was markedly increased after 20 min (lanes 3, 5, 10, and 12), and substantial amounts of higher molecular mass conjugates accumulated after

60 min (lanes 4, 6, 11, and 13). In contrast to reactions lacking Rbx1, where <5% of Sic1 was conjugated (lane 9), >85% of Sic1 was converted to GST-Ub^{RA} conjugates in the presence of Rbx1 and FLAG-Skp1 complexes (lanes 11 and 13). To examine the extent of activation and the concentration dependence of Rbx1 activation, we purified SCF^{Cdc4} complexes from insect cells coexpressing increasing levels of MYC-Rbx1 and then assayed for Sic1 ubiquitin-conjugating activity (Fig. 4D). In the absence of Rbx1, low levels of conjugates were observed (lane 1). Increased quantities of Rbx1 led to increased levels of ubiquitination, with the maximal extent of activation approaching 20-fold at the point where MYC-Rbx1 assembly into SCF^{Cdc4} complexes was saturated and stoichiometric (lanes 6 to 9). This estimate represents a lower limit of the extent to which Rbx1 can increase the rate of accumulation of Sic1-GSTUb^{RA} conjugates because a large fraction of the phosphorylated Sic1 substrate was depleted at the end of reactions (lane 9). Immunoblot analysis of these complexes revealed that the levels of Cdc53, Cdc4, and Skp1 were constant throughout the Rbx1 titration. It is likely that incorporation of small amounts of insect cell Rbx1 protein contributed to the previous observation of Sic1 ubiquitination activity by SCF^{Cdc4} complexes in vitro (*15*, *16*).

Our findings have implications for the functions of both the VHL complex and Rbx1. First, our observation that Rbx1 is a component of both the VHL and SCF^{Cdc4} complexes extends their marked structural similarity and raises the possibility that the VHL complex, and perhaps other Cullin complexes, may function as ubiquitin ligases for as yet unidentified target proteins. Such a function for the VHL complex could explain the remarkably pleiotropic phenotypes associated with VHL mutations. Second, our finding that Rbx1 is a component of the SCF^{Cdc4} complex, together with the observations that Rbx1 is required for Cln1 ubiquitination by an SCF^{Grr1} complex (*23*), indicates that, like Cdc53/CUL1 and Skp1, Rbx1 functions as a common SCF subunit. Thus, it seems likely that Rbx1 regulates ubiquitination by SCF complexes containing additional F-box proteins, including the mammalian β-TRCP protein, which directs ubiquitination of the human immunodeficiency virus–1 coreceptor CD4 and the transcriptional regulators IκB and β-catenin (*25*–*28*).

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7. The VHL complex was purified from a postnuclear supernatant prepared from the livers of 360 male Sprague-Dawley rats (~3 kg of liver). Details of the purification are available at www.sciencemag.org/feature/data/991128.shl.
8. The VHL complex was fractionated by 13% SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were visualized by staining the gel with Coomassie blue, excised, and subjected to in-gel reduction, S-carboxyamidomethylation, and tryptic digestion. Using 10% of the digestion mixture, we determined peptide sequences in a single run by microcapillary reversed-phase chromatography coupled to the electrospray ionization source of a quadrupole ion trap mass spectrometer (Finnigan LCQ, San Jose, CA). The ion trap's online data-dependent scans allowed the automatic acquisition of high-resolution spectra to determine charge state and exact mass, and tandem mass spectrometry spectra for sequence information. The relative collision energy was 35% and isolation width was 2.5 dalton. Identification of human and mouse expressed sequence tags that encoded the peptide sequences NHIMDLCIECQAN, QVCPLDNREWEFQK, WNAVAL, and WLK was facilitated with the algorithm SEQUEST [J. K. Eng, A. L. McCormick, J. R. Yates III, *J. Am. Soc. Mass Spectrom.* **5**, 976 (1994)] and by programs developed in the Harvard Microchemistry Facility [H. S. Chittum *et al.*, *Biochemistry* **37**, 10866 (1998)]. IMAGE Consortium cDNA clones [G. Lennon, C. Auffray, M. Polymeropoulos, M. B. Soares, *Genomics* **33**, 151 (1996)] encoding the complete 108–amino acid ORFs of human (H71993) and mouse (W66989 and AA260889) Rbx1 were obtained from Research Genetics, (Huntsville, AL), and the nucleotide sequences of both strands were determined. Human (AF140598) and mouse (AF140599) cDNAs encoded identical polypeptides of 108 amino acids.
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10. To express recombinant proteins in insect cells, Rbx1 and VHL were subcloned into pBacPAK8 with NH_2-terminal His tags and NH_2-terminal MYC and COOH-terminal FLAG tags, respectively. CUL1 was introduced into the same vector with a COOH-terminal HA tag, CUL2 was introduced into pBacPAK-His1 with NH_2-terminal His and HA tags, and recombinant baculoviruses were generated with the BacPAK baculovirus expression system (Clontech). The baculovirus vectors encoding *S. cerevisiae* Cdc53 [A. R. Willems *et al.*, *Cell* **86**, 453 (1996)] and Elongins B and C have been described (*29*). Sf21 or Hi5 cells were cultured in Sf-900 II SFM with 5% fetal calf serum at 27°C and infected with the indicated recombinant baculoviruses. Sixty hours after infection, cells were collected and lysed in ice-cold buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1 mM dithiothreitol, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, and 5 μg/ml each of leupeptin, antipain, pepstatin A, and aprotinin. Lysates were centrifuged at 10,000*g* for 20 min at 4°C. The supernatants were used for immunoprecipitations.
11. Sources of antibodies were as follows: Anti-T7 and anti-HSV, Novagen; anti-HA (12CA5) and anti–c-MYC (9E10), Boehringer-Mannheim; anti-FLAG (M2), Eastman Kodak; anti–Elongin C monoclonal antibody (mAb), Transduction Laboratories; anti-VHL mAb (Ig32), Pharmingen; anti-Sic1 (yN-19 and yC-19) and anti-Cdc53 (yC-17), Santa Cruz Biotechnology. Anti-Elongin B rabbit polyclonal antibodies have been described previously (*29*). Anti-Sic1, Cdc4, and Skp1 were from (*15*). Immunoblotting and immunoprecipitations were performed as in (*29*).
12. For preparation of recombinant proteins in *Escherichia coli*, full-length mouse Rbx1 was expressed in pRSET B (Invitrogen) with NH_2-terminal 6-histidine and MYC epitope tags. Human VHL was expressed in pRSET B with NH_2-terminal 6-histidine and COOH-terminal FLAG epitope tags. Purification of recombinant proteins from inclusion bodies and expression constructs for Elongins B and C have been described (*29*).
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21. Construction of yeast strains: The *RBX1* gene was disrupted in MCY453 (*Mata/MATα his3Δ-200/his3Δ-200 can1^R/can1^R cyh2^R/cyh2^R ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2*) by replacing the complete *RBX1* ORF (YOL133w) with the *HIS3* gene (*20*). For rescue of the *rbx1* deletion strain (MCY557) with mammalian *RBX1*, wild-type and mutant mammalian *RBX1* genes were fused to the GAL1,10 promoter in the plasmid YEp352-GAL. These plasmids were transformed into MCY557, and Ura$^+$ transformants were selected. Random spores were germinated on galactose medium minus histidine and uracil and allowed to grow for 4 days at 30°C. The resulting colonies were tested for mating. To confirm that rescue was due to the presence of the *RBX1* expression plasmid, cells were tested for the ability to grow in FOA (5-fluoro-orotic acid) after prolonged growth in medium containing uracil. The *rbx1-1* ts allele was isolated as described (*23*). Strains carrying either a wild-type copy of *RBX1* or *rbx1-1* were transformed with either an empty GAL vector (pHY316) or construct expressing Sic1 under control of the GAL1,10 promoter (pCB24) (*14*). Transformants were streaked to selective media containing either glucose or galactose at 30°C.
22. Less mRbx1 was immunoprecipitated from yeast containing the endogenous *RBX1* gene (Fig. 3A, lane 3) than from yeast deleted for *RBX1* (lane 1), even though similar amounts of Cdc53 were precipitated. mRbx1 was highly overexpressed in these cells and was likely present in large excess over the endogenous Cdc53.
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24. Hi5 cells (1×10^6) infected with the indicated baculoviruses were lysed as described (*15*, *23*) and immunoprecipitated with immobilized anti-MYC or anti-Flag. Complexes and lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Cdc34-dependent Sic1 ubiquitination assays were performed was described with Cln1/Cdc28-phosphorylated Sic1 as substrate (*15*). Sic1 ubiquitination assays in fractionated yeast lysates were performed essentially as described (*31*), except that FLAG-Skp1–Cdc4 complexes (200 ng) purified from insect cells with immobilized anti-FLAG were added to yeast lysates to stimulate Sic1 ubiquitination activity.
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