quencing reactions with purified PCR products were performed by using Big Dye Terminator chemistry and forward or reverse primers in separate sequencing reactions (Applied Biosystems, Foster City, CA). Reactions were analyzed by using a 3700 Sequence Analyzer (Applied Biosystems). Sequence traces were automatically analyzed by using PhredPhrap and Polyphred (*47*, *48*). For SNPs identified through this analysis, PCR Invader assays (Third Wave Technologies, Madison, WI) were designed and tested on 90 samples from the Polymorphism Discovery Resource panel (PDR90) (*49*). Successful assays were subsequently used to analyze samples from our study. Genotypes were assigned automatically by cluster analysis (M. Olivier *et al.*, in preparation). Differences among genotypes were analyzed by one-way ANOVA using STATVIEW 4.1 software (Abacus Concepts, Inc., Berkeley, CA). SNPs 1 to 4 are available in dbSNP under accession numbers ss3199913, ss3199914, ss3199915, and ss3199916, respectively.

35. Subjects were a combined subset of 501 healthy, nonsmoking Caucasian individuals aged >20 years (429 men, 72 women) who had participated in previous dietary intervention protocols (*50*, *51*) (R. M. Krauss *et al.*, unpublished data). All subjects had been free of chronic disease during the previous 5 years and were not taking medication likely to interfere with lipid metabolism. In addition, they were required to have plasma total cholesterol concentrations <6.74 mmol/liter (260 mg/dl), triacylglycerol <5.65 mmol/l (500 mg/dl), resting blood pressure <160/105 mm Hg, and body weight <130% of ideal. Each participant signed a consent form approved by the Committee for the Protection of Human Subjects at E. O. Lawrence Berkeley National Laboratory, University of California, Berkeley, and participated in a medical interview. Fasting blood samples were obtained from subjects eating their usual diets, and after 4 to 6 weeks of consuming diets containing high fat (35 to 46% energy) and low fat (20 to 24% energy) (*50*, *51*). Plasma lipid and lipoprotein measurements were performed as previously described (*50*, *51*). In addition, on the high- and low-fat diets, total lipoprotein mass was measured by analytic ultracentrifugation (*50*, *51*).
36. Of the 501 individuals in the original study, 388 were successfully genotyped by PCR amplification for the Sst I polymorphism as previously described (*16*, *28*).
37. To genotype the C/T SNP3 polymorphisms upstream of *APOAV*, oligonucleotides AV6-F-5′-GATTGATTCAAGATGCATTTAGGAC-3′ and AV6-R-5′-CCCCAGGAACTGGAGCGAAATT were used to amplify a 187-bp fragment from genomic DNA. The penultimate base in AV6-R was changed to T to create a Mse I site (TTAA) in the common allele. The PCR reactions were performed in 20 μl volumes containing 50 mmol/liter KCl, 10 mmol/liter tris (pH 8.3), 1.5 mmol/liter $MgCl_2$, 0.2 mmol/liter of each dNTP, 1 U of *Taq* DNA polymerase, and 200 pmol/liter of each primer. DNA was amplified under the following conditions: initial denaturation of 96°C for 2 min, followed by 32 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 3 min. PCR product (20 μl) was digested with 10 U of Mse I (New England Biolabs) at 37°C for 3 hours. The PCR products were size-fractionated on 3% agarose gels, stained with ethidium bromide, and visualized on an ultraviolet transilluminator.
38. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990).
39. K. Osoegawa *et al.*, *Genome Res.* **10**, 116 (2000).
40. I. Dubchak *et al.*, *Genome Res.* **10**, 1304 (2000).
41. G. G. Loots *et al.*, *Science* **288**, 136 (2000).
42. C. Mayor *et al.*, *Bioinformatics* **16**, 1046 (2000).
43. A. Lupas, M. Van Dyke, J. Stock, *Science* **252**, 1162 (1991).
44. H. Nielsen, J. Engelbrecht, S. Brunak, G. von Heijne, *Protein Eng.* **10**, 1 (1997).
45. C. C. Allain, L. S. Poon, C. S. Chan, W. Richmond, P. C. Fu, *Clin. Chem.* **20**, 470 (1974).
46. E. M. Beasley, R. M. Myers, D. R. Cox, L. C. Lazzeroni, *PCR Applications* (Academic Press, San Diego, CA, 1999).
47. D. A. Nickerson, V. O. Tobe, S. L. Taylor, *Nucleic Acids Res.* **25**, *2745 (1997)*.
48. B. Ewing, P. Green, *Genome Res.* **8**, 186 (1998).
49. C. A. Mein *et al.*, *Genome Res.* **10**, 330 (2000).
50. R. M. Krauss, D. M. Dreon, *Am. J. Clin. Nutr.* **62**, 478S (1995).
51. D. M. Dreon, H. A. Fernstrom, P. T. Williams, R. M. Krauss, *Arterioscler. Thromb. Vasc. Biol.* **17**, 707 (1997).
52. Animals were killed, and tissues were harvested for either total RNA isolation by using the RNAeasy-midi protocol (Qiagen) or for poly(A)$^+$ mRNA isolation by using the FastTrack 2.0 system (Invitrogen, Carlsbad, CA). About 10 μg of total RNA or 2 μg of poly(A)$^+$ mRNA were separated in 1.0% agarose by gel electrophoresis and the RNA was transferred to a charged nylon membrane (Ambion, Austin, TX). The RNA blots were hybridized with [α-^{32}P]dCTP random-primed *apoAV* probes in ULTRAhyb buffer (Ambion). Probe templates were generated by PCR amplification of liver cDNA with degenerate primers degApoAV-F2-5′-GCGCGTGGTGGGRGAAGACA-3′ and degApoAV-R2-TCGCGCAGCTGGTCCAGGTT-3′. Filters were washed in 2× saline sodium citrate at room temperature for 20 min and in 0.1× SSC at 42°C for 20 min, followed by autoradiography visualization.
53. M. Olivier, unpublished observations.
54. R. M. Krauss, unpublished observations.
55. R. C. Lewontin, *Genetics* **120**, 849 (1988).
56. We thank H. Hobbs, J. Fruchart, A. Plump, C. Prange-Pennacchio and members of the Rubin laboratory for thoughtful discussions; E. Gong, K. Houston, K. Lewis, W. Dean, J.-F. Cheng, I. Dubchak, J. Schwartz, V. Afzal, and X. Yang for technical support; V. Bustos, K. Sheppard, D. Zierten, A. de Witte, R. Freudenberg, J. Bushard, A. Almendras, and A. Indap for assistance with sequencing and genotyping; and P. Blanche, L. Holl, and J. Orr for performing lipoprotein measurements. This work was supported by the National Dairy Promotion and Research Board in cooperation with the National Dairy Council and NIH-NHBLI grant HL-18574 (R.M.K., E.M.R.), the NIH-NHLBI Programs for Genomic Application Grant HL66681 (E.M.R.), through the U.S. Department of Energy under contract no. De-AC03-76SF00098 (E.M.R.), HL-53917 (J.C.C.), and an appointment to the Alexander Hollaender Distinguished Postdoctoral Fellowship Program sponsored by the U.S. Department of Energy, Office of Biological and Environmental Research, and administered by the Oak Ridge Institute for Science and Education (L.A.P.).

30 July 2001; accepted 4 September 2001

Phosphorylation-Dependent Ubiquitination of Cyclin E by the SCF^{Fbw7} Ubiquitin Ligase

Deanna M. Koepp,[1,2,3] Laura K. Schaefer,[1,2,3]* Xin Ye,[1]* Khandan Keyomarsi,[4] Claire Chu,[1] J. Wade Harper,[1] Stephen J. Elledge[1,2,3]†

Cyclin E binds and activates the cyclin-dependent kinase Cdk2 and catalyzes the transition from the G_1 phase to the S phase of the cell cycle. The amount of cyclin E protein present in the cell is tightly controlled by ubiquitin-mediated proteolysis. Here we identify the ubiquitin ligase responsible for cyclin E ubiquitination as SCF^{Fbw7} and demonstrate that it is functionally conserved in yeast, flies, and mammals. Fbw7 associates specifically with phosphorylated cyclin E, and SCF^{Fbw7} catalyzes cyclin E ubiquitination in vitro. Depletion of Fbw7 leads to accumulation and stabilization of cyclin E in vivo in human and *Drosophila melanogaster* cells. Multiple F-box proteins contribute to cyclin E stability in yeast, suggesting an overlap in SCF E3 ligase specificity that allows combinatorial control of cyclin E degradation.

Passage through the cell cycle is controlled by the activity of cyclin-dependent kinases (CDKs) (*1*). Cyclin E is the regulatory subunit of Cdk2 and controls the G_1 to S phase transition, which is rate-limiting for proliferation. Cyclin E is tightly regulated by ubiquitin-mediated proteolysis, which requires phosphorylation on Thr^{380} and Cdk2 activation (*2*–*4*). Failure to properly regulate cyclin E accumulation can lead to accelerated S phase entry (*5*), genetic instability (*6*), and tumorigenesis (*7*). Elucidating the mechanism controlling cyclin E destruction has important implications for understanding control of cell proliferation during development and its subversion by tumorigenesis.

The formation of polyubiquitin-protein conjugates, which are recognized and destroyed by the 26*S* proteasome, involves three components that participate in a cascade of ubiquitin transfer reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a specificity factor (E3) called a ubiquitin ligase (*8*). E3s control the specificity of target protein selection and therefore are key to controlling individual target protein abundance.

The SCF (Skp1/Cullin/F-box protein) comprises a large family of modular E3s that control ubiquitination of many substrates in a phosphorylation-dependent manner (*9*). SCF complexes contain four subunits: Skp1, Cul1 (Cdc53), Rbx1, and an F-box–containing pro-

[1]Department of Biochemistry and Molecular Biology, [2]Department of Molecular and Human Genetics, [3]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX, 77030, USA. [4]Department of Experimental Radiation Oncology, M. D. Anderson Cancer Center, Houston, TX 77030, USA

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: selledge@bcm.tmc.edu

tein. F-box proteins, over 50 of which have been identified in mammals (*10*, *11*), bind Skp1 through the F-box motif (*12*) and mediate substrate specificity of SCF complexes by binding substrates through protein-protein interaction domains, often WD40 repeats or leucine-rich repeats (LRRs) (*13*, *14*).

Several observations suggest that accumulation of cyclin E might be controlled through the SCF pathway. Cyclin E, like many SCF substrates, requires phosphorylation for destruction, and mice lacking Cul1 accumulate cyclin E (*15*, *16*). Because Cul3 mutant mice also show increased amounts of cyclin E (*17*), it is not clear if the effects of either cullin are direct. Stability of cyclin E expressed in *Saccharomyces cerevisiae* depends on phosphorylation of Thr^{380}, suggesting a conserved mechanism in yeast and mammals (*3*). Therefore, we exploited the genetics of *S. cerevisiae* to explore the contribution of SCF to cyclin E ubiquitination. We used a stability assay to perform a pulse-chase analysis of cyclin E protein in wild-type and *skp1-11*, *cdc34-2*, or *cdc53-1* mutants. To prevent cell cycle position effects, we arrested cells in S phase by addition of 200 mM hydroxyurea throughout the experiment. Cyclin E was unstable in wild-type cells but stabilized in SCF mutant cells (Fig. 1A). We examined cyclin E stability in yeast F-box protein mutant strains *cdc4-1*, *grr1*, *ydr219*, *yjl149*, *yml088/ufo1*, *ynl230/ela1*, *ynl311*, and *yor080/dia2*. Cyclin E was stabilized in *cdc4-1* strains to an extent similar to that seen with core SCF mutants and was also stabilized in *yor080* mutants (Fig. 1A). Cdc4 and Yor080 contain WD40 and LRR motifs, respectively. We incubated recombinant SCF^{Cdc4} and SCF^{Yor080} complexes with recombinant cyclin E–Cdk2, E1, Cdc34 (E2), Ub, and adenosine triphosphate (ATP) (Fig. 1B). Ubiquitination of cyclin E was detected with both complexes in an F-box– and ubiquitin-dependent manner (Fig. 1B). Ubiquitination was also stimulated by phosphorylated cyclin E as it was largely prevented when catalytically inactive cyclin E–$Cdk2^{KD}$ complexes were used as substrate (Fig. 1B).

To find the mammalian F-box protein that recognizes cyclin E, we surveyed previously identified F-box proteins (*11*) for those that bound cyclin E either after coexpression in insect cells or in vitro using ^{35}S-methionine–labeled translation products and immobilized glutathione *S*-transferase (GST)–cyclin E–CDK2 complexes. Seventeen F-box proteins were tested, including 16 that contained either WD40 or LRR motifs (*18*). Of these, only the WD40-containing Fbw7 (*19*) bound specifically to GST–cyclin E–Cdk2 but not to GST alone (Fig. 1C) (*20*). This interaction was specific

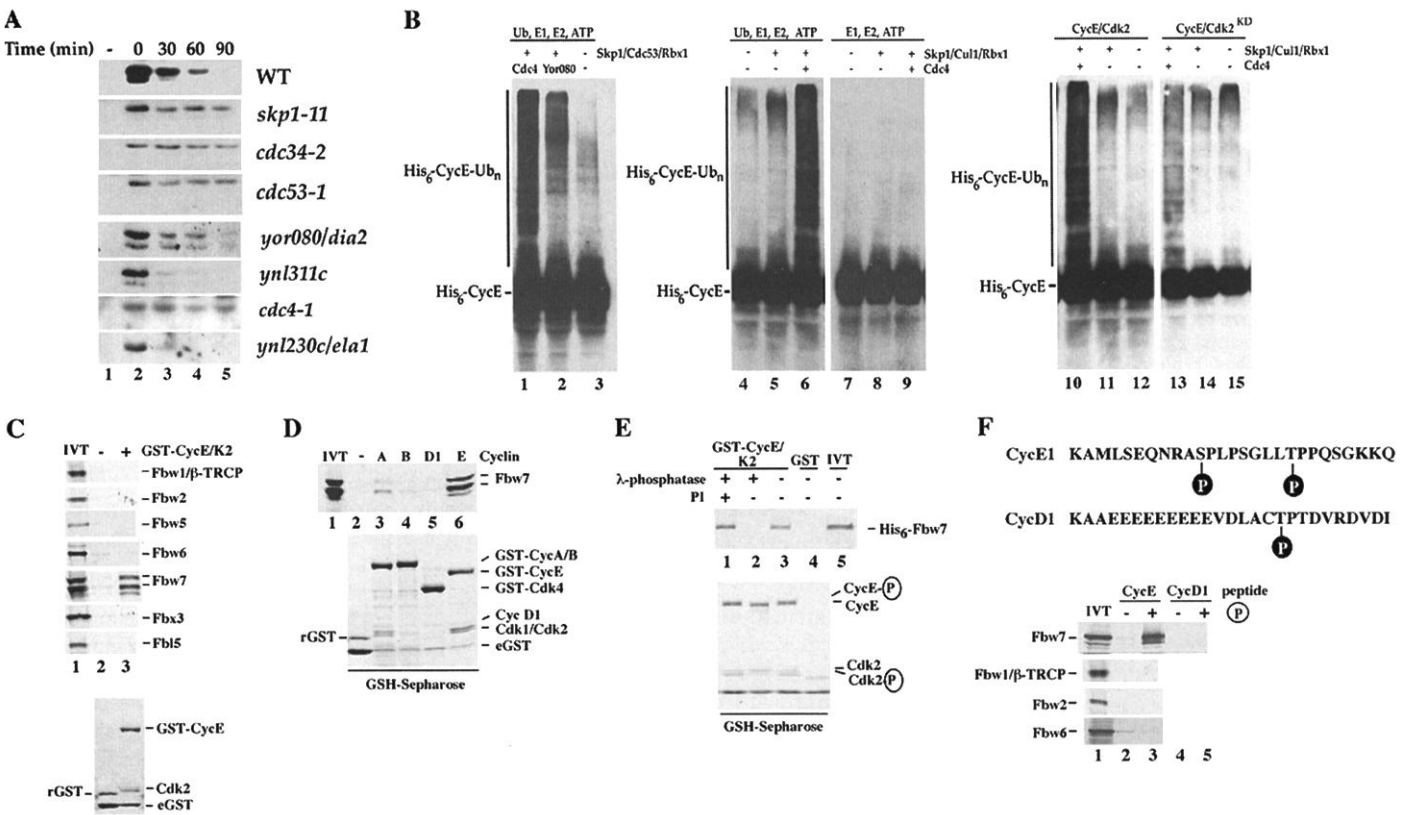

Fig. 1. Interaction between cyclin E and SCF components in yeast and mammalian cells. (**A**) Stabilization of cyclin E in *skp1-11*, *cdc34-2*, *cdc53-1*, *cdc4-1*, and *yor080* mutants (*12*, *30*). Strains of the indicated genotypes were grown in medium containing raffinose; cyclin E expression was induced for 1 hour by galactose addition and at time = 0 was repressed by addition of glucose. Cells were harvested at the indicated times, and the abundance of cyclin E was determined by immunoblotting. Extracts from uninduced cells are shown in lane 1. WT, wild type. (**B**) Cyclin E is ubiquitinated in vitro by SCF complexes. SCF^{Cdc4} or SCF^{Yor080} complexes were purified from insect cells (*13*) and supplemented with ubiquitin (Ub), E1, Cdc34 (E2), and ATP, as indicated, before addition of His_6–cyclin E–Cdk2 purified from insect cells (*13*). (**C**) GST–cyclin E–Cdk2 binds Fbw7. Immobilized GST–cyclin E–Cdk2 (lane 3) or GST (lane 2) was incubated with in vitro–translated F-box proteins (*31*, *32*). Lane 1 contains in vitro translation (IVT) product (33% of input). The bottom panel shows GST–cyclin E–Cdk2 and GST as detected by Coomassie staining. The positions of endogenous insect cell GST protein (eGST) and recombinant GST (rGST) are indicated. (**D**) Fbw7 preferentially binds cyclin E–Cdk2. The indicated Cdk complexes (lanes 2 to 6) were purified from insect cells and used for in vitro binding with Fbw7 as above. Cyclins were fused to GST for affinity purification, except for cyclin D1 where GST-Cdk4 is used. (**E**) Phosphorylation-dependent association of Fbw7 with cyclin E–Cdk2. Immobilized GST–cyclin E–Cdk2 was treated with λ-phosphatase in the presence (lane 1) or absence (lane 2) of phosphatase inhibitors (PI) before in vitro binding to His_6-Fbw7. Untreated GST–cyclin E–Cdk2 (lane 3) and GST (lane 4) were used as controls. Binding reactions were performed as in (C). (**F**) Immobilized cyclin E– or cyclin D–derived peptides with or without phosphorylation were incubated with Fbw7, Fbw1 (β-TRCP), Fbw2, and Fbw6 IVT products as in (C). The peptide sequence and sites of phosphorylation (P) are indicated (*33*).

for cyclin E as Fbw7 did not interact tightly with other cyclin-Cdk complexes (Fig. 1D). The interaction between Fbw7 and cyclin E was phosphorylation-dependent (Fig. 1E). Furthermore, Fbw7 bound specifically to a phosphopeptide containing the region of cyclin E required genetically for ubiquitination (Fig. 1F). Thus, the properties of Fbw7 are consistent with the predicted properties of a cyclin E ubiquitin ligase.

The mouse and human Fbw7 cDNA encodes a protein of 627 amino acids containing seven WD40 repeats (Fig. 2, A and B). The presence of stop codons in all three reading frames of the 5′ untranslated region (UTR) indicates that the encoded open reading frame (ORF) is full-length. Database searches revealed substantial sequence similarity with *Caenorhabditis elegans sel-10*, which is involved in the presenilin (*sel-12*) and Notch/lin-12 pathways (*21*), and the predicted protein encoded by *Drosophila melanogaster* CG15010 (*DmFbw7*). Among *S. cerevisiae* F-box proteins, Fbw7 is 28% identical to Cdc4 (Fig. 2A). The relationship between *sel-10* and a partial cDNA containing two COOH-terminal WD40 repeats from Fbw7 was noted previously (*21*). The extreme NH_2-terminus of Fbw7 contains a 23-residue stretch (residues 7 to 29) of highly hydrophobic amino acids recognized by the SMART protein analysis program as a transmembrane domain (*22*).

To examine the importance of the WD40 motifs in cyclin E recognition, we searched for basic residues located on the surface of the β-propeller structure that are conserved in Hs-Fbw7, Cdc4, Sel-10, and DmFbw7 but not in other Fbw proteins. Such residues would be candidates for phosphorylation-dependent interaction with ubiquitination targets. Arg^{417}, Arg^{457}, and Arg^{495}, located in WD40 repeats 3, 4, and 5, met these criteria (Fig. 2, A and D). These residues were independently replaced with alanine, and the resulting proteins were tested for binding to GST–cyclin E in vitro. Mutation of Arg^{417} or Arg^{457} abolished binding to cyclin E, whereas mutation of Arg^{495} reduced binding (Fig. 2C).

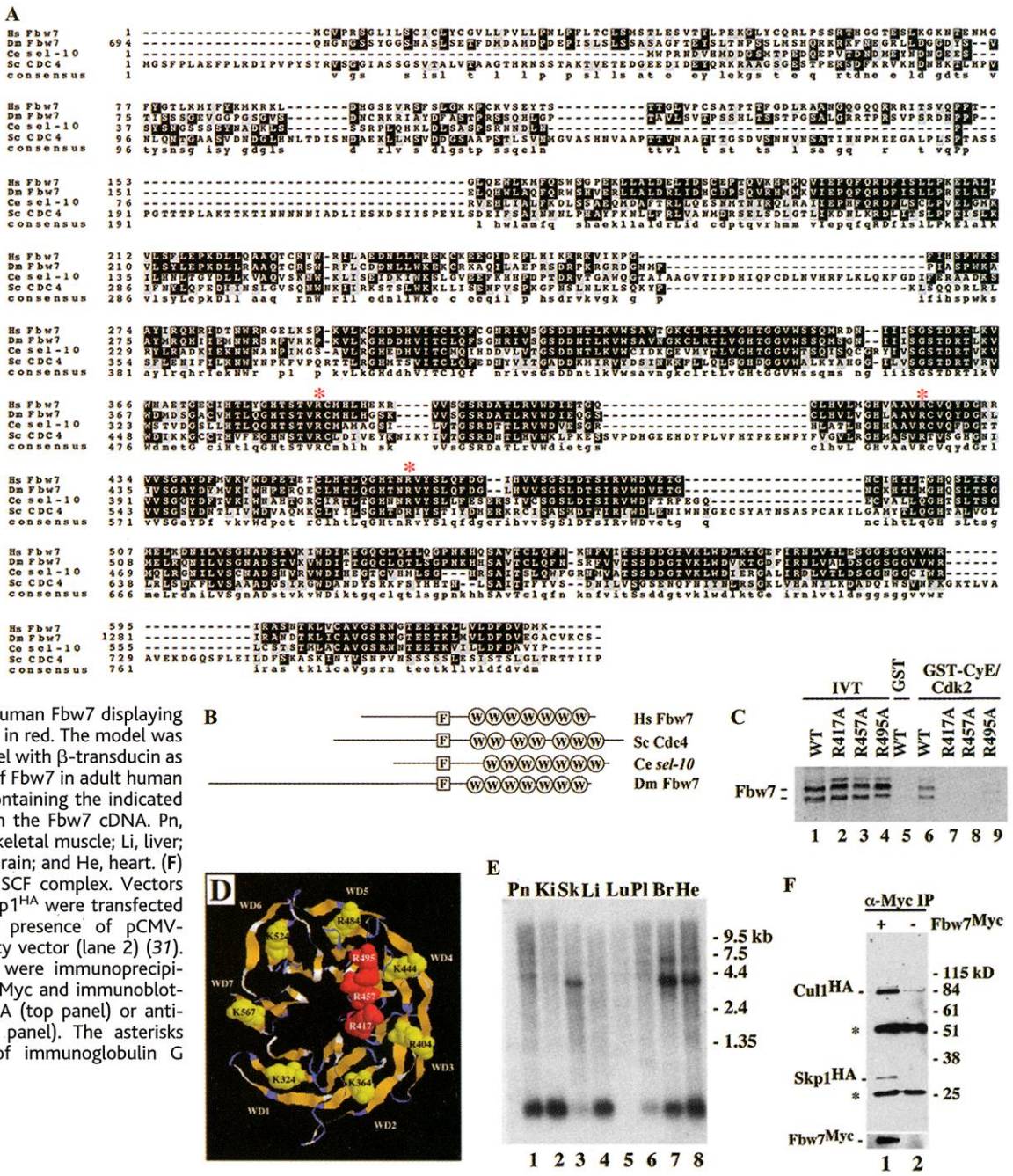

Fig. 2. Characterization of the WD40-repeat–containing F-box protein, Fbw7. (**A**) Conservation between human (Hs) Fbw7 and *C. elegans* (Ce) *sel-10*, *S. cerevisiae* (Sc) Cdc4, and *D. melanogaster* (Dm) Fbw7 (*33*). Identical residues are shaded black and similarities are shaded gray. Asterisks indicate conserved arginine residues required for cyclin E binding. (**B**) Domain structures of Fbw7 homologs. F, F-box; W, WD40 repeat. (**C**) Three surface arginines on Fbw7 are required for binding cyclin E. Wild-type (WT) and mutant Fbw7 IVT products were used for binding with GST–cyclin E–Cdk2 (lanes 6 to 9) or GST (lane 5). One-third of the input is shown (lanes 1 to 4). (**D**) Model of the β-propeller structure of human Fbw7 displaying Arg^{417}, Arg^{457}, and Arg^{495} in red. The model was generated with Swissmodel with β-transducin as template. (**E**) Expression of Fbw7 in adult human tissues. Northern blots containing the indicated mRNAs were probed with the Fbw7 cDNA. Pn, pancreas; Ki, kidney; Sk, skeletal muscle; Li, liver; Lu, lung; Pl, placenta; Br, brain; and He, heart. (**F**) Fbw7 assembles into an SCF complex. Vectors expressing $Cul1^{HA}$ and $Skp1^{HA}$ were transfected into 293T cells in the presence of $pCMV\text{-}Fbw7^{Myc}$ (lane 1) or empty vector (lane 2) (*31*). After 48 hours, extracts were immunoprecipitated with antibodies to Myc and immunoblotted with antibodies to HA (top panel) or antibodies to Myc (bottom panel). The asterisks indicate the positions of immunoglobulin G heavy and light chains.

Fbw7 mRNA is abundant in adult brain, heart, and skeletal muscle, tissues with a high percentage of terminally differentiated cells (Fig. 2E). Cotransfection of vectors encoding Myc-tagged Fbw7 with hemagglutinin (HA)-tagged Cul1 and HA-tagged Skp1 in 293T cells allowed detection of Fbw7 in SCF complexes, consistent with involvement of Fbw7 in ubiquitination (Fig. 2F).

We tested cyclin E ubiquitination in reticulocyte lysates in which either Fbw7 or Fbw2 had been translated. Ubiquitinated forms of cyclin E were observed in the presence of Fbw7 but not Fbw2 (Fig. 3A). Fbw7-dependent ubiquitination of cyclin E was also achieved in more purified systems. His_6-Fbw7 was affinity-purified on immobilized GST–cyclin E–Cdk2 (Fig. 3, B and C) or antibodies to His_6 (Fig. 3D) and used in ubiquitination reactions. Cyclin E ubiquitination was dependent on Fbw7 (Fig. 3, B and C) and was stimulated by Cul1-Rbx1 (Fig. 3, B to D). A small fraction of Fbw7 was associated with endogenous Cul1 in reticulocyte lysates (*20*). The pattern of conjugates was distinctly different when a form of ubiquitin that cannot undergo polyubiquitination (GST-Ub^{RA}) was included in the reaction mixture (Fig. 3C), indicating that the larger forms of cyclin E are ubiquitin conjugates. The ubiquitination reaction was also stimulated by phosphorylation of cyclin E (Fig. 3D) and was reduced when the cyclin E Thr^{380} → Ala (T380A) mutant was used as substrate (Fig. 3E).

If Fbw7 is rate-limiting for controlling cyclin E abundance, overexpression of Fbw7 should lead to decreased amounts of cyclin E. To test this, we transfected 293T cells with vectors encoding cytomegalovirus (CMV) promoter–driven cyclin E, Cdk2, and either Fbw7 or empty vector and assayed cyclin E amounts by immunoblotting. Cells cotransfected with Fbw7 reproducibly had smaller amounts of cyclin E but constant amounts of Cdk2 (Fig. 4A).

Conversely, inhibition of Fbw7 should lead to increased accumulation of cyclin E. To test this, we used the small interfering RNA (siRNA) technique to reduce expression of Fbw7 in HeLa cells (*23*). Cells transfected with a double-stranded RNA (dsRNA) oligo corresponding to Fbw7 showed increased accumulation of cyclin E when compared with cells transfected with a control dsRNA oligo (Fig. 4B). Amounts of Cdk2 and bulk Cdk2 activity remained unaffected (Fig. 4B) (*20*). The amount of p27 was similar in both Fbw7- and green fluorescent protein (GFP)–inhibited cells at the 48-hour time point, indicating that the accumulation of cyclin E in Fbw7-inhibited cells was not substantially influenced by p27 (*20*). To assess the effect of Fbw7 on cyclin E stability, we used the siRNA-inhibited cells for a pulse-chase analysis of cyclin E (*2*). Cells were labeled in vivo with ^{35}S-methionine, samples were taken at the indicated times after replacement with medium containing unlabeled methionine, and cyclin E was immunoprecipitated (Fig. 4C). In the GFP siRNA cells, cyclin E was unstable, whereas in Fbw7-inhibited cells, cyclin E remains stable for the course of the experiment. Immunoblotting of the immunoprecipitates indicated that cyclin E amounts remained constant throughout the experiment.

We also used the RNA interference (RNAi) technique to ablate Fbw7 in *D. melanogaster* (S2) cells (*24*). Transfection of S2 cells with dsRNAs corresponding to various portions of the *DmFbw7* gene reduced amounts of DmFbw7 mRNA (Fig. 4D) and increased accumulation of cyclin E protein but not that of a control protein, Mle1 (Fig. 4D). In contrast, amounts of cyclin E mRNA were unaltered or slightly reduced, indicating that DmFbw7 regulates cyclin E through a posttranscriptional mechanism. Control dsRNAs had no effect on DmFbw7 or cyclin E (Fig. 4E). RNAi with the COOH-terminal fragment of Fbw7 was less efficient in destabilizing Fbw7 mRNA; thus, smaller increases in cyclin E accumulation were observed.

In this report, we show that SCF^{Fbw7}-related ligases control the stability of cyclin E in a manner conserved through evolution. The finding that different E3s can control cyclin E levels in yeast may have implications for control of cell proliferation in mammals. Such a role would allow multiple signals to be independently integrated through different E3s to control cyclin E levels and cell proliferation. This could allow tissues to exert combinatorial control of proliferation and differentiation, consistent with the tissue-specific expression of

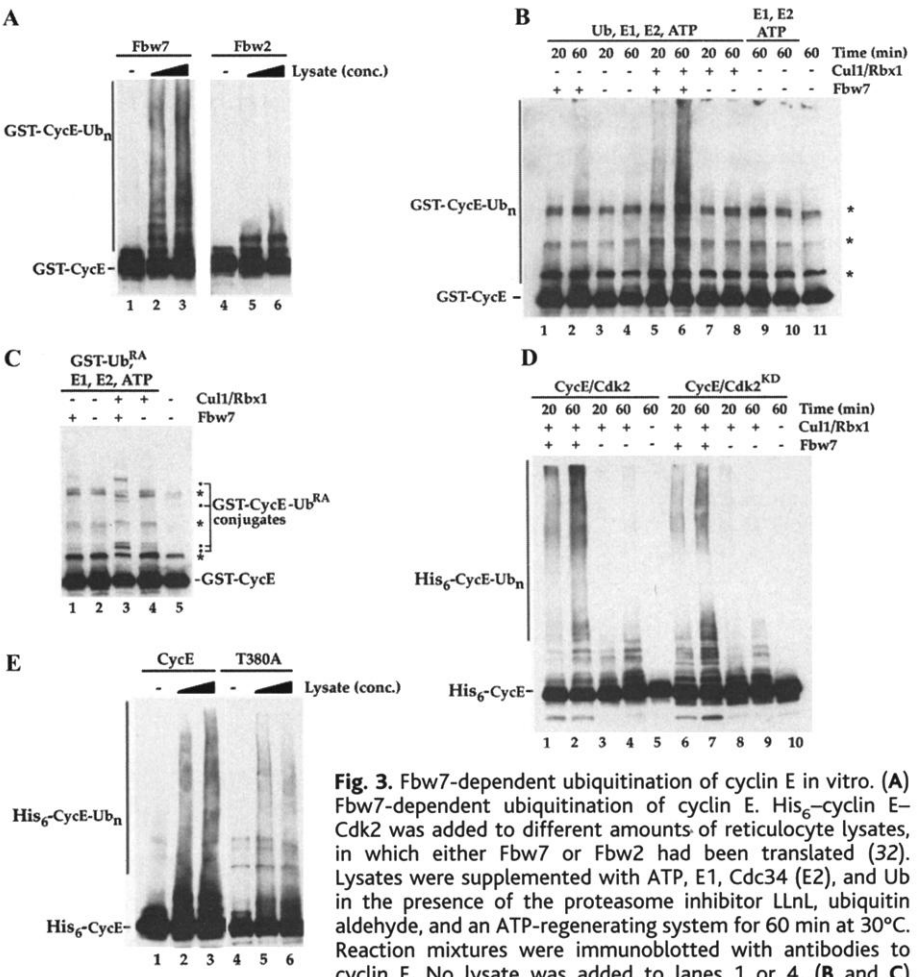

Fig. 3. Fbw7-dependent ubiquitination of cyclin E in vitro. (**A**) Fbw7-dependent ubiquitination of cyclin E. His_6–cyclin E–Cdk2 was added to different amounts of reticulocyte lysates, in which either Fbw7 or Fbw2 had been translated (*32*). Lysates were supplemented with ATP, E1, Cdc34 (E2), and Ub in the presence of the proteasome inhibitor LLnL, ubiquitin aldehyde, and an ATP-regenerating system for 60 min at 30°C. Reaction mixtures were immunoblotted with antibodies to cyclin E. No lysate was added to lanes 1 or 4. (**B** and **C**) Ubiquitination of GST–cyclin E by prebound His_6-Fbw7. (B) Immobilized GST–cyclin E–Cdk2 was incubated with reticulocyte extracts in the presence or absence of Fbw7. Beads were supplemented with E1, Cdc34 (E2), ATP, and either ubiquitin (Ub; 100 μg/ml) or GST-Ub^{RA} (100 μg/ml). Where indicated, 50 ng of a purified Cul1-Rbx1 complex was added. The asterisks indicate the positions of three proteins that cross react with the monoclonal antibodies to cyclin E. (C) As in (B), but GST-Ub^{RA} was used in place of ubiquitin. (**D**) Cyclin E phosphorylation enhances ubiquitination of cyclin E by SCF^{Fbw7}. Reticulocyte lysates with or without His_6-Fbw7 were immunoprecipitated with antibodies to His tag, supplemented with cyclin E–Cdk2 (or cyclin E–$Cdk2^{KD}$), E1, Cdc34 (E2), ubiquitin, and ATP and incubated at room temperature for the indicated time. Samples were treated as in (B). (**E**) Phophorylation of Thr^{380} enhances ubiquitination of cyclin E. Reactions were performed as in (A), but cyclin E T380A was also used as substrate.

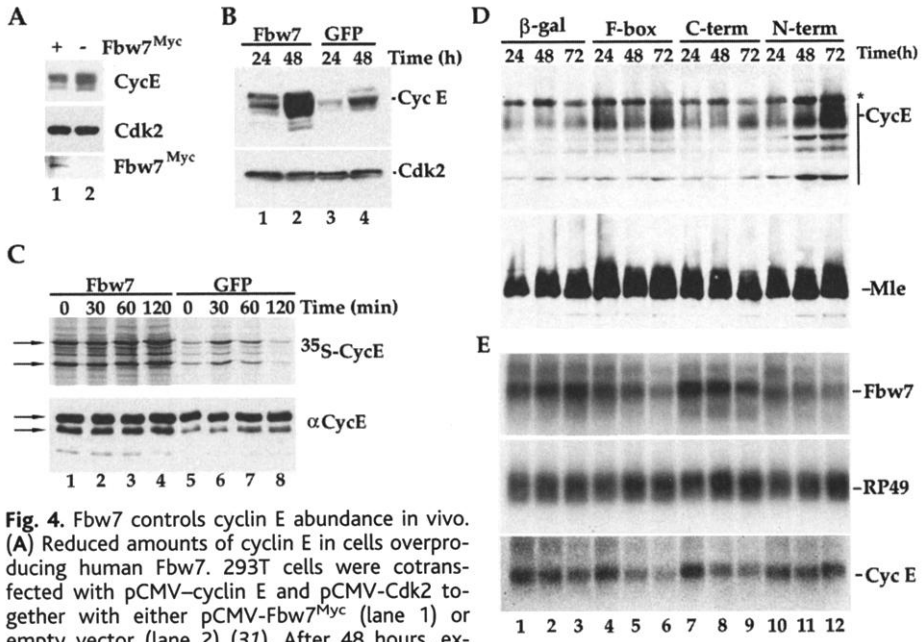

Fig. 4. Fbw7 controls cyclin E abundance in vivo. (**A**) Reduced amounts of cyclin E in cells overproducing human Fbw7. 293T cells were cotransfected with pCMV–cyclin E and pCMV-Cdk2 together with either pCMV-Fbw7^{Myc} (lane 1) or empty vector (lane 2) (*31*). After 48 hours, extracts were prepared and immunoblotted with antibodies to Cdk2, cyclin E, or Myc. (**B**) Accumulation of cyclin E in HeLa cells transfected with Fbw7 siRNA but not GFP siRNA. Cells were transfected as described (*23*, *34*). At the indicated times, cells were harvested and cell lysates were generated. Samples were immunoblotted with antibodies to cyclin E or Cdk2. (**C**) Cyclin E is stable in Fbw7-inhibited cells. Cells were transfected as in (B), and pulse-chase analysis was performed as described (*2*). Medium containing unlabeled methionine was added at time = 0. Samples were also immunoblotted with monoclonal antibodies to cyclin E (bottom panel). Arrows indicate the two major forms of cyclin E. (**D** and **E**) Accumulation of *DmCycE* in response to ablation of *DmFbw7* by RNA interference. S2 cells were transfected with dsRNA corresponding to the NH_2-terminal (N-term), COOH-terminal (C-term), or F-box region of *DmFbw7* or against β-galactosidase (β-gal) as a control (*34*). At the indicated times, cells were harvested and used to generate protein extracts and total RNA. (D) Cell extracts were immunoblotted with polyclonal antibodies against *DmCycE* or *maleless* (Mle). (E) Messenger RNA was subjected to Northern blotting with probes directed toward *DmFbw7*, *DmCycE*, or a ribosomal RNA (RP49).

Fbw7. Cells lacking the F-box protein Skp2 also accumulate cyclin E (*25*). However, this effect may be an indirect result of the accumulation of the Skp2 substrate, p27 (*26*, *27*). Individual E3s often control the ubiquitination of multiple substrates (*9*); therefore, controlling accumulation of cyclin E through expression of a particular E3 may limit the function of other signaling pathways as a consequence. Thus, using different E3s to control cyclin E might lead to regulation of different constellations of signaling pathways in a tissue-specific manner. It is likely that Fbw7 controls the ubiquitination of other proteins in addition to cyclin E. Putative substrates include Notch and Presenilin proteins, as the *C. elegans* homolog *sel-10* has been implicated in the control of both Notch and Presenilin signaling (*21*, *28*).

As a negative regulator of cyclin E, Fbw7 is a potential tumor suppressor. Consistent with this, we have observed that amounts of Fbw7 mRNA are decreased in breast tumor lines that have increased amounts of cyclin E (see supplemental Web figure 1 on *Science* Online at www.sciencemag.org/cgi/content/full/294/5540/173/DC1). Thus far, we have not identified mutations in the Fbw7 gene in these or other tumors. However, Fbw7 maps to 4q32, a site of loss of heterozygosity in a number of cancers (*29*). Additional studies will be required to resolve Fbw7's role in tumorigenesis.

References and Notes

1. C. J. Sherr, J. M. Roberts, *Genes Dev.* **13**, 1501 (1999).
2. B. E. Clurman, R. J. Sheaff, K. Thress, M. Groudine, J. M. Roberts, *Genes Dev.* **10**, 1979 (1996).
3. K. A. Won, S. I. Reed, *EMBO J.* **15**, 4182 (1996).
4. S. V. Ekholm, S. I. Reed, *Curr. Opin. Cell Biol.* **12**, 676 (2000).
5. M. Ohtsubo, J. M. Roberts, *Science* **259**, 1908 (1993).
6. C. H. Spruck, K. A. Won, S. I. Reed, *Nature* **401**, 297 (1999).
7. K. Keyomarsi, D. Conte Jr., W. Toyofuku, M. P. Fox, *Oncogene* **11**, 941 (1995).
8. A. Hershko, H. Heller, S. Elias, A. Ciechanover, *J. Biol. Chem.* **258**, 8206 (1983).
9. D. M. Koepp, J. W. Harper, S. J. Elledge, *Cell* **97**, 431 (1999).
10. C. Cenciarelli *et al.*, *Curr. Biol.* **9**, 1177 (1999).
11. J. T. Winston, D. M. Koepp, C. Zhu, S. J. Elledge, J. W. Harper, *Curr. Biol.* **9**, 1180 (1999).
12. C. Bai *et al.*, *Cell* **86**, 263 (1996).
13. D. Skowyra, K. L. Craig, M. Tyers, S. J. Elledge, J. W. Harper, *Cell* **91**, 209 (1997).
14. Y. G. Hsiung *et al.*, *Mol. Cell. Biol.* **21**, 2506 (2001).
15. M. J. Dealy *et al.*, *Nature Genet.* **23**, 245 (1999).
16. Y. Wang *et al.*, *Curr. Biol.* **9**, 1191 (1999).
17. J. D. Singer, M. Gurian-West, B. Clurman, J. M. Roberts, *Genes Dev.* **13**, 2375 (1999).
18. F-box proteins tested for cyclin E binding include Fbw1 (β-TRCP), Fbw2 (MD6), Fbw5, Fbw6, Fbw7, Fbl1 (Skp2), Fbl2, Fbl3a, Fbl4, Fbl5, Fbl6, Fbl7, Fbl8, Fbl11, Fbl12, and Fbx3. The Fbw6 construct for in vitro translation was generated from an expressed sequence tag (EST) previously named Fbx29 (GenBank accession number AF176707) (*11*).
19. We previously identified the F-box motif in EST clone AI836688 as Fbx30 (*11*). Further sequence analysis of a longer cDNA (GenBank accession number AY033553) revealed WD40 repeats, and it was renamed Fbw7 according to convention (*10*, *11*).
20. D. M. Koepp, L. K. Schaefer, X. Ye, K. Keyomarsi, J. W. Harper, S. J. Elledge, unpublished results.
21. E. J. Hubbard, G. Wu, J. Kitajewski, I. Greenwald, *Genes Dev.* **11**, 3182 (1997).
22. See http://smart.embl-heidelberg.de.
23. S. M. Elbashir *et al.*, *Nature* **411**, 494 (2001).
24. S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, *Nature* **404**, 293 (2000).
25. K. Nakayama *et al.*, *EMBO J.* **19**, 2069 (2000).
26. A. C. Carrano, E. Eytan, A. Hershko, M. Pagano, *Nature Cell Biol.* **1**, 193 (1999).
27. C. Spruck *et al.*, *Mol. Cell* **7**, 639 (2001).
28. G. Wu, E. J. Hubbard, J. K. Kitajewski, I. Greenwald, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15787 (1998).
29. S. Knuutila *et al.*, *Am. J. Pathol.* **155**, 683 (1999).
30. Description of the yeast F-box protein mutants will be presented elsewhere. The Fbw7 ORF lacking the stop codon was amplified by polymerase chain reaction from the EST 3347354 and inserted into pCR2.1 and pcDNA3.1 Myc-His. The His_6-Fbw7 vector was generated similarly except that the amplified ORF contained a stop codon.
31. 293T cells were transfected with Lipofectamine (Invitrogen). For association of cyclin E–Cdk2 and other cyclins with F-box proteins in vitro, immobilized GST-cyclin-Cdk or GST was incubated (1 hour, 4°C) with ^{35}S-methionine–labeled in vitro–translated F-box proteins and washed four times before electrophoresis. In some experiments, GST–cyclin E–Cdk2 was treated with 400 units of λ-phosphatase for 60 min at 30°C and then washed twice before binding.
32. SCF complexes were assembled by coexpression of Flag-Skp1, Cdc53, Rbx1, and either Cdc4 or Yor080 in insect cells and used as described (*13*). Some SCF^{Cdc4} complexes were assembled on GSH-Sepharose (Amersham Pharmacia) with human GST-Cul, Skp1, and Rbx1. His_6-tagged E1 enzyme and Cdc34-E2 were purified from yeast and bacteria, respectively. For ubiquitination in crude extracts, reticulocyte lysates programmed with either Fbw7 or Fbw2 were supplemented with an ATP regeneration system, E1 (100 ng), Cdc34 (E2) (300 ng), ubiquitin (5 μg), and His_6-cyclin E-Cdk2. For ubiquitination of GST–cyclin E prebound to SCF^{Fbw7}, GST–cyclin E–Cdk2 was incubated with reticulocyte lysate programmed with Fbw7 or lysate lacking Fbw7 at 4°C (60 min). Washed complexes were supplemented as described above. To examine ubiquitination by immune complexes containing His_6-Fbw7, we supplemented in vitro–translated His_6-Fbw7 immobilized on antibody to His tag beads as described above.
33. 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.
34. RNA interference was performed as described (*24*) except that Effectene (Qiagen) was used for transfection. dsRNAs corresponded to nucleotides 1 to 505 (NH_2-terminal), 2678 to 3159 (F-box), and 3469 to 3981 (COOH-terminal) of the Fbw7 coding region. The siRNA oligo corresponded to nucleotides 713 to 735 of the human Fbw7 coding region.

35. We thank C. Lehner, M. Kuroda, T. Orr-Weaver, R. Duronio, M. Tyers, J. Roberts, I. Greenwald, A. Newman, H. Zheng, and S. Reed for gifts of reagents, plasmids, and helpful discussions and D. Liu for technical assistance. D.M.K. is a fellow with the Helen Hay Whitney Foundation. This work was supported by grants from the NIH and the Department of Defense. S.J.E. is an Investigator with the Howard Hughes Medical Institute.

8 August 2001; accepted 13 August 2001
Published online 30 August 2001;
10.1126/science.1065203
Include this information when citing this paper.