Reconstitution of G_1 Cyclin Ubiquitination with Complexes Containing SCF^{Grr1} and Rbx1

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Control of cyclin levels is critical for proper cell cycle regulation. In yeast, the stability of the G_1 cyclin Cln1 is controlled by phosphorylation-dependent ubiquitination. Here it is shown that this reaction can be reconstituted in vitro with an SCF E3 ubiquitin ligase complex. Phosphorylated Cln1 was ubiquitinated by SCF (Skp1-Cdc53–F-box protein) complexes containing the F-box protein Grr1, Rbx1, and the E2 Cdc34. Rbx1 promotes association of Cdc34 with Cdc53 and stimulates Cdc34 auto-ubiquitination in the context of Cdc53 or SCF complexes. Rbx1, which is also a component of the von Hippel–Lindau tumor suppressor complex, may define a previously unrecognized class of E3-associated proteins.

Cyclins are regulatory subunits of cyclin-dependent kinases (CDKs) and are the rate-limiting components in many cell cycle transitions (*1*). G_1 cyclins control S-phase entry, and their abundance is tightly controlled by regulated transcription, translation, and protein stability—the latter through ubiquitin-mediated proteolysis. The levels of mammalian G_1 cyclins D1 and E are regulated by phosphorylation-dependent ubiquitination (*2–4*). In the yeast *Saccharomyces cerevisiae*, the stability of the G_1 cyclins Cln1 and Cln2 are also controlled by phosphorylation-dependent ubiquitination (*5*). Genetic data implicate an SCF (Skp1-Cdc53–F-box protein) E3 ubiquitin ligase and the E2 Cdc34 in Cln turnover (*6–11*), with the F-box protein Grr1 acting as a receptor of phosphorylated Clns (*6*, *12*). However, SCF^{Grr1} complexes isolated from insect cells do not support Cln1 ubiquitination (*12*), possibly owing to the absence of a critical component.

To further elucidate the role of SCF^{Grr1} in Cln ubiquitination, we fractionated extracts from *GRR1* and *grr1*Δ strains by ion exchange chromatography (*9*). Cln1 ubiquitination activity eluted between 50 and 250 mM KCl (F250) (Fig. 1A, lane 3) (*13*). This activity required the addition of E1, Cdc34, ATP, and ubiquitin (Ub) and Cln1 autophosphorylation by its associated Cdc28 kinase (Fig. 1B, lanes 9 to 12) (*8*, *14*), consistent with the requirements for Cln1 destruction in vivo. F250 fractions from *grr1*Δ mutant extracts were completely defective in Cln1 ubiquitination (Fig. 1B, lanes 5 to 8). Insect cell–derived SCF^{Grr1} complexes purified through a FLAG-tag on Skp1 lacked Cln1 ubiquitination activity (Fig. 1C, lane 4). Both Skp1/Grr1 and SCF^{Grr1} complexes, but not Skp1 alone, restored Cln1 ubiquitination activity to *grr1*Δ F250 fractions (Fig. 1C, lane 6 to 8), demonstrating that Grr1 plays an active role in Cln1 ubiquitination and that insect cell–derived SCF^{Grr1} complexes were missing one or more components.

We next investigated the role of Rbx1, which stimulates Sic1 ubiquitination in vitro (*15*). If Rbx1 were a general SCF component, it should be required for all SCF substrates. Because disruption of the *RBX1* gene was lethal, we generated a temperature-sensitive *RBX1* mutation (*rbx1-1*) (*16*) that contained two changes, $Lys^{72} \rightarrow Arg$ (K72R) and $Cys^{81} \rightarrow Arg$ (C81R). At the nonpermissive temperature, more than 90% of *rbx1-1* cells displayed a multibudded phenotype (Fig. 2A), similar to that of cells with mutations in *SKP1* and *CDC53* (*6*,*11*,*17*). *CLN2* overproduction was toxic in *rbx1-1* mutants (Fig. 2B) (*16*) as it is in *skp1* and *cdc53* mutants, which are defective in Cln ubiquitination (*6*, *7*). F250 fractions from the *rbx1-1* mutant were profoundly defective in Cln1 ubiquitination (Fig. 2C, lanes 1 to 6).

We next determined whether Rbx1 could assemble into active SCF^{Grr1} complexes (*18*, *19*). Grr1 assembled with Cdc53, Rbx1, and

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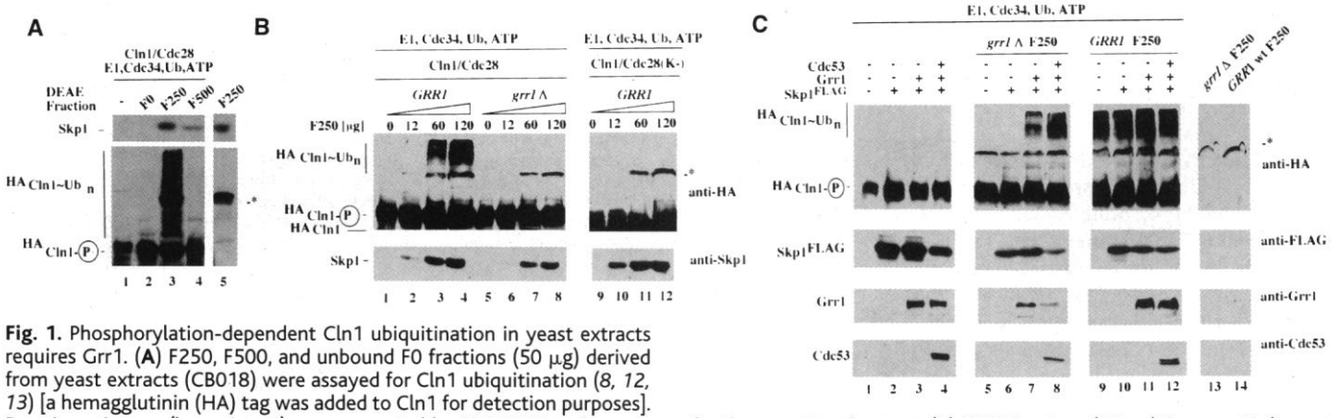

Fig. 1. Phosphorylation-dependent Cln1 ubiquitination in yeast extracts requires Grr1. (**A**) F250, F500, and unbound F0 fractions (50 μg) derived from yeast extracts (CB018) were assayed for Cln1 ubiquitination (*8*, *12*, *13*) [a hemagglutinin (HA) tag was added to Cln1 for detection purposes]. Reaction mixtures (lanes 1 to 4) were separated by SDS-PAGE and immunoblotted with antibodies to HA (anti-HA) to reveal Cln1 ubiquitin conjugates, and blots reprobed with anti-Skp1. The asterisk indicates a yeast protein in F250 (lane 5) that cross-reacts with anti-HA, and "P" inside a circle designates phosphate. (**B**) F250 fractions (75 μg) from *GRR1* (YM4575) or *grr1*Δ (YM4576) strains were assayed for HA-Cln1 ubiquitination with phosphorylated HA-Cln1/Cdc28 (lanes 1 to 8) or unphosphorylated HA-Cln1/Cdc28(K-) (lanes 9 to 12). Blots were probed with anti-Skp1 to control for the quantity of extract. (**C**) F250 fractions (75 μg) from *grr1*Δ (lanes 5 to 8) or *GRR1* (lanes 9 to 12) strains were supplemented with anti-FLAG immunoprecipitates containing FLAG-Skp1, FLAG-Skp1/Grr1, or FLAG-Skp1/Cdc53/Grr1 and assayed for HA-Cln1 ubiquitination (60 min). Skp1 complexes were also assayed in the absence of yeast extracts (lanes 1 to 4). The quantities of Skp1, Grr1, and Cdc53 were 1 μg, 100 ng, and 200 ng, respectively. Blots were reprobed with anti-FLAG, anti-Grr1, and anti-Cdc53 (Santa Cruz Biotechnology).

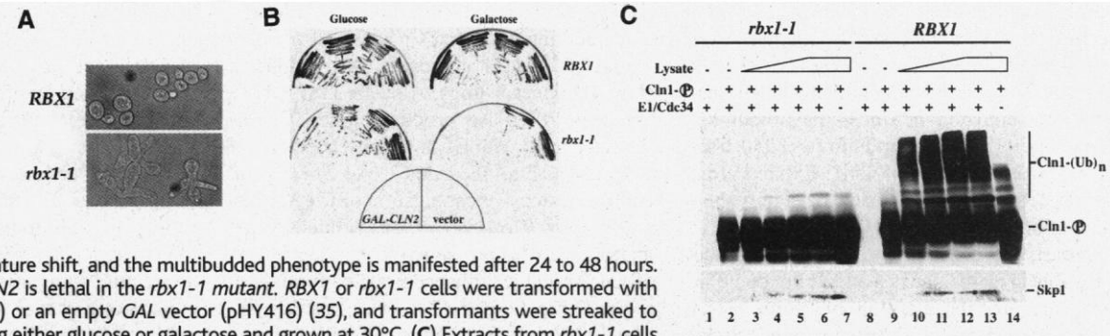

Fig. 2. A temperature-sensitive allele of *RBX1* interacts genetically with *CLN2* and is defective for Cln1 ubiquitination in vitro. (**A**) *rbx1-1* cells are elongated and multibudded at the restrictive temperature (38°C). Elongated buds appear 3 hours after the temperature shift, and the multibudded phenotype is manifested after 24 to 48 hours. (**B**) Overexpression of *CLN2* is lethal in the *rbx1-1 mutant*. *RBX1* or *rbx1-1* cells were transformed with a *GAL-CLN2* (pMT634) (*6*) or an empty *GAL* vector (pHY416) (*35*), and transformants were streaked to selective media containing either glucose or galactose and grown at 30°C. (**C**) Extracts from *rbx1-1* cells are defective for Cln1 ubiquitination (*13*). Amounts of F250 proteins were 25 μg (lanes 3, 10), 50 μg (lanes 4 and 11), 75 μg (lanes 5 and 12), and 100 μg (lanes 6 and 13). Lanes 1 and 8 lack substrate. Lanes 2 and 9 lack F250. Lanes 7 and 14 lack E1 and Cdc34. The bottom panel shows the same blot reprobed for Skp1 as a control for protein amounts.

Skp1 (Fig. 3A, lane 3) and MYC3-tagged Rbx1 bound to Grr1 independent of the other SCF subunits (lane 5). Rbx1 also binds to Cdc4 (*15*) and β-translucin repeat-containing protein (β-TRCP) (*14*), indicating that it can interact with multiple F-box proteins, possibly through the F box. Rbx1/SCF^{Grr1} complexes were examined for Clnl ubiquitination in vitro (Fig. 3B) (*19*). Phosphorylated Cln1 was efficiently ubiquitinated by this complex in a time-dependent (lanes 1 to 4), Cdc34-dependent (lane 5), and Cln1 phosphorylation–dependent (lanes 7 and 8) manner. When a form of ubiquitin that does not undergo polyubiquitin chain formation [glutathione S-transferase (GST)–Ub^{RA}] was used, discrete Cln1 conjugates were found only in the presence of Rbx1 (Fig. 3C, lanes 3 to 5 and 9), indicating that Cln1 ubiquitination in vitro by SCF^{Grr1} requires Rbx1. These results demonstrate that Grr1 is the specificity determinant for recognition of phosphorylated Cln1 (*12*) and that Rbx1 is a general component of SCF complexes.

Given that Rbx1 is an integral component of the SCF complex, we investigated its role in SCF function by examining SCF-E2 assembly. Cdc34 weakly associates with Cdc53 produced in insect cells (*7*, *12*). Because Rbx1 binds Cdc53 (*15*), we examined whether it affected the interaction of Cdc34 with Cdc53. Anti–MYC-Cdc53 immune complexes from insect cells expressing MYC-Cdc53 and Cdc34 contain small amounts of Cdc34 (Fig. 4A, lane 11). When Rbx1 was coexpressed, the amount of Cdc34 associated with MYC-Cdc53 was substantially increased (lane 12). Furthermore, MYC3-Rbx1 bound to Cdc34 when coexpressed in insect cells in the presence or absence of Cdc53 (Fig. 4A, lane 8). In a purified system, bacterial Cdc34 associated preferentially with Rbx1/Cdc53 complexes (*14*). These data suggest that Rbx1 may acts as an adapter to recruit the E2 Cdc34 to Cdc53 or SCF complexes. The observed interaction of Cdc34 and Cdc53 in insect cells may be due to inclusion of small amounts of insect Rbx1.

When Cdc34 is charged with ubiquitin by E1 and ATP, it undergoes limited auto-ubiquitination (Fig. 4C, lane 6) (*20*), but it cannot transfer ubiquitin to other substrates including Sic1 (*12*) and Cln1 (Fig. 3C, lane 2) in the absence of the appropriate SCF complex. In contrast, Cdc34 is extensively polyubiquitinated by yeast F250 extracts in Cln1 ubiquitination reactions (Fig. 4C, lanes 2 and 3), although Cdc34 ubiquitination in this setting does not require the presence of Cln1 (lane 1). Cdc34 polyubiquitination activity in F250 fractions migrates with a peak centered at ~250 kD upon gel filtration that largely coincides with the pattern of Skp1 elution (Fig. 4B, lanes 4 to 7) (*21*). Both Cdc4 protein and Cln1 ubiquitin-ligase activity overlap with the peak of Cdc34 polyubiquitination activity and Skp1. One possible interpretation of these data is that Cdc34 auto-ubiquitination reflects its intrinsic ubiquitin-conjugating activity and that association of Cdc34 with SCF

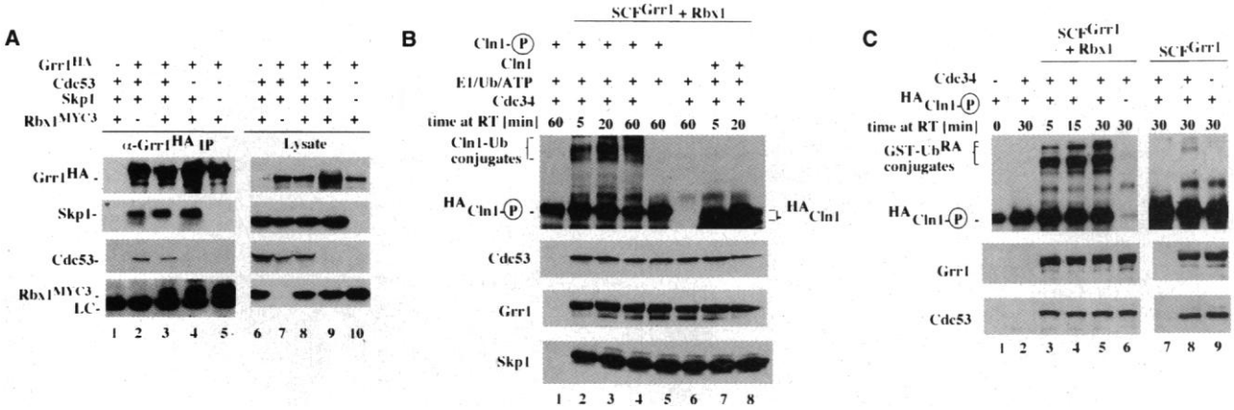

Fig. 3. Reconstitution of Cln1 ubiquitination requires Rbx1. (**A**) Lysates from insect cells infected with the indicated baculoviruses were immunoprecipitated (IP) with immobilized anti-HA (*19*). Washed complexes (lanes 1 to 5) and lysates (lanes 6 to 10) were separated by SDS-PAGE before immunoblotting with the indicated antibodies. LC, immunoglobulin G light chain. (**B**) Phosphorylation and Cdc34-dependent ubiquitination of HA-Cln1 by an Rbx1/SCF^{Grr1} complex. Hi5 cells were coinfected with baculoviruses encoding FLAG-Skp1, Cdc53, Grr1, and Rbx1, and complexes were purified with anti-FLAG (lanes 2 to 8). Complexes were supplemented with Cdc34, E1, ubiquitin, ATP, and phosphorylated (lanes 2 to 5) or unphosphorylated HA-Cln1 (lanes 7 and 8) and incubated at room temperature (RT) (*19*). Immunoblots of reaction mixtures were probed with anti-HA and stripped blots were reprobed for Cdc53, Grr1, and Skp1. (**C**) Rbx1 is required for HA-Cln1 ubiquitination by SCF^{Grr1} complexes. SCF^{Grr1} complexes with (lanes 3 to 6) or without (lanes 7 to 9) Rbx1 were purified from insect cells through a FLAG tag on Skp1 and assayed for HA-Cln1 ubiquitination (*19*). GST-Ub^{RA} conjugates were detected with anti-HA, and blots were reprobed with anti-Grr1 and anti-Cdc53 as a control for protein amounts. The quantities of Grr1 and Cdc53 in (B) and (C) were 400 ng and 200 ng, respectively.

complexes activates its intrinsic ubiquitin-conjugating activity toward both itself and SCF-bound substrates. To examine whether Rbx1 plays a role in Cdc34 auto-ubiquitination, we compared the Cdc34 polyubiquitination activity of *RBX1* and *rbx1-1* F250 fractions in the presence of Cln1. Extracts from *RBX1* cells contained robust Cdc34 polyubiquitination activity (Fig. 4C, lanes 1 to 3). In contrast, the activity in *rbx1-1* cell extracts was low (Fig. 4C, lanes 4 and 5) but could be enhanced by the addition of mouse Rbx1 (lane 9). Cdc34 polyubiquitination activity was also markedly reduced in extracts from *cdc53-1* and *cdc53-2* mutants but not from *grr1*Δ strains (*14*).

To examine whether Rbx1 promotes Cdc34 auto-ubiquitination in vitro, we purified SCF^{Cdc4} complexes with and without Rbx1 and assayed them using GST-Ub^{RA} as the ubiquitin source. In the absence of Rbx1, a doublet migrating at the position of a single GST-Ub^{RA}/Cdc34 conjugate was produced (Fig. 4D, lanes 1 to 5). A single GST-Ub^{RA} conjugate was also produced when Cdc34 was incubated with E1 in the absence of SCF complexes (Fig. 4E, lanes 1 and 2) (*14*). In contrast, extensive conjugation of Cdc34 was produced by Rbx1/SCF^{Cdc4}, as defined by the appearance of more slowly migrating forms of Cdc34 and an enhanced rate of formation of the first conjugate (Fig. 4D, lanes 6 to 8). The inclusion of Sic1 did not alter the appearance of these conjugates (Fig. 4D; compare lanes 8 and 9). Anti–MYC-Cdc53 immune complexes, in the presence or absence of Skp1, generated small amounts of the first GST-Ub^{RA} conjugate (Fig. 4E, lanes 3 to 6). In contrast, association of Rbx1 with MYC-Cdc53 and MYC-Cdc53/Skp1 complexes led to increased rates and extent of Cdc34 ubiquitination (Fig. 4E, lanes 7 to 10). Inclusion of Cdc4 into Rbx1/MYC-Cdc53/Skp1 complexes resulted in a further increase in Cdc34 auto-ubiquitination activity (lanes 11 and 14). These data indicate that Rbx1, in a complex with Cdc53 or with a fully assembled SCF complex, can stimulate Cdc34 auto-ubiquitination. In isolation, yeast (*14*) or mouse-MYC-Rbx1 (Fig. 4E, lanes 13 and 14) have only a mild stimulatory effect on Cdc34 ubiquitination; however, mouse MYC-Rbx1 significantly stimulates Cdc34 ubiquitination in the presence of Cdc53 (lanes 15 and 16), similar to that seen with MYC-Cdc53/Rbx1 complexes (lanes 7 and 8). Stimulation of Cdc34 auto-ubiquitination is not specific to Cdc4 because it is also observed with SCF^{Grr1} complexes (*14*). We speculate that the Rbx1-mediated recruitment of Cdc34

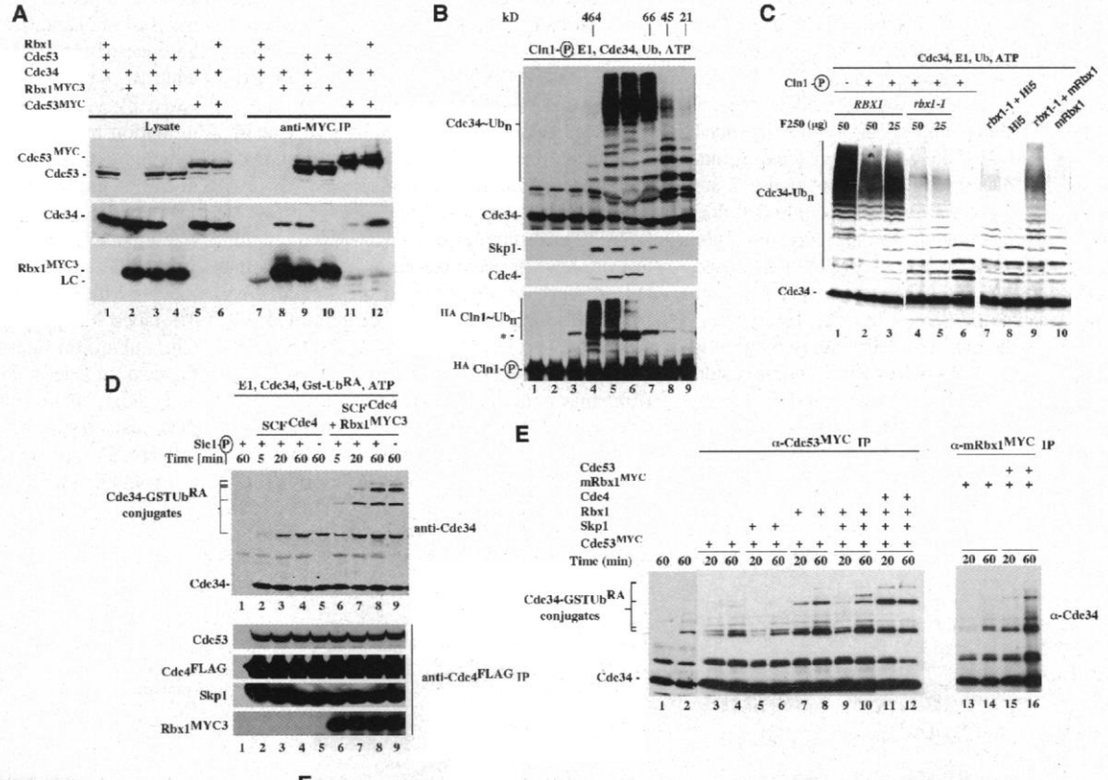

Fig. 4. Rbx1 interacts with Cdc34 and is required for efficient polyubiquitination of Cdc34. (**A**) Rbx1 binds Cdc34 and stimulates association of Cdc53 with Cdc34 in insect cells. Hi5 cells were infected with the indicated baculoviruses, and anti-MYC immune complexes (lanes 7 to 12) or crude lysates (lanes 1 to 6) were separated by SDS-PAGE before blotting with anti-Cdc34, anti-Cdc53, or anti-MYC. (**B**) Fractionation of Cdc34 polyubiquitination activity during gel filtration. Yeast F250 proteins were separated by gel filtration (*21*) and pooled fractions were assayed for Cln1 ubiquitination (lanes 1 to 9) (*13*). Immunoblots were reprobed with anti-Cdc34, anti-Cdc4, and anti-Skp1. The asterisk indicates a yeast protein that cross-reacts with anti-HA. (**C**) Extracts from *rbx1-1* cells are defective for Cdc34 polyubiquitination. Ubiquitination reactions (*13*) were resolved by SDS-PAGE and immunoblotted with anti-Cdc34. Lanes 7 and 9 contain 25 μg of *rbx1-1* lysate supplemented with 10 μg of lysate from Hi5 cells expressing mouse MYC-Rbx1 (lane 9) or a control lysate (lane 7). Samples in lanes 8 and 10 lacked F250 proteins. (**D**) Rbx1/SCF complexes stimulate Cdc34 auto-ubiquitination. Hi5 cells were coinfected with Skp1, Cdc53, and FLAG-Cdc4 with (lanes 6 to 9) or without (lanes 2 to 5) MYC3-Rbx1 and anti-FLAG complexes assayed for Cdc34 ubiquitination (*19*). Lanes 2 to 4 and 6 to 8 contain phosphorylated Sic1 (50 ng) (*12*). Reaction mixtures were immunoblotted and probed for SCF components. (**E**) Hi5 cells were infected with baculoviruses encoding MYC-Cdc53, Skp1, Cdc4, and Rbx1 (lanes 3 to 12) or with mouse MYC-Rbx1 and Cdc53 (lanes 13 to 16), and anti-MYC immune complexes were assayed for Cdc34 ubiquitination with GST-Ub^{RA} (*19*). Immunoblots of assay samples were probed with anti-Cdc34. MYC-Cdc53 and mouse MYC-Rbx1 levels were constant (*14*). (**F**) Alignment of *S. cerevisiae* R-box–containing proteins. Black boxes, identities; shaded boxes, similarities. 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.

into an SCF complex facilitates activation of Cdc34's intrinsic conjugating activity. Rbx1 may also function as a ubiquitin carrier, a possibility that remains to be tested.

Rbx1 is a highly conserved protein (*15*) that plays an essential role in SCF function in part by recruiting Cdc34. Consistent with this, *rbx1* mutants have been independently found that are synthetically lethal with *cdc34* mutants (*22*). The closest Rbx1 homolog in yeast is the essential anaphase-promoting complex (APC) subunit Apc11 (*23*), which may act in a parallel fashion, binding to the Cdc53 homolog Apc2 and recruiting an E2 into the APC, making the APC a distantly related member of the SCF family of E3s. Sequences related to the R-box motif in Rbx1 can be found in several other *S. cerevisiae* proteins associated with ubiquitination, including Hrd1, Rad18, and Ubr1, which have been implicated in 3-hydroxy-3-methylglutary–coenzyme A reductase ubiquitination (*24*), DNA repair (*25*), and the N-end rule pathway (*26*), respectively, and in four uncharacterized open reading frames (Fig. 4F). Rad18 and Ubr1 form independent complexes with the E2 Rad6 (*27–29*), which controls the N-end rule pathway, DNA repair, and telomeric silencing (*25–27*). Most R-box proteins are much larger than Rbx1 and may themselves contain substrate recognition domains, as has been demonstrated for Ubr1 (*29*). In plants, the Prt1 R-box protein is genetically implicated in the N-end rule pathway (*30*). In mammals, Mdm2, a p53 E3, also contains an R-box–related motif (*14*) that is required for its function (*31*). Currently, ubiquitin-carrying HECT-domain proteins are the only well characterized class of E3s not linked to R-box proteins. Thus, E3s may fall into two main classes: the SCF class that require R-box proteins and the HECT class that do not. The finding that mammalian Rbx1 is a component of the von Hippel–Lindau (VHL)–Elongin B/C–Cul2 complex (*15*) suggests that these complexes may also be involved in the transfer of ubiquitin or ubiquitin-like proteins.

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13. Yeast extracts from isogenic YM4575 (*GRR1*) or YM4576 (*grr1Δ*) strains (*32*) or CB018 (*pep4::HIS3*) were fractionated on DEAE-Sephacryl (*33*). Proteins eluting with 250 and 500 mM KCl were collected (F250 and F500, respectively). Immunoblot analysis revealed Cdc34 only in the F500 fraction. Twenty-microliter ubiquitination reactions (30 to 60 min, 25°C) contained 500 nM Cdc34, 300 nM E1, 2 mM adenosine triphosphate (ATP), an ATP-regenerating system, 20 μM ubiquitin, 0 to 120 μg of F250, and 50 ng of active or kinase-defective (K-) HA-Cln1/GST-Cdc28 (*12*). Cln1 autophosphorylation was performed at 25°C in the presence of 1 mM ATP for 30 min. Reaction mixtures were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and products were visualized by immunoblotting with ECL detection (Amersham). SCF complexes were purified from insect cells 40 hours after infection with antibodies to tagged proteins (*12*). A baculovirus encoding untagged Grr1 (in pVL1392) was produced with Baculogold (Pharmingen). Rabbit anti-Grr1 was generated with the peptide MDQDNNNHND-SNRL (see legend to Fig. 4 for amino acid abbreviations). *RBX1* and *rbx1-1* (*16*) extracts were prepared from cells shifted to 38°C for 16 hours. Strains (*MATa RBX1::HIS3 ura3 leu2 trp1 lys2 his3Δ200 can1-100 cyh2*) were maintained by a plasmid containing either a wild-type (pDK101) or mutant (pDK102) version of *RBX1* in pRS314.
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16. *RBX1* was disrupted by inserting the *LEU2* gene into the Nsi I site in the *RBX1* open reading frame in pDK100 (containing a genomic *RBX1* fragment in pRS316). A Sac I–Sph I fragment from pDK100 was used to transform Y80 (*MATa can1-100 ade2-1 his3-11, 15 leu 2-3, 112 trp1-1 ura3-1*) carrying pDK100 to generate the disruption strain. Strains that could not grow without pDK100 were chosen, and integration of the *LEU2* gene was confirmed by Southern (DNA) blot analysis. The *rbx1-1* allele was generated by polymerase chain reaction mutagenesis (*34*) with primers flanking the open reading frame. The plasmid was rescued from a temperature-sensitive strain, retested, and sequenced.
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19. For SCF assembly, we coinfected Hi5 cells (2×10^6) with recombinant baculoviruses. Forty hours later, cells were lysed in 50 mM tris-HCl (pH 7.5) containing 100 mM NaCl, 0.5% NP-40, 2 mM dithiothreitol, 0.1 mM $Zn(OAC)_2$, and protease inhibitors (Boehringer-Mannheim). Cleared lysates were immunoprecipitated for 1 to 2 hours with 10 μl of beads containing immobilized antibodies. Proteins were separated by SDS-PAGE and immunoblotted. HA-Cln1 and Cdc34 ubiquitination was performed at 25°C with 10 μl of agarose beads containing SCF complexes with or without Rbx1 supplemented with 500 nM Cdc34, 300 nM E1, 4 mM ATP, 20 μM ubiquitin (or 40 μM GST-Ub^{RA}), and 50 ng of HA-Cln1/GST-Cdc28. Reaction mixtures were separated by SDS-PAGE and immunoblotted.
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Male Homosexuality: Absence of Linkage to Microsatellite Markers at Xq28

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Several lines of evidence have implicated genetic factors in homosexuality. The most compelling observation has been the report of genetic linkage of male homosexuality to microsatellite markers on the X chromosome. This observation warranted further study and confirmation. Sharing of alleles at position Xq28 was studied in 52 gay male sibling pairs from Canadian families. Four markers at Xq28 were analyzed (DXS1113, BGN, Factor 8, and DXS1108). Allele and haplotype sharing for these markers was not increased over expectation. These results do not support an X-linked gene underlying male homosexuality.

Previous studies have suggested that there is a genetic component in male sexual orientation. These include controlled family studies that have shown an increased frequency of homosexual brothers of homosexual index subjects as compared to heterosexual index subjects (*1*, *2*) and twin studies, which have shown increased concordance for homosexu-