normal development. The mutations that disrupt its function *in vivo* reduce binding to Cyclin E and delay a decay in levels of Cyclin E. It has previously been shown that Cyclin E can be degraded in mammalian cells by direct interaction with a cullin[17–19]. Our findings, together with the observation that mutations in the *C. elegans* genes *cul1* and *lin-23* (which encode a cullin and an F-box protein respectively) have increased cell divisions[20,21], highlight the importance of SCF-mediated degradation in regulating cell proliferation through Cyclin E. Because *ago* RNA is expressed in a dynamic pattern, our results indicate that degradation of Cyclin E is not constitutive *in vivo*. Dynamic expression of Ago provides another mechanism by which cyclin/cdk activity and cell proliferation can be regulated during development. Finally, we implicate impaired proteolysis of Cyclin E in the pathogenesis of human cancers. □

Methods

Fly stocks

All crosses were conducted at 25 °C. *w; FRT80B* males were mutagenized with ethylmethanesulphonate (EMS), then crossed to *y w eyFLP;FRT80B P[mini-w, arm-LacZ]* virgin females (stocks a gift of J. Treisman). Males with more white than red eye tissue were selected and maintained as balanced stocks. Alleles of *archipelago* isolated were ago^1, ago^3 and ago^4. Other stocks were *y w hsFLP;FRT80B P[πMyc] P[w y], w;FRT80B P[mini-w] P[UbiGFP]/TM6B* (a gift of B. Edgar), *GMR-p35* (a gift of K. White) and *w;* $cycE^{JP}$ (a gift of H. Richardson).

Microscopy, immunohistochemistry, flow cytometry

Adult eyes were photographed submerged in mineral oil. Imaginal disc tissue of the indicated genotypes was fixed and stained for Cyclin E and β-galactosidase as described previously[3]. Images were collected on a Carl Zeiss Axiovert 100M Confocal microscope. The mouse monoclonal antibody to *Drosophila* type I Cyclin E and the Cyclin B antibody were gifts of H. Richardson and C. Lehner, respectively. The antisense *Drosophila cyclin E* probe was derived from full-length type I *cyclin E* complementary DNA. The antisense *ago* probe was derived from a full-length cDNA. Flow cytometry on third-instar larval wing discs was performed as described previously[3]. For *ago* loss-of-function FACS analysis, the following genotype was used: *y w hsFLP; FRT80B* ago^3*/FRT80B P[mini-w] P[UbiGFP]*.

Molecular biology

For GST-fusion proteins, PCR fragments corresponding to the C-terminal 660 amino acids of the wild-type or *ago* mutant open reading frames were cloned in-frame into the pGEX-2T vector (Amersham Pharmacia). Following induction, equal amounts of intact GST–AgoΔN fusion proteins were incubated with 100 μg of S2 whole-cell extract from cells transfected using the CELLFECTIN reagent (Gibco BRL) with *Drosophila cdk2* and Myc-epitope-tagged *Drosophila* type I *cyclin E* cDNAs cloned into pIE1–4 insect expression vectors (Novagen). Myc-tagged cyclin E protein was detected in western blots using the 9E10 anti-Myc tag monoclonal antibody; human Cyclin E was detected in lysates of human ovarian cancer cell lines synchronized in G1/S by incubation for 36 h in 2 mM thymidine with the HE12 anti-cyclin E monoclonal antibody (both antibodies were a gift of E. Harlow). For northern analysis, 10 μg of total cellular RNA was probed with a 0.9-kilobase *Bam*H1-*Xmn*1 fragment of the human *cyclin E* cDNA.

Characterization of human *ago*

The α and β forms of human *ago* were identified in BAC067826 by exon-prediction programmes. The existence of both forms was confirmed by RT-PCR. An in-frame termination codon is present 75 nucleotides upstream from the initiating ATG in the β cDNA. We were unable to locate an upstream in-frame termination codon in the α cDNA. These sequences have been deposited in GenBank. Human *ago* was amplified by RT-PCR in six overlapping fragments. PCR products were resolved by gel electrophoresis and sequenced directly with the BigDyeTerminator kit (Applied Biosystems) and analysed on an ABI300 genetic analyser. In addition to eight primary tumours, the cancer cell lines analysed were: breast (MCF7ADR, MDAMB435, T47D, BT483, MDAMB436, MDAMB453, MDAMB468, MDAMB415, MDAMB231, MDAMB175, MDAMB157, HS157, HS467T, HS496T, HS578T, UACC893, BT549), ovarian (ES-2, IGROV-1, MDAH2774, OV1063, OVCAR3, OVCAR4, OVCAR5, OVCAR8, SKOV3, SW626), lung (NCIH460, NCI522, HOP92), central nervous system (SF295, SNB19, U251), leukaemia (CCRF-CEM, K562, MOLT4, RPMI-8226, SR), colon (COLO205, HCT116, HCT15), renal (786-0, ACHN, CAKI-1, SN12C, U031), melanoma (LOXMVII, M14, SKMEL2, UACC62) and osteosarcoma (U2OS, SAOS2). The wild-type controls were EBV-immortalized cell lines from normal individuals[22].

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to I.K.H. (e-mail: hariharan@helix.mgh.harvard.edu). The sequences for human Ago have been deposited in GenBank under accession numbers AF411971 (α form) and AF411972 (β form).

Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line

Heimo Strohmaier*, Charles H. Spruck*, Peter Kaiser*, Kwang-Ai Won*, Olle Sangfelt† & Steven I. Reed*

** Department of Molecular Biology, MB-7, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA*
† Department of Oncology/Pathology, CCK R8:03, Karolinska Hospital, 171 76 Stockholm, Sweden

Cyclin E, one of the activators of the cyclin-dependent kinase Cdk2, is expressed near the G_1–S phase transition and is thought to be critical for the initiation of DNA replication and other S-phase functions[1–3]. Accumulation of cyclin E at the G_1–S boundary is achieved by periodic transcription coupled with regulated proteolysis linked to autophosphorylation of cyclin E[4]. The proper timing and amplitude of cyclin E expression seem to

be important, because elevated levels of cyclin E have been associated with a variety of malignancies[5,6] and constitutive expression of cyclin E leads to genomic instability[7]. Here we show that turnover of phosphorylated cyclin E depends on an SCF-type protein-ubiquitin ligase that contains the human homologue of yeast Cdc4, which is an F-box protein containing repeated sequences of WD40 (a unit containing about 40 residues with tryptophan (W) and aspartic acid (D) at defined positions). The gene encoding hCdc4 was found to be mutated in a cell line derived from breast cancer that expressed extremely high levels of cyclin E.

We and others have previously demonstrated that human cyclin E is targeted for ubiquitin-mediated proteolysis in both mammalian and yeast cells by phosphorylation of residue Thr 380 (refs 8, 9). Because the characteristics of cyclin E turnover were found to be similar in mammalian and yeast cells, we used yeast to elucidate the cellular machinery that targets cyclin E for proteolysis in human cells. In yeast, a protein-ubiquitin ligase system known as SCF has been shown to target a number of proteins for ubiquitin-mediated proteolysis in a phosphorylation-dependent manner[10–12]. SCF consists of four subunits: Skp1, Cdc53/Cul-1, Roc1 and one of a family of F-box proteins, which determine substrate specificity[13]. The suggestion that SCF might be involved in turnover of cyclin E is consistent with the observation that levels of cyclin E are elevated in *Cul1*-deficient embryos[14,15]. To determine whether SCF is indeed involved in cyclin E turnover in yeast, *cdc53* and *skp1* thermosensitive mutants were compared with a wild-type strain for cyclin E turnover at the restrictive temperature (Fig. 1a). Cyclin E was stabilized in both the *cdc53* and *skp1* thermosensitive mutants, indicating that SCF activity is required for cyclin E turnover in yeast. The ubiquitin-conjugating (E2) enzyme that usually works in concert with SCF in yeast is Cdc34. Accordingly, *cdc34* mutants were also found to stabilize cyclin E (Fig. 1a). In yeast, the three best-characterized F-box proteins are Cdc4, Grr1 and Met30. Whereas cyclin E turned over at the wild-type rate in a *met30* mutant, it was stabilized in a *cdc4* mutant (Fig. 1a). Thus, in yeast, ubiquitination of cyclin E is most probably mediated by the concerted action of the ubiquitin-conjugating enzyme Cdc34 and the protein-ubiquitin ligase SCF^{Cdc4}. One caveat with this interpretation is that stabilization of the CDK-inhibitor Sic1 in a *cdc4* mutant might prevent phosphorylation of cyclin E, thereby conferring stabilization indirectly. We found, however, that the turnover rate

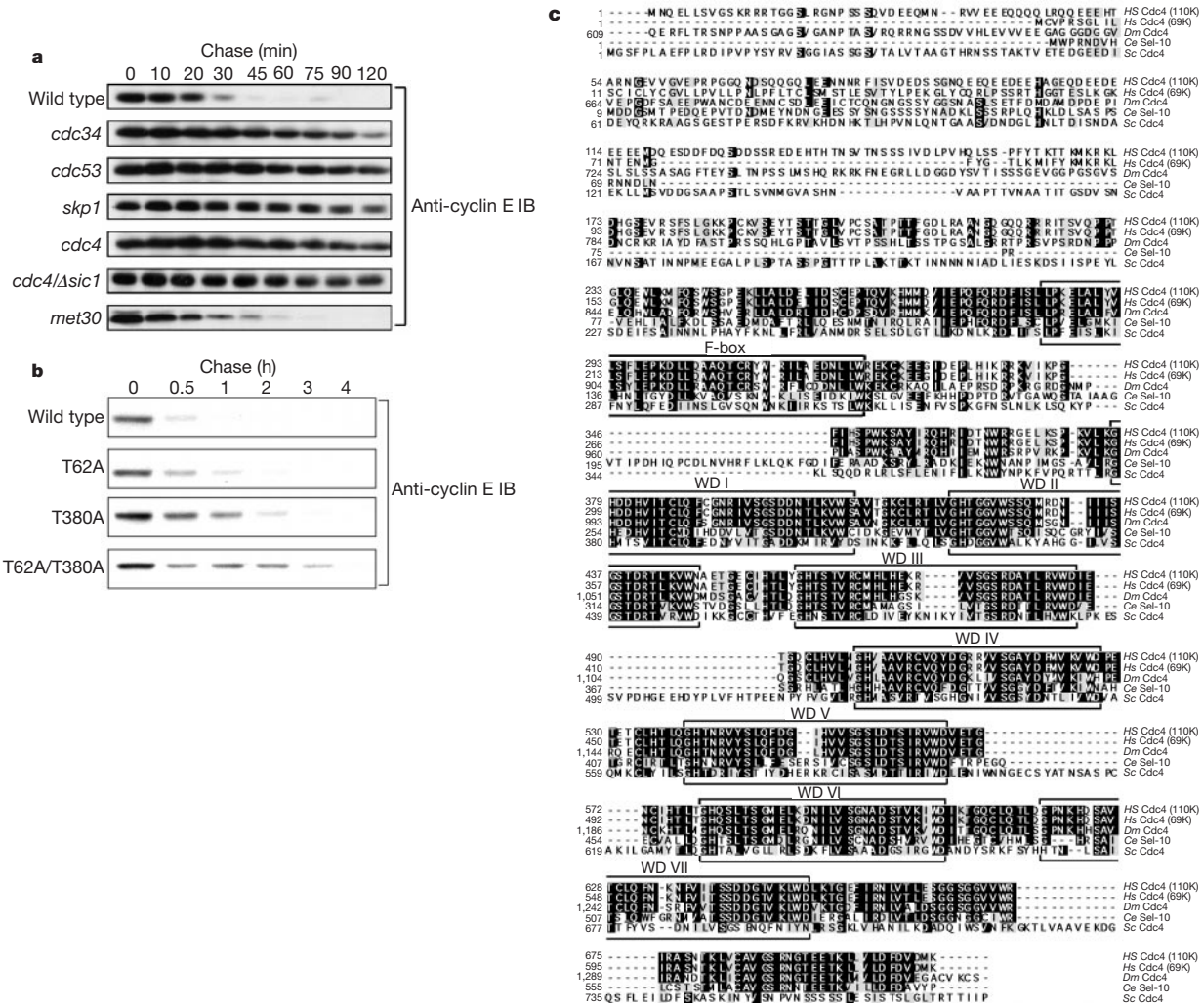

Figure 1 Human cyclin E is targeted for ubiquitin-dependent degradation by the SCF^{Cdc4} protein-ubiquitin ligase in yeast. **a**, Cyclin E was expressed from the inducible *GAL1* promoter in a wild-type strain and in various thermosensitive SCF mutant strains at the restrictive temperature, and the turnover of cyclin E was followed by immunoblotting (IB). **b**, Wild-type cyclin E and phosphorylation-site mutants of cyclin E were expressed from the *GAL1* promoter in yeast. Analysis was as in **a**. **c**, Protein sequence alignment showing (from top to bottom) the two hCdc4 isoforms (*Hs* 110K and 69K), a homologue from *Drosophila* (*Dm* Cdc4), a homologue from *C. elegans* (*Ce* Sel-10), and yeast Cdc4 (*Sc* Cdc4). Identical amino acids are highlighted in black, and conserved substitutions are highlighted in grey. The F-box and the seven WD40 repeats are boxed.

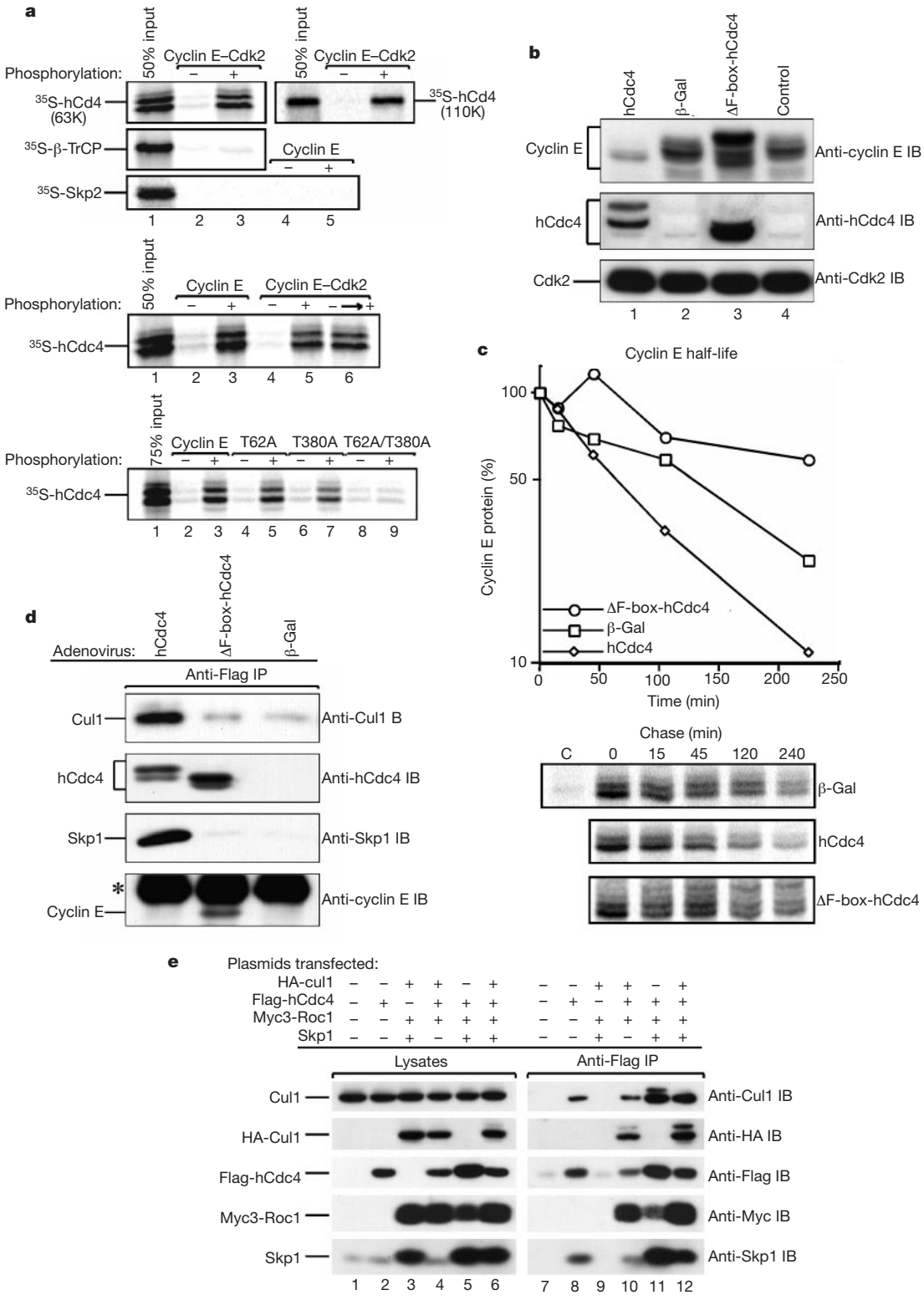

Figure 2 Human Cdc4 assembles into SCF complexes with Cul1, Skp1 and Roc1 *in vivo*, and regulates cyclin E turnover through specific association with phosphorylated cyclin E. **a**, *In vitro* translated, ^{35}S-labelled hCdc4 (63K and 110K forms), β-TrCP and Skp2 were assayed for binding to either free or Cdk2-bound GST-tagged cyclin E purified from SF9 insect cells on glutathione beads (lanes 1–5, top and middle panels). Dephosphorylation of cyclin E was performed after purification with λ phosphatase. ^{35}S-labelled hCdc4 was also tested for binding to cyclin E that had been dephosphorylated followed by rephosphorylation by its associated kinase, Cdk2, in the presence of 1 mM ATP (lane 6, middle panel), and for binding to single phosphorylation-site mutants (T62A, T380A) as well as the double mutant (T62A/T380A) (bottom panel). **b**, Thymidine-arrested KB cells were transduced with recombinant adenoviruses expressing hCdc4 (lane 1), a ΔF-box hCdc4 (lane 3) or a control virus (β-galactosidase) (lane 2). Lane 4 is an uninfected control. **c**, ^{35}S-methionine pulse-chase analysis of cyclin E in adenovirally transduced KB cells described in **b**. **d**, Immunoprecipitation (IP) of adenovirally transduced wild-type and mutant hCdc4 from KB cells described in **b**, and analysis of coprecipitated proteins by western blotting. In the bottom panel, the asterisk corresponds to the immunoglobulin-γ (IgG) heavy chain. **e**, 293T cells were transfected with the indicated plasmids and lysates were used for anti-Flag immunoprecipitations. Immune complexes (lanes 7–12) or crude lysates (lanes 1–6) from each transfection were analysed for the presence of Cul1, Skp1, Roc1 and hCdc4 by immunoblotting (IB).

was unchanged in a *cdc4/sic1* double mutant relative to the *cdc4* mutant (Fig. 1a), confirming that Cdc4 is indeed the critical F-box protein for cyclin E degradation in yeast.

Close scrutiny of half-life data obtained for the T380A mutant and comparison with data for wild-type cyclin E in SCF mutants suggested that the T380A mutant may still be susceptible to SCF-mediated ubiquitination and proteolysis. Accordingly, mutations were constructed at other potential phosphorylation sites. We found that the T62A mutation rendered cyclin E slightly more stable than the wild type (Fig. 1b). Significantly, the double mutant (T62A/T380A) was more stable than the T380A mutant (Fig. 1b). This suggests that T62 is a secondary phosphorylation site involved in ubiquitination and turnover of cyclin E.

We found a human expressed-sequence tag (EST) in the EST database of GenBank (http://www.ncbi.nlm.nih.gov) that, when translated, had significant homology to yeast Cdc4 (Fig. 1c). Analysis of hCdc4 complementary DNAs from a number of cell lines and the genomic structure of the *hCDC4* locus indicated that

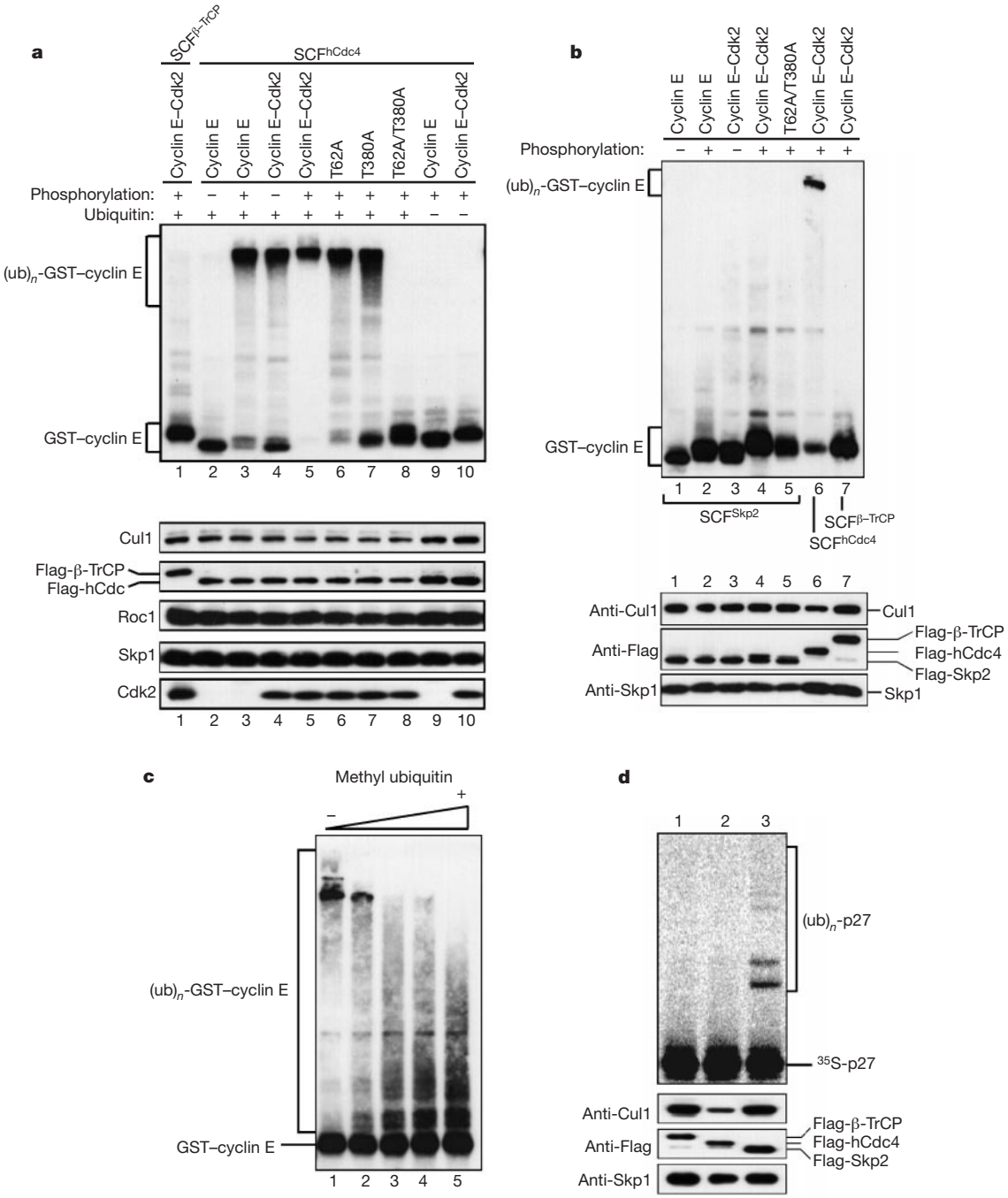

Figure 3 SCF^{hCdc4} ubiquitinates cyclin E in a phosphorylation-dependent manner *in vitro*. **a**, Anti-Flag immunoprecipitates from 293T cells transfected with either SCF^{hCdc4} (lanes 2–10) or $SCF^{\beta\text{-}TrCP}$ (lane 1) were assayed for the ability to ubiquitinate cyclin E and phosphorylation-site mutants (top panel). The bottom panel shows anti-Flag immunoprecipitates. **b**, Comparison of immunoprecipitated SCF^{Skp2} (lanes 1–5), SCF^{hCdc4} (lane 6) and $SCF^{\beta\text{-}TrCP}$ (lane 7) for the ability to ubiquitinate cyclin E (top panel). ub, ubiquitin. Immunoblots for Cul1, Skp1 and Flag-tagged F-box proteins are shown (bottom panel). **c**, Effect of increasing concentrations of methylated ubiquitin on the mobility of poly-ubiquitinated cyclin E species. **d**, Comparison of the ability of immunoprecipitated SCF complexes to ubiquitinate $p27^{Kip1}$.

two alternatively spliced variants exist that encode proteins with different amino termini (Fig. 1c, top two lines). A search for related proteins in other species suggested that homologues exist in *Drosophila melanogaster* as well as *Caenorhabditis elegans* (Fig. 1c). *In vitro* translation of the *hCDC4* cDNA produced a polypeptide with a relative molecular mass of about 63,000 (M_r 63K). However, further analysis of *hCDC4* transcripts and genomic structure indicated that two alternative forms of the protein exist. The 63K polypeptide derived from the initial hCdc4 EST is a slightly truncated version of a 69K species, which appears to be quite tissue specific (see below). The prevalent species in most tissues, consisting of 707 amino acids, runs aberrantly on SDS gels at an apparent M_r of 110K (see below). To generate the 110K form, one large upstream coding exon is substituted for the first exon of the 69K species (data not shown). We found that expression of the 110K form of hCdc4 could partially rescue a *cdc4* mutation in the yeast *Saccharomyces cerevisiae* (data not shown), consistent with these proteins being functional and structural homologues.

To determine whether hCdc4 interacts specifically with phosphorylated cyclin E, hCdc4 translated *in vitro* (the 63K and 110K forms) was incubated with glutathione beads bound to either free glutathione *S*-transferase (GST)–cyclin E complexes or GST–cyclin E–Cdk2 complexes. In parallel samples, cyclin E was either phosphorylated or dephosphorylated. hCdc4 binds to phosphorylated free or Cdk2-bound cyclin E, but not to dephosphorylated cyclin E, regardless of Cdk2 binding (Fig. 2a). In contrast, *in vitro* translated β-TrCP, another human WD40-repeat-containing F-box protein, bound to neither phosphorylated nor dephosphorylated cyclin E (Fig. 2a). It has been suggested that the human F-box protein Skp2, which contains leucine-rich repeats rather than WD40 repeats, targets cyclin E for ubiquitin-dependent degradation[16,17]. However, *in vitro* translated Skp2 bound to neither phosphorylated nor dephosphorylated cyclin E, either free or bound to Cdk2 (Fig. 2a). hCdc4 (63K) translated *in vitro* was assayed further for the ability to bind to cyclin E phosphorylation site mutant proteins. Cyclin E (T380A) was subjected to phosphorylation and dephosphorylation and assayed for hCdc4 binding as had been done for wild-type cyclin E. Binding of this mutant protein was reduced but not eliminated (Fig. 2a). However, on the basis of our analysis presented in Fig. 1b, we assayed both the T62A (single) and T62A/T380A (double) mutants for hCdc4 binding. Cyclin E (T62A) was only slightly reduced in hCdc4 binding (Fig. 2a). However, cyclin E (T62A/T380A) was completely defective in binding (Fig. 2a), consistent with the *in vivo* half-life data (Fig. 1b).

To determine whether expression of hCdc4 *in vivo* has an impact on cyclin E turnover, KB cells were transduced with an hCdc4 recombinant adenovirus. Endogenous levels of cyclin E were

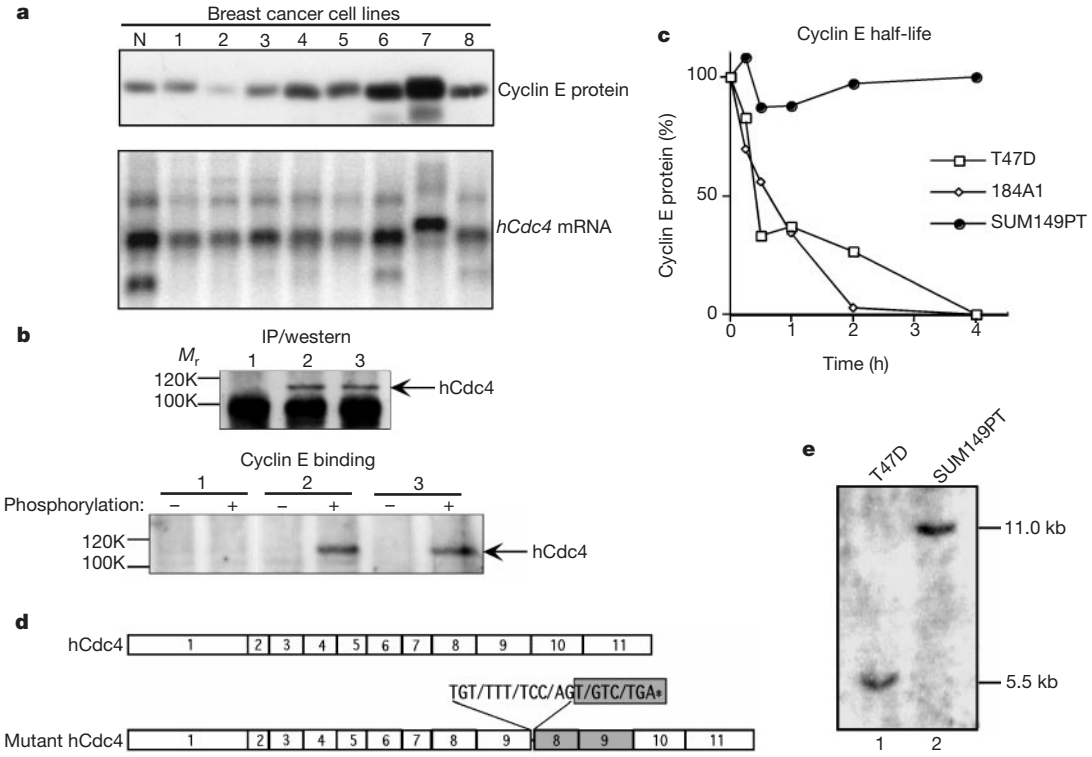

Figure 4 Aberrant hCdc4 mRNA, loss of hCdc4 protein and loss of heterozygosity in cell lines derived from breast cancer with high cyclin E expression. **a**, Eight randomly chosen breast-cancer-derived cell lines (lanes 1–8) and a breast epithelial cell line (184A1, lane N) were analysed for cyclin E expression by immunoblotting (top panel), and for hCdc4 transcripts by northern blot analysis (bottom panel). Cell lines: lane 1, MDA-MB-435S; lane 2, T47D; lane 3, BT-549; lane 4, ZR-75-1; lane 5, MDA-MB-436; lane 6, MDA-MB-157; lane 7, SUM149PT; lane 8, SK-BR-3. **b**, The breast epithelial cell line 184A1 (lane 2) and the breast-cancer-derived cell lines SUM149PT (lane 1) and T47D (lane 3) were analysed for expression of hCdc4 protein by anti-hCdc4 immunoprecipitation (IP) followed by immunoblotting using specific anti-hCdc4 antibodies (top panel) or by incubating crude lysates prepared from these cell lines with either dephosphorylated (control) or phosphorylated GST–cyclin E immobilized on glutathione beads followed by SDS–PAGE and western blotting with anti-hCdc4 antibodies (bottom panel). The heavy band migrating ahead of hCdc4 in the immunoprecipitation–immunoblot experiment corresponds to IgG heavy chain–light chain heterodimers. **c**, ^{35}S-methionine pulse-chase analysis was performed to measure the turnover rate of cyclin E in the indicated cell lines. **d**, Structure of SUM149PT hCdc4 cDNA. Exons are numbered. Shaded exons are duplicated in tandem resulting from a tandem genomic duplication of the region containing exons 8 and 9. Spliced intronic sequences in the cDNA are shown, which lead to a chain termination at the beginning of the duplicated exon 8. **e**, Southern blot analysis of genomic DNA from breast-cancer-derived cell lines T47D and SUM149PT.

dramatically reduced compared with control adenovirus transductions (Fig. 2b). Conversely, when an adenovirus expressing an F-box-deleted (ΔF-box) and thereby, most probably, dominant negative hCdc4 allele, was transduced into the same cell line, a significant accumulation of cyclin E was observed (Fig. 2b). In addition, the bulk of accumulated cyclin E was hyperphosphorylated on the basis of SDS–polyacrylamide gel electrophoresis (SDS–PAGE) mobility (Fig. 2b), consistent with a block in the degradation specifically of phosphorylated cyclin E. ^{35}S-methionine pulse-chase experiments were performed on parallel adenoviral transductions (Fig. 2c). Transduction of wild-type hCdc4 led to a decrease in cyclin E half-life, whereas transduction of the dominant negative hCdc4 allele led to an increase in cyclin E half-life (Fig. 2c). Thus hCdc4 levels are rate limiting for cyclin E turnover. To confirm that the ΔF-box version of hCdc4 indeed had the appropriate characteristics to behave as a dominant negative, we showed by immunoprecipitation that it binds specifically to phosphorylated cyclin E *in vivo* but not to components of SCF (Fig. 2d).

To confirm that wild-type hCdc4 is part of an SCF complex, Flag-tagged hCdc4 was introduced into 293T cells by transfection. Analysis of anti-Flag immunoprecipitates indicated that hCdc4 is associated with both endogenous and co-transfected core components of human SCF (Fig. 2e).

To determine whether SCF^{hCdc4} can ubiquitinate cyclin E in a phosphorylation-dependent manner, expression plasmids for Flag-hCdc4, as well as the other three components of SCF, were cotransfected into 293T cells. Anti-Flag immunoprecipitates were then tested for their ability to ubiquitinate phosphorylated cyclin E. Immunoprecipitated SCF^{hCdc4} was capable of efficiently ubiquitinating phosphorylated cyclin E, either free or bound to Cdk2 (Fig. 3a, b). Addition of methylated ubiquitin increased the mobility of the cyclin E derivatives (Fig. 3c), confirming that the modification is indeed ubiquitination. In parallel experiments using Flag-tagged β-TrCP and Skp2, respectively, anti-Flag immunoprecipitates were incapable of efficiently ubiquitinating either phosphorylated or dephosphorylated cyclin E even though SCF complexes were formed (Fig. 3a, b). In contrast, immunoprecipitated SCF^{Skp2} could ubiquitinate phosphorylated $p27^{Kip1}$, one of its established targets (Fig. 3d). Consistent with the binding studies described above, SCF^{hCdc4}-mediated ubiquitination of cyclin E (T62A) was slightly reduced compared with wild-type cyclin E, ubiquitination of cyclin E (T380A) was moderately reduced, and the double mutant was not ubiquitinated at all (Fig. 3a). Thus hCdc4 is incorporated into an SCF complex that efficiently ubiquitinates phosphorylated but not unphosphorylated cyclin E. These data, taken together with the ability of transduced wild-type and dominant negative hCdc4 to affect dramatically the steady-state levels of cyclin E *in vivo*, strongly suggest that SCF^{hCdc4} represents the predominant pathway mediating turnover of cyclin E in mammalian cells.

A tissue RNA blot indicated that hCdc4 is expressed in most, if not all, tissues. In most tissues, the predominant messenger RNA species is about 5.5 kilobases (kb) long (data not shown). However, some tissues strongly expressed a 4-kb mRNA as the predominant form, in particular brain and skeletal muscle. The fact that hCdc4 is expressed at high levels in non-proliferating tissues suggests a function in addition to turnover of cyclin E, because cyclin E expression should be limited to tissues undergoing cell division. These data also suggest that the 5.5-kb mRNA, which is ubiquitously expressed, encodes the F-box protein responsible for targeting cyclin E. Using exon-specific probes, we have shown that the 5.5-kb mRNA encodes the 110K hCdc4 isoform, whereas the 4-kb species encodes the 69K species (data not shown). Analysis of hCdc4 expression in synchronized HeLa cells indicated that neither hCdc4 mRNA nor protein is regulated during the cell cycle (data not shown).

Levels of cyclin E are elevated in many types of human malignancy[5,6]. Furthermore, dysregulation of cyclin E levels has been directly linked to genomic instability[7] and tumorigenesis in model systems[18]. To determine whether loss of hCdc4 might account for elevated levels of cyclin E, we first analysed a panel of cell lines derived from breast cancer for cyclin E levels (Fig. 4a). Two such cell lines (MDA-MB-157 and SUM149PT, lanes 6 and 7, respectively) exhibited significant elevation of cyclin E above the level observed in 184A1 (ref. 19), an immortalized, non-transformed breast epithelial cell line (Fig. 4a, lane N). One of the cell lines expressing high levels of cyclin E (MDA-MB-157) has been shown previously to contain a genomic amplification of the cyclin E locus[20]. Northern blot analysis using the hCdc4 cDNA as a probe (Fig. 4a) indicated that the 184A1 breast epithelial cell line contained a predominant hybridizing mRNA species at approximately 5.5 kb and a species with lower abundance at about 4 kb. Most of the breast cancer cell lines expressed only the 5.5-kb species. However, one cell line (SUM149PT) that exhibited high levels of cyclin E expressed an mRNA species of reduced mobility. The lack of any of the mRNA species characteristic of hCdc4 suggests a mutational lesion and, furthermore, loss of heterozygosity.

To determine whether the aberrant mRNA species in the SUM149PT cell line corresponded to a loss or alteration of hCdc4 protein, hCdc4 was concentrated from lysates either by adsorption to immobilized phosphorylated cyclin E or by immunoprecipitation with anti-hCdc4 antibody. The concentrated hCdc4 was then, in each case, subjected to SDS–PAGE and western blotting with anti-hCdc4 antibody (Fig. 4b). The 184A1 cell line and a breast cancer cell line that does not exhibit elevated cyclin E levels (T47D) contained the 110K hCdc4 isoform, which was detected either by binding specifically to phosphorylated cyclin E or by immunoprecipitation with anti-hCdc4 antibody. However, SUM149PT expressed no hCdc4-crossreactive protein capable of being immunoprecipitated or that bound to phosphorylated cyclin E within the limit of detection. ^{35}S-methionine pulse-chase experiments support this interpretation in that cyclin E has an extended half-life in the SUM149PT cell line compared with 184A1 and T47D cell lines (Fig. 4c).

To determine the nature of the mutation at the *hCDC4* locus in the SUM149PT cell line, the presumptive protein-coding region of the cDNA was sequenced and found to contain a direct repeat of exons 8 and 9 separated by 11 base pairs of intronic sequence (Fig. 4d). This mutation would be predicted to result in chain termination, eliminating the last four (of seven) WD40 repeats, presumably rendering the resulting polypeptide nonfunctional. Indeed, translation *in vitro* of the cDNA isolated from the SUM149PT cell line produced a truncated product that did not bind to phosphorylated cyclin E (data not shown). The loss of heterozygosity and internal genomic duplication at the *hCDC4* locus was confirmed by Southern blotting (Fig. 4e). DNA was cleaved with *Sst*I, which cuts in intronic sequences immediately downstream of exon 7, and with *Eco*RV, which cuts in intronic sequences immediately downstream of exon 10, and probed with a genomic fragment containing exons 8 and 9. The predicted wild-type fragment is 5.5 kb long, whereas that of the mutant is close to 11 kb. This finding and the implication of elevated cyclin E in carcinogenesis suggests that hCdc4 may be a tumour suppressor associated with some types of malignancy, including breast cancer. hCdc4 was identified independently on the basis of homology to the *Drosophila archipelago* gene product, also shown to regulate cyclin E proteolysis, and given the name human Ago in that study[21]. □

Methods

Plasmids and baculovirus constructions

A human EST encoding part of the hCdc4 gene was amplified from HeLa mRNA by polymerase chain reaction with reverse transcription (RT-PCR) using two sequence-

specific oligonucleotide primers, Pcr1 (5′-gcaagcttatgggtttctacggcacatt-3′, forward), and Pcr2 (5′-atgggccctgctcttcacttcatgtcc-3′, reverse), and TA cloned into pCR2.1 (Invitrogen). The sequence of the cloned cDNA was verified in its entire length (1.7 kb) by sequencing and found to match the sequence published in the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank, Genbank accession number BAA91986.1). For further details, see Supplementary Information. A mammalian transfection plasmid expressing N-terminal Flag-tagged hCdc4 protein was constructed by subcloning into pFLAG-CMV2 (Sigma). For expression in *Escherichia coli*, hCdc4 was tagged at the N terminus with a RGS.His epitope through subcloning into pQE-10 (Qiagen). Complementary DNAs encoding hCdc4 and a ΔF-box mutant that had been deleted for its F-box by a two-step PCR protocol, as well as the cDNA coding for β-galactosidase, were cloned into pDV46.

Recombinant adenoviruses were generated by co-transfecting the recombinant plasmids and pBHG10 (ref. 22) into 293 cells using the calcium phosphate precipitation method. The β-TrCP and Skp2 clones were gifts of F. Mercurio and M. Pagano, respectively, and were cloned into pFLAG-CMV2 to obtain β-TrCP and Skp2 tagged at their N termini with the Flag epitope. The mammalian transfection plasmid pCDNA3-Cul1-HA was a gift of R. Klausner, and pCDNA3-3MYCROC1 as well as pCDNA3-hSkp1 were gifts of Y. Xiong. Baculovirus expressing cyclin E with a GST tag at its N terminus was a gift of B. Sarcevic. Recombinant baculoviruses expressing GST-tagged versions of cyclin E phosphorylation site mutants (T62A, T380A, T62A/T380A) were generated using the pFastBac-system (Gibco BRL) according to the manufacturer's protocol. Baculovirus encoded proteins were expressed in SF9 insect cells grown in Ex-Cell 401 media (JRH) supplemented with 2% fetal bovine serum.

Analysis of cyclin E turnover in yeast

All yeast strains are isogenic to 15DaubΔ, a *bar1Δura3Δns*, a derivative of BF264-15D (ref. 23). Several thermosensitive *skp1* mutants with different cell cycle arrest phenotypes were constructed by a combination of PCR mutagenesis and *in vivo* gap repair similar to the procedure described by Muhlrad *et al.*[24]. The mutant shown in Fig. 1 (*skp1-24*) arrested with 1C DNA content and a multi-budded phenotype. To analyse turnover of cyclin E in various yeast mutants expressing cyclin E from the inducible *GAL1* promoter, cells were grown in YEP-raffinose at 25 °C to an absorbance at wavelength 600 nm of 0.3. Cells were then shifted to 35 °C and after 30 min galactose was added to a final concentration of 2% to induce the *GAL1* promoter. To terminate cyclin E expression after 60 min, cells were collected on filters and transferred to YEPD media and incubation was continued at 35 °C. Extracts were prepared from aliquots taken after the periods indicated and analysed for cyclin E by western blot. For further details, see Supplementary Information.

Cell culture and immunological techniques

A panel of cell lines derived from breast cancer was obtained from the American Type Culture Collection (ATCC) and the University of Michigan Breast Cell/Tissue Bank and Database; the cells were grown in media recommended by the suppliers. HeLa, KB (human epidermoid carcinoma) and 293T cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified 37 °C incubator with 5% CO_2. 293T cells were transfected with various combinations of plasmids in 10-cm dishes by the calcium phosphate precipitation method. Forty hours after transfection, cells were lysed and subjected to immunoprecipitation followed by immunoblotting. For further details, see Supplementary Information.

Pulse-chase, northern and Southern blot analyses

Pulse-chase experiments were performed on thymidine-arrested cells as described[25]. Thymidine-arrested KB cells were co-transduced with adenovirus encoding cyclin E (resulting in a five- to tenfold elevation over endogenous levels) and virus encoding either wild-type hCdc4, a ΔF-box hCdc4, or control β-galactosidase. Viral transductions were incubated for 24 h before pulse-chase. Immunoprecipitations were performed with a monoclonal anti-cyclin E antibody (HE172). Quantification of bands was performed with ImageQuant software (Molecular Dynamics). For northern blot analysis, 2 μg of poly(A)$^+$ RNA was isolated from asynchronously growing cultures according to the manufacturer's protocol (Qiagen) and run on a 1% formaldehyde agarose gel as described[26]. The gel was blotted onto Zeta-Probe GT genomic membrane (Bio-Rad) and hybridized with a radiolabelled hCdc4 probe followed by autoradiography. For Southern blot analysis, 10 μg of DNA was digested with *Sst*I and *Eco*RV, run on an 0.8% agarose gel, blotted and probed with a genomic fragment corresponding to exons 8 and 9.

In vitro binding

Complementary DNAs encoding various hCdc4 isoforms and mutants, β-TrCP and Skp2 were translated *in vitro* into ^{35}S-methionine-labelled proteins by a T7 transcription/translation system (Promega). GST-tagged cyclin E and various cyclin E phosphorylation-site mutants were expressed in baculovirus-infected SF9 insect cells and adsorbed on glutathione beads. Bound proteins were analysed by SDS–PAGE followed by autoradiography. For further details, see Supplementary Information.

In vitro ubiquitination assay

Recombinant SCF complexes containing different Flag-tagged F-box proteins were isolated from transfected 293T cells. Equal amounts of SCF immune complexes were mixed with cyclin E protein for 30 min on ice to allow binding. Aliquots of this mixture were then added to ubiquitination reactions in a total volume of 30 μl containing 15 μg of bovine ubiquitin (Sigma), 0.5 μg of yeast E1 enzyme (Boston Biochem), 1 μg of human 6xHis-Cdc34 purified from bacteria, and an ATP-regenerating system (1 mM ATP, 20 mM creatine phosphate, 0.1 mg ml^{-1} creatine kinase) in ubiquitination reaction buffer[25] supplemented with 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulphonylflouride, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and 2 μg ml^{-1} pepstatin. The Cdk2-inhibitor roscovitine (Biomol; 100 μM final concentration) was added to reactions containing dephosphorylated cyclin E as substrate. Reactions were incubated at 30 °C for 2 h, terminated by boiling for 5 min with SDS sample buffer, and analysed by SDS–PAGE followed by immunoblotting using anti-cyclin E antibodies. The ubiquitination assay using p27 as a substrate was performed as described[25].

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to S.I.R. (e-mail: sreed@scripps.edu). The GenBank accession number for the hCdc4 cDNA encoding the isoform with M_r 110K is AY049984.

Supplementary Information for "Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line" Nature, V413, 316

Methods

Plasmids and baculovirus constructions

A human EST encoding part of the hCdc4 gene was amplified from HeLa mRNA by RT-PCR using two sequence-specific oligonucleotide primers, Pcr1 (5'-gcaagcttatgggtttctacggcacatt-3', forward), and Pcr2 (5'-atgggccctgctcttcacttcatgtcc-3', reverse), and TA-cloned into pCR2.1 (Invitrogen). The sequence of the cloned cDNA was verified in its entire length (1.7 Kb) by sequencing and found to match the sequence published in the NCBI database (Genbank accession no: BAA91986.1). The complete coding region corresponding to this cDNA was determined by 5' RACE. Probing a Northern blot with sequences corresponding to specific exons, it was found that this cDNA corresponds to a 4 kb hCdc4 mRNA expressed at high levels in only some cell types. A second cDNA was identified by scanning upstream genomic sequences for probable exons and using these exons to probe a Northern blot. This cDNA was found to correspond to a ubiquitous 5.5 kb hCdc4 mRNA. Human cDNAs encoding the two full-length isoforms of the hCdc4 protein were amplified from HeLa mRNA by RT-PCR using sequence-specific oligonucleotide primers, Pcr4 (5'-cttttggaaatgaatcaggaa-3', forward) and Pcr2 (5'-atgggccctgctcttcacttcatgtcc-3', reverse) for the cDNA encoding the 110 kDa isoform, and Pcr5 (5'-catgtatgtatgtgtgtcccg-3', forward) and Pcr2 for the cDNA encoding the 69 kDa isoform, and subsequently TA-cloned into pCR2.1 (Invitrogen). The sequences of the cloned cDNAs were verified in their entire lengths (2.2 kb, and 1.9 kb, respectively) by sequencing. The cDNA encoding the 110 kDa isoform was deposited in GenBank (accession number: AY049984). A mammalian transfection plasmid expressing N-terminal Flag-tagged hCdc4 protein was constructed by subcloning into pFLAG-CMV2 (Sigma). For expression in *E. Coli*, hCdc4 was tagged at the N-terminus with a RGS.His epitope through subcloning into pQE-10 (Qiagen). Complementary DNAs encoding hCdc4 and a DF-box-mutant that had been deleted for its F-box using a two-step PCR protocol, as well as the cDNA coding for b-galactosidase were cloned into pDV46. Recombinant adenoviruses were generated by co-transfecting the recombinant plasmids and pBHG10[22] into 293 cells using the calcium phosphate precipitation method. The b-TrCP and Skp2 clones were gifts from Dr. F. Mercurio (Signal Pharmaceuticals, San Diego), and Dr. M. Pagano (Department of Pathology and Kaplan Comprehensive Cancer Center, New York University, New York), respectively, and were cloned into pFLAG-CMV2 to obtain b-TrCP and Skp2 tagged at their N-termini with the Flag-epitope. The mammalian transfection plasmid pCDNA3-Cul1-HA was a gift from Dr. R. Klausner (NIH, Bethesda, Maryland), and pCDNA3-3MYCROC1 as well as pCDNA3-hSkp1 were gifts from Dr. Y. Xiong (Lineberger Cancer Center, University of North Carolina at Chapel Hill). Baculovirus expressing cyclin E with a GST-tag at its N-terminus was a gift from Dr. B. Sarcevic (The Garvan Institute, Sydney, Australia). Recombinant baculoviruses expressing GST-tagged versions of cyclin E phosphorylation site mutants (T62A, T380A, T62A/T380A) were

generated using the pFastBac-system (Gibco BRL) according to the manufacturer's protocol. Baculovirus encoded proteins were expressed in SF9 insect cells grown in Ex-Cell 401 media (JRH) supplemented with 2% fetal bovine serum.

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All yeast strains are isogenic to 15DaubD , a *bar1D ura3D ns*, a derivative of BF264-15D[23]. Several temperature sensitive *skp1* mutants with different cell cycle arrest phenotypes were constructed by a combination of PCR mutagenesis and *in vivo* gap-repair similar to the procedure described by Muhlrad et al.[24]. The mutant shown in Figure 1 (*skp1-24*) arrested with 1C DNA content and a multi-budded phenotype. To analyze cyclin E turnover in various yeast mutants expressing cyclin E from the inducible *GAL1* promoter, cells were grown in YEP-raffinose at 25˚C to an OD_{600}= 0.3. Cells were then shifted to 35˚C and after 30 min galactose was added to a final concentration of 2% to induce the *GAL1* promoter. To terminate cyclin E expression after 60 min, cells were collected on filters and transferred to YEPD media and incubation was continued at 35˚C. Extracts were prepared from aliquots taken after the periods indicated and analyzed for cyclin E by Western blotting using monoclonal anti-cyclin E antibodies (HE12). Cells were lysed in RIPA buffer (1% deoxycholic acid, 1% Triton-X-100, 0.1% SDS, 250 mM NaCl, 50 mM Tris-HCl pH 7.5). A similar protocol was used to analyze the turnover of cyclin E phosphorylation site mutants in yeast except that a strain was used where human CDK2 was substituted for the endogenous Cdk Cdc28[8].

Cell culture and immunological techniques

A panel of breast cancer-derived cell lines were obtained from ATCC and the University of Michigan Breast Cell/Tissue Bank and Database and grown in media recommended by the suppliers. HeLa, Kb (human epidermoid carcinoma), and 293T cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified 37˚C incubator with 5% CO_2. 293T cells were transfected with various combinations of plasmids in 10 cm-dishes using the calcium phosphate precipitation method. Forty hours post-transfection, cells were lysed, as indicated, into either mammalian cell lysis buffer 1, MCLB1 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) or mammalian cell lysis buffer 2, MCLB2 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25% NP-40, 10% glycerol, 1 mM EDTA, 1 mM DTT), supplemented with 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulfonylflouride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin. After centrifugation at 4° C (14.000 rpm, 15 min) lysates (1 mg/0.5 ml) were subjected to immunoprecipitation using 10 mg anti-Flag antibody immobilized on agarose beads (M2, Sigma) for 2 hours at 4° C. Immune complexes were washed three times with lysis buffer and subsequently used for ubiquitination reactions or analyzed by immunoblotting. Polyclonal antibodies against human Cdc4 were generated in rabbits after injection of recombinant Cdc4 protein produced in bacteria. Antibodies were affinity-purified using nitrocellulose-bound antigen. Commercially obtained antibodies used in this study: Mouse monoclonal anti-Flag antibody (M2, Sigma), mouse monoclonal anti-HA antibody (HA.11, Babco), rabbit polyclonal anti-Myc antibody (A-14, Santa Cruz), rabbit polyclonal anti-Cdk2 antibody (M2, Santa Cruz), and rabbit polyclonal antibodies against Cul1, Skp1, and ROC1 (Neomarkers).

Pulse-chase, Northern and Southern blot analysis

Pulse-chase experiments were performed on thymidine arrested cells as described[25]. Thymidine arrested Kb cells were co-transduced with adenovirus encoding cyclin E (resulting in a 5-10 fold elevation over endogenous levels) and virus encoding either wild-type hCdc4, an F-box deleted hCdc4, or control b-galactosidase. Viral transductions were incubated for 24 hours prior to pulse-chase. Immunoprecipitations were performed with a monoclonal anti-cyclin E antibody (HE172). Quantitation of bands was performed using ImageQuant software (Molecular Dynamics). For

Northern blot analysis, 2 mg of poly[A]+ RNA was isolated from asynchronously growing cultures according to the manufacturer's protocol (Qiagen) and run on a 1% formaldehyde agarose gel as described[26]. The gel was blotted onto Zeta-Probe GT genomic membrane (Bio-Rad) and hybridized with a radiolabeled hCdc4 probe followed by autoradiography. For Southern blot analysis, 10: g of DNA was digested with *Sst*I and *Eco*RV, run on 0.8% agarose gels, blotted and probed with a genomic fragment corresponding to Exons 8 and 9.

In vitro binding

Complementary cDNAs encoding various hCdc4 isoforms and mutants, b-TrCP, and Skp2 were *in vitro* translated into ^{35}S methionine-labeled proteins using a T7 transcription/translation system (Promega). GST-tagged cyclin E and various cyclin E phosphorylation site mutants were expressed in baculovirus infected SF9 insect cells and adsorbed on glutathione beads for 1 hour at 4° C after lysing the cells into GST-lysis buffer (50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 0.5% Tween-20, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) supplemented with 5 mM sodium flouride, 1 mM sodium orthovanadate, 10 mM b-glycerophosphate, 1 mM phenylmethyl sulfonylflouride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin. After washing the beads first with lysis buffer and then with wash buffer (50 mM HEPES-NaOH pH 7.5, 0.01% Tween-20, 10% glycerol, 1 mM EDTA, 1 mM DTT) they were subjected to either phosphorylation reaction in kinase assay buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$) in the presence of 1 mM ATP or dephosphorylation reaction using l–phosphatase (NEB) for 1 hour at 30° C. The beads were washed three times with binding buffer (20 mM Tris-HCl pH 7.6, 200 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT) prior to incubation with in vitro translated [^{35}S] methionine-labeled proteins in 200 ml binding buffer for 2 hours at 4° C. Bound proteins were analyzed by SDS-PAGE followed by autoradiography.

In vitro ubiquitination assay

Recombinant SCF complexes containing different Flag-tagged F-box proteins were isolated from transfected 293T cells lysed into mammalian cell lysis buffer 2 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25% NP-40, 10% glycerol, 1 mM EDTA, 1 mM DTT), supplemented with 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulfonylflouride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin) using anti-Flag antibodies immobilized on agarose beads. Individual immune complexes were washed three times with lysis buffer and three times with ubiquitination reaction buffer (20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM DTT). GST-tagged cyclin E and cyclin E phosphorylation site mutants purified from baculovirus infected SF9 insect cells on glutathione beads were subjected to either phosphorylation or dephosphorylation followed by elution of the bound proteins with elution buffer (50 mM HEPES-NaOH pH 7.5, 0.01% Tween-20, 10% glycerol, 1 mM EDTA, 1 mM DTT) containing 15 mM reduced glutathione. Eluates from multiple elution steps were pooled and concentrated on Centricon-30 spin columns (Amicon) and stored in aliquots at –80° C. Equal amounts of SCF immune complexes were mixed with different eluted cyclin E proteins for 30 min on ice to allow binding. Subsequently, aliquots of this mixture were added to ubiquitination reactions in a total volume of 30 ml containing 15 mg of bovine ubiquitin (Sigma), 0.5 mg of yeast E1 enzyme (Boston Biochem), 1 mg of human 6xHis-Cdc34 purified from bacteria, and an ATP-regenerating system (1 mM ATP, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase) in ubiquitination reaction buffer[25] supplemented with 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulfonylflouride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin. The CDK2-inhibitor roscovitine (Biomol; 100 mM final concentration) was added to reactions containing dephosphorylated cyclin E as substrate. Reactions were incubated at 30° C for 2 hours, terminated by boiling for 5 min with SDS-sample buffer, and analyzed by SDS-PAGE followed by immunoblotting using anti-cyclin E antibodies. The ubiquitination assay using p27 as a substrate was performed as described[25].

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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.
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54. R. M. Krauss, unpublished observations.
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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

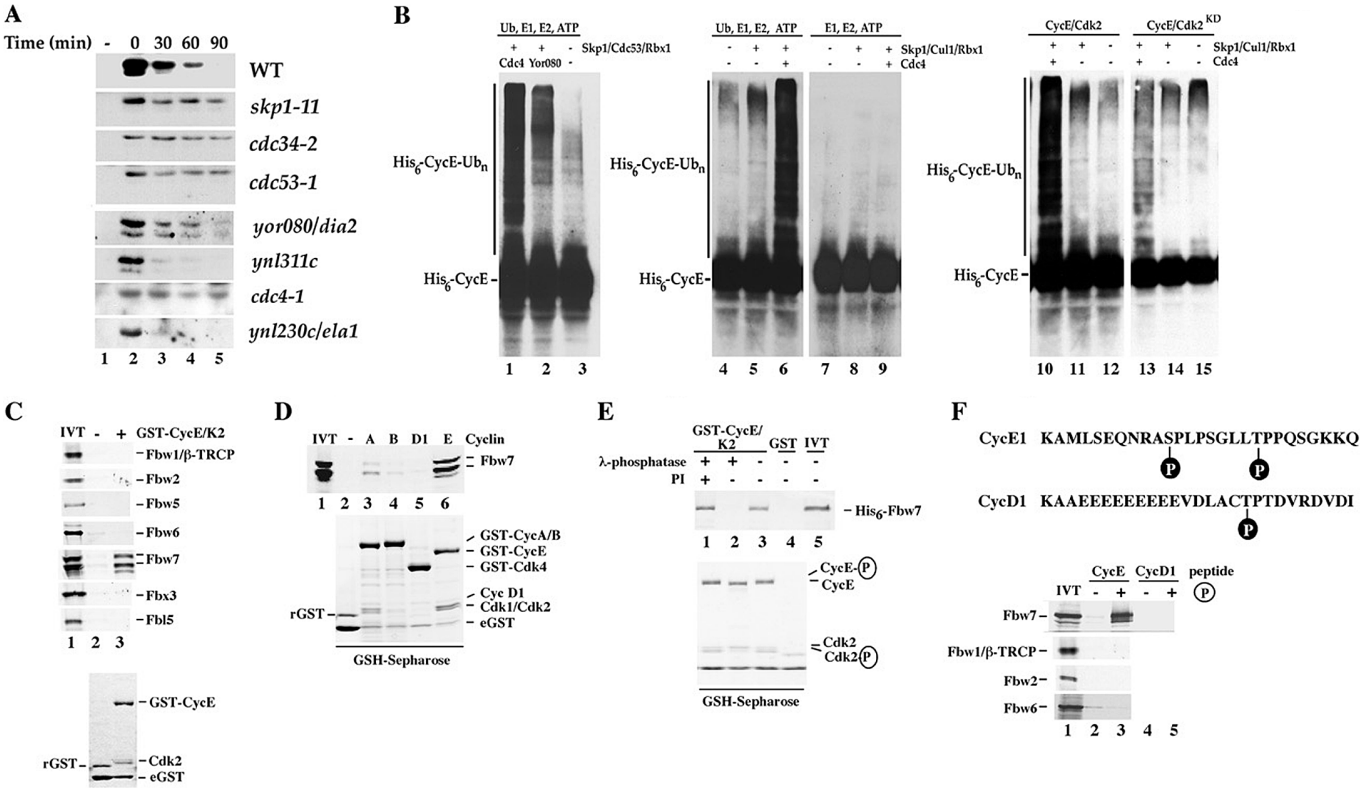

Fig. 1. Interaction between cyclin E and SCF components in yeast and mammalian cells. (**A**) Stabilization of cyclin E in *skp1-11*, *cdc34-2*, *cdc53-1*, *cdc4-1*, and *yor080* mutants (*12*, *30*). Strains of the indicated genotypes were grown in medium containing raffinose; cyclin E expression was 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).

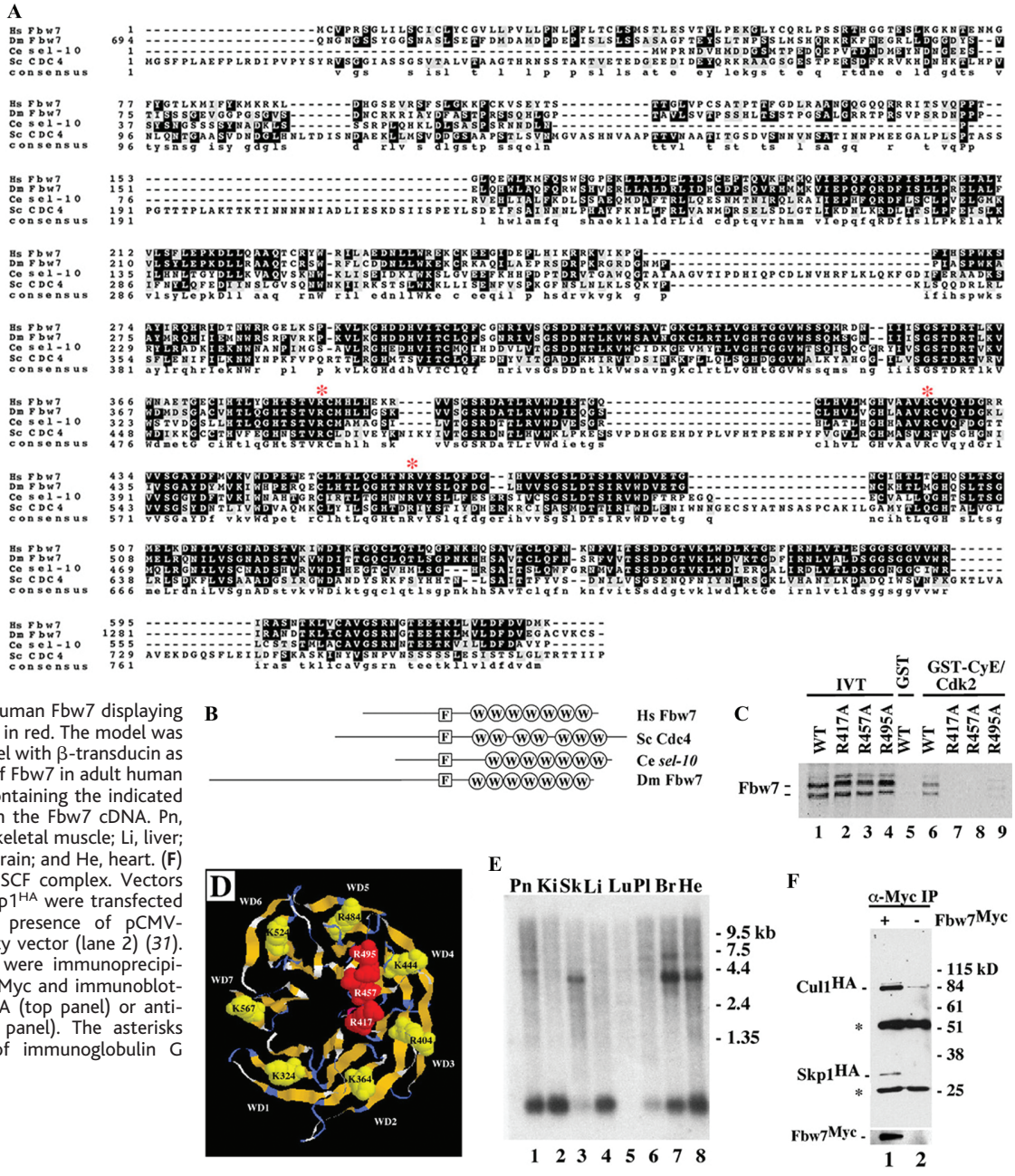

Fig. 2. Characterization of the WD40-repeat–containing F-box protein, Fbw7. (**A**) Conservation between human (Hs) Fbw7 and *C. elegans* (Ce) *sel-10*, *S. cerevisiae* (Sc) Cdc4, and *D. melanogaster* (Dm) Fbw7 (*33*). Identical residues are shaded black and similarities are shaded gray. Asterisks indicate 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 $Thr^{380} \rightarrow$ 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

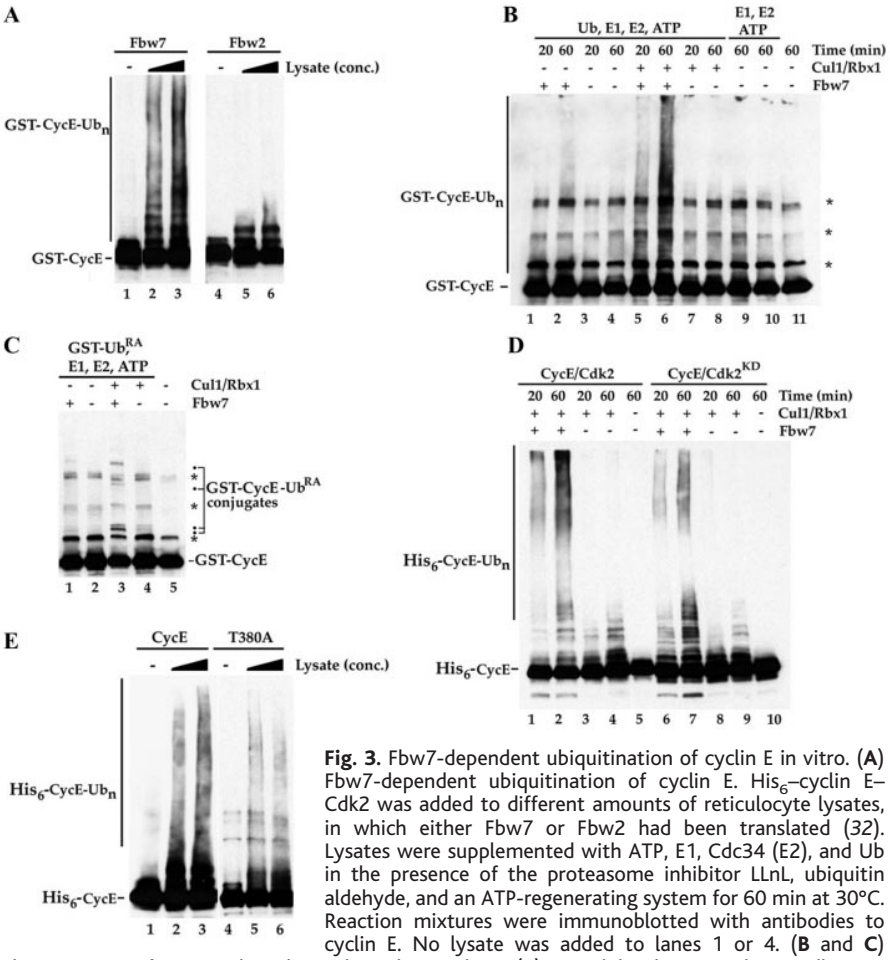

Fig. 3. Fbw7-dependent ubiquitination of cyclin E in vitro. (**A**) Fbw7-dependent ubiquitination of cyclin E. His_6–cyclin E–Cdk2 was added to different amounts of reticulocyte lysates, in which either Fbw7 or Fbw2 had been translated (*32*). Lysates were supplemented with ATP, E1, Cdc34 (E2), and Ub 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.

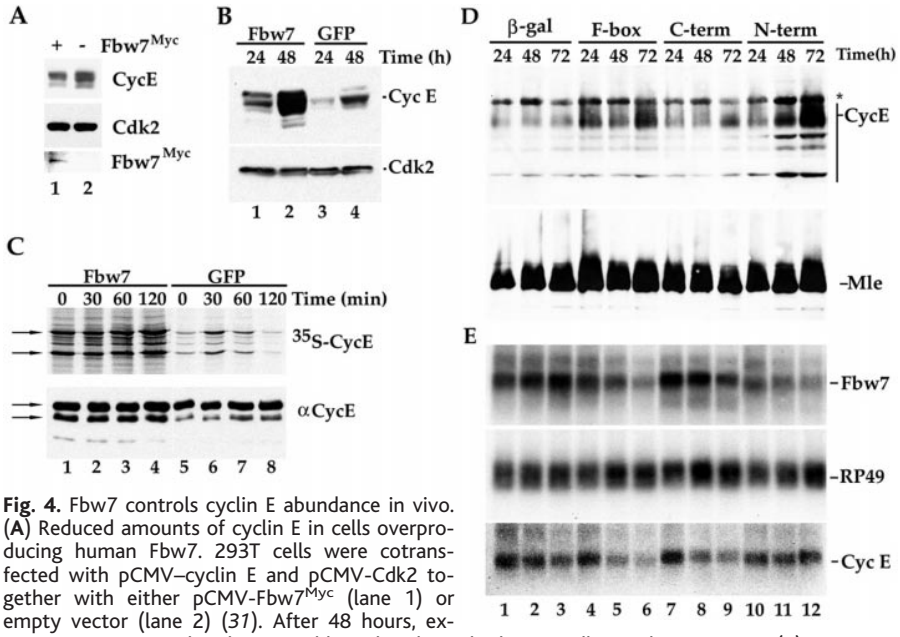

Fig. 4. Fbw7 controls cyclin E abundance in vivo. (**A**) Reduced amounts of cyclin E in cells overproducing human Fbw7. 293T cells were cotransfected with pCMV–cyclin E and pCMV-Cdk2 together with either pCMV-Fbw7^{Myc} (lane 1) or empty vector (lane 2) (*31*). After 48 hours, extracts were prepared 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.

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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*).
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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.

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