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

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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

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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

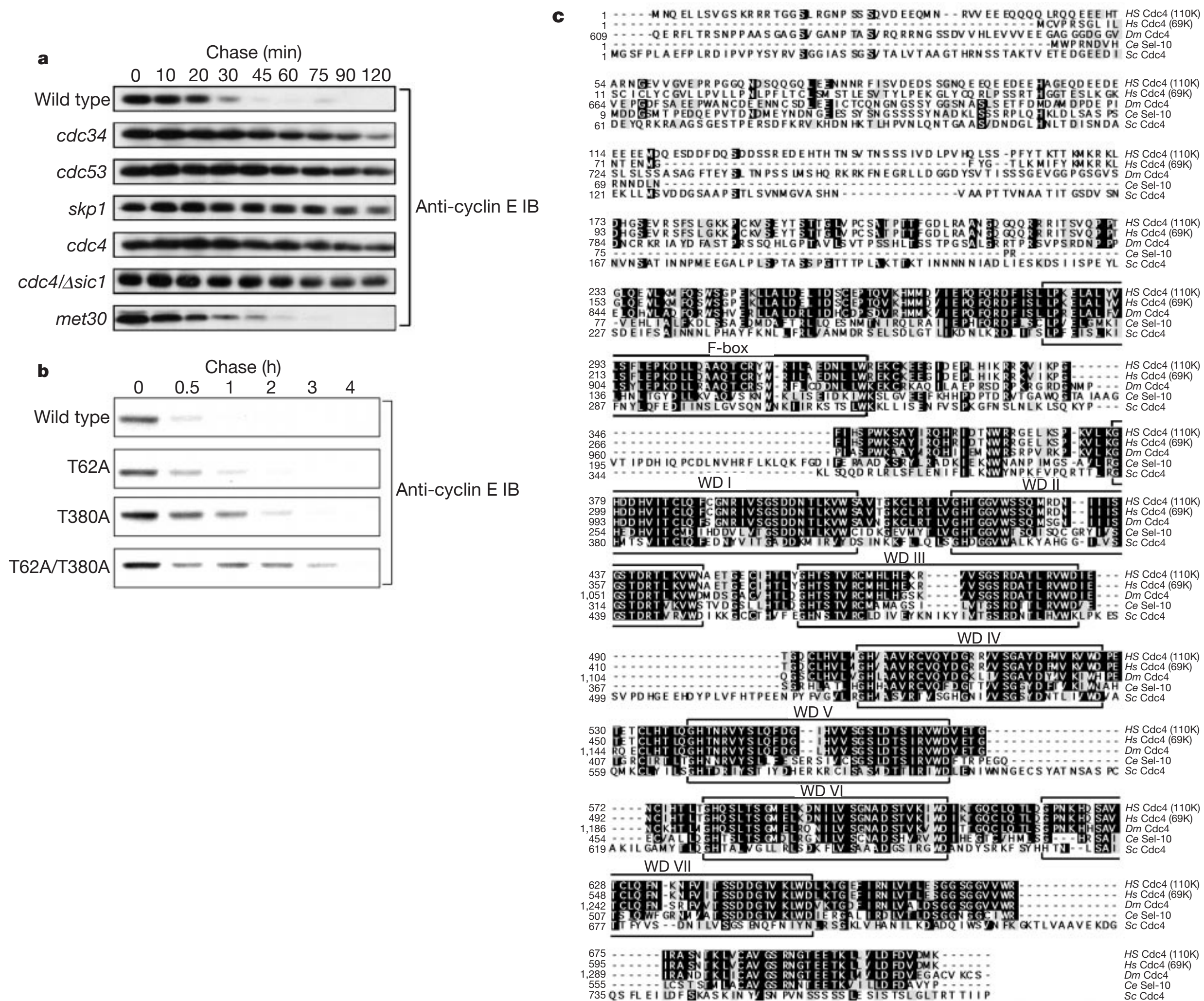

Figure 1 Human cyclin E is targeted for ubiquitin-dependent degradation by the SCF^{Cdc4} protein-ubiquitin ligase in yeast. **a**, Cyclin E was expressed from the inducible *GAL1* promoter in a wild-type strain and in various thermosensitive SCF mutant strains at the restrictive temperature, and the 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.

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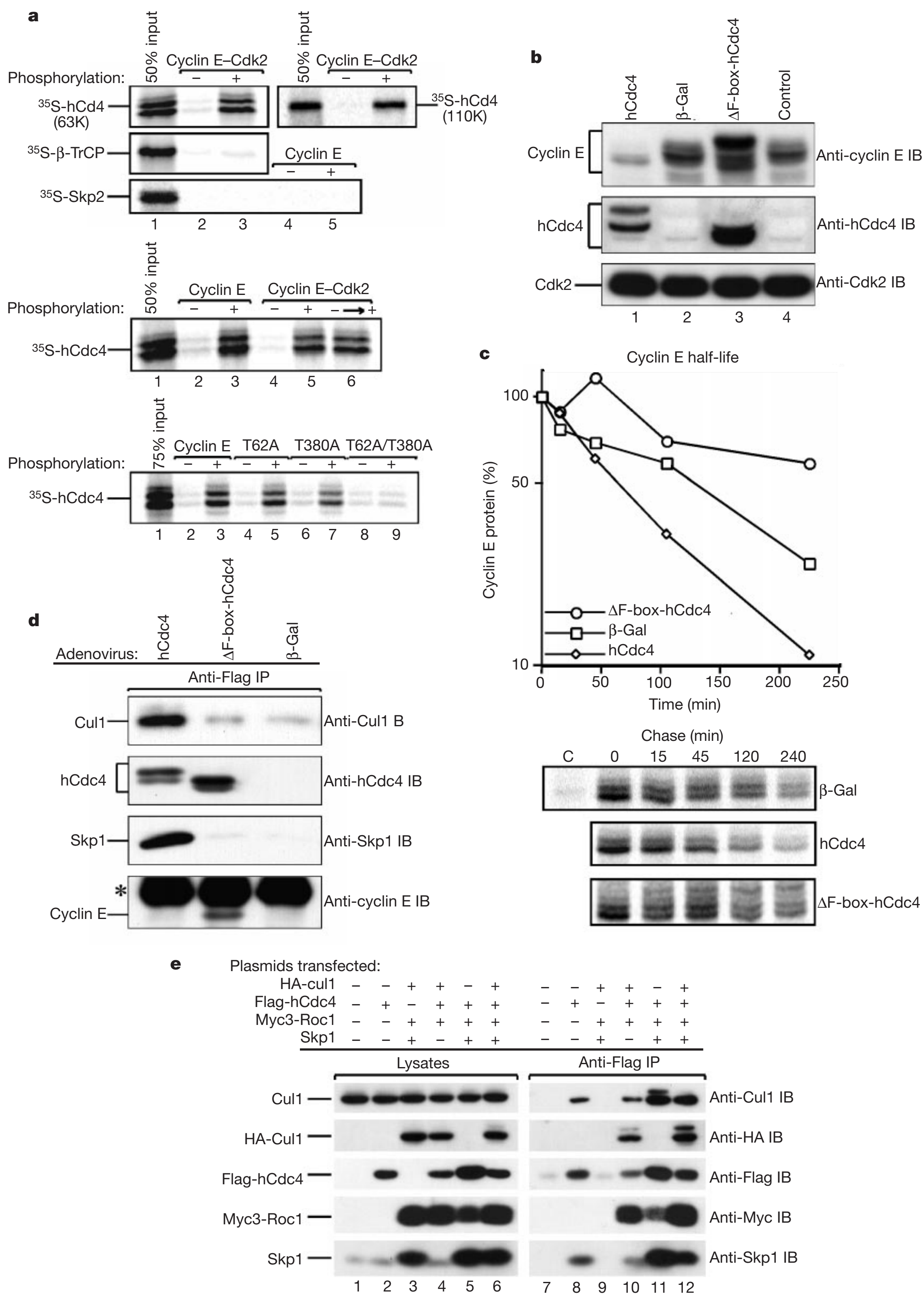

Figure 2 Human Cdc4 assembles into SCF complexes with Cul1, Skp1 and Roc1 *in vivo*, and regulates cyclin E turnover through specific association with phosphorylated cyclin E. **a**, *In vitro* translated, ^{35}S-labelled hCdc4 (63K and 110K forms), *β*-TrCP and Skp2 were assayed for binding to either 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).

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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

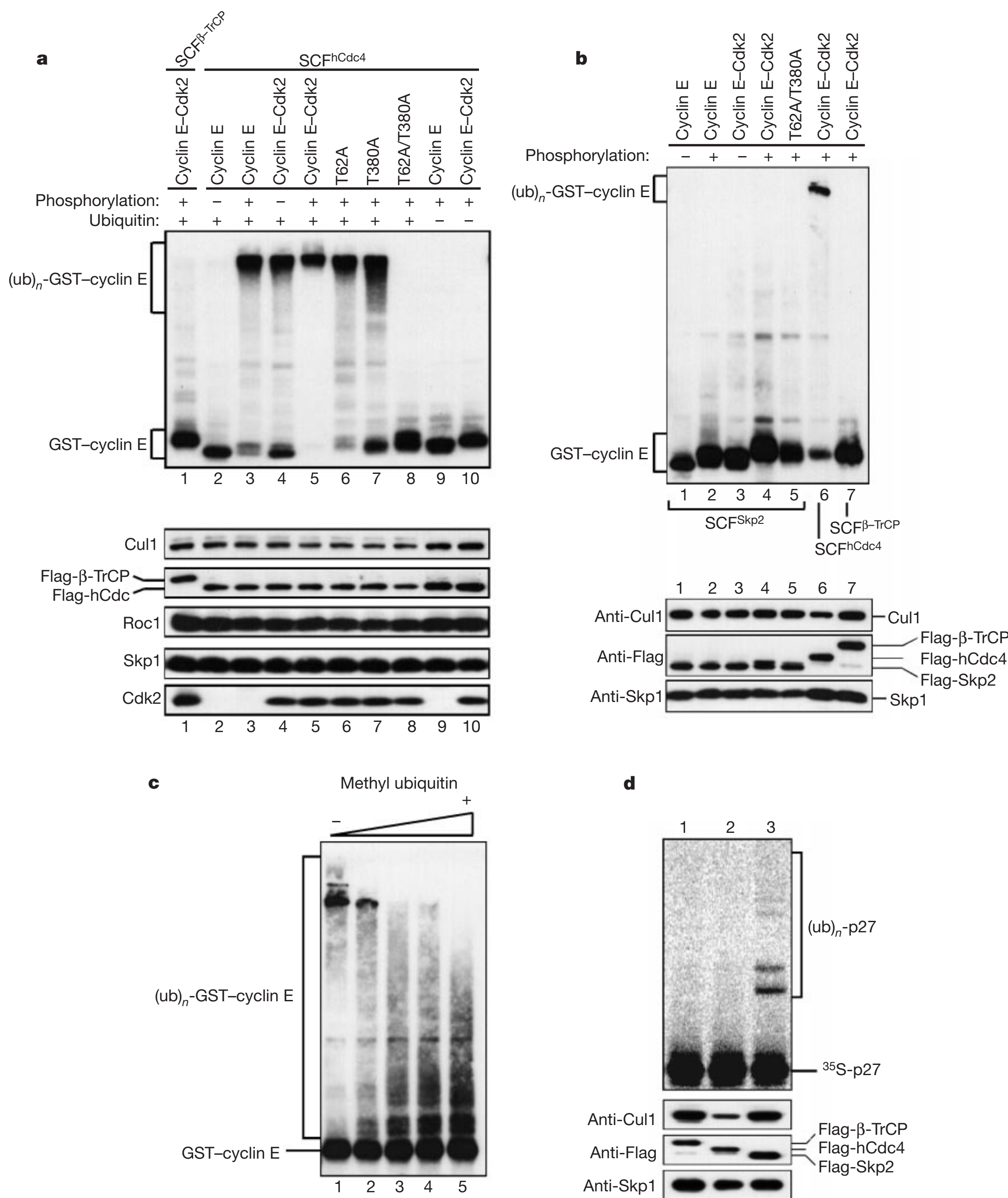

Figure 3 SCF^{hCdc4} ubiquitinates cyclin E in a phosphorylation-dependent manner *in vitro*. **a**, Anti-Flag immunoprecipitates from 293T cells transfected with either SCF^{hCdc4} (lanes 2–10) or SCF$^{\beta\text{-TrCP}}$ (lane 1) were assayed for the ability to ubiquitinate cyclin E and phosphorylation-site 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}.

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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

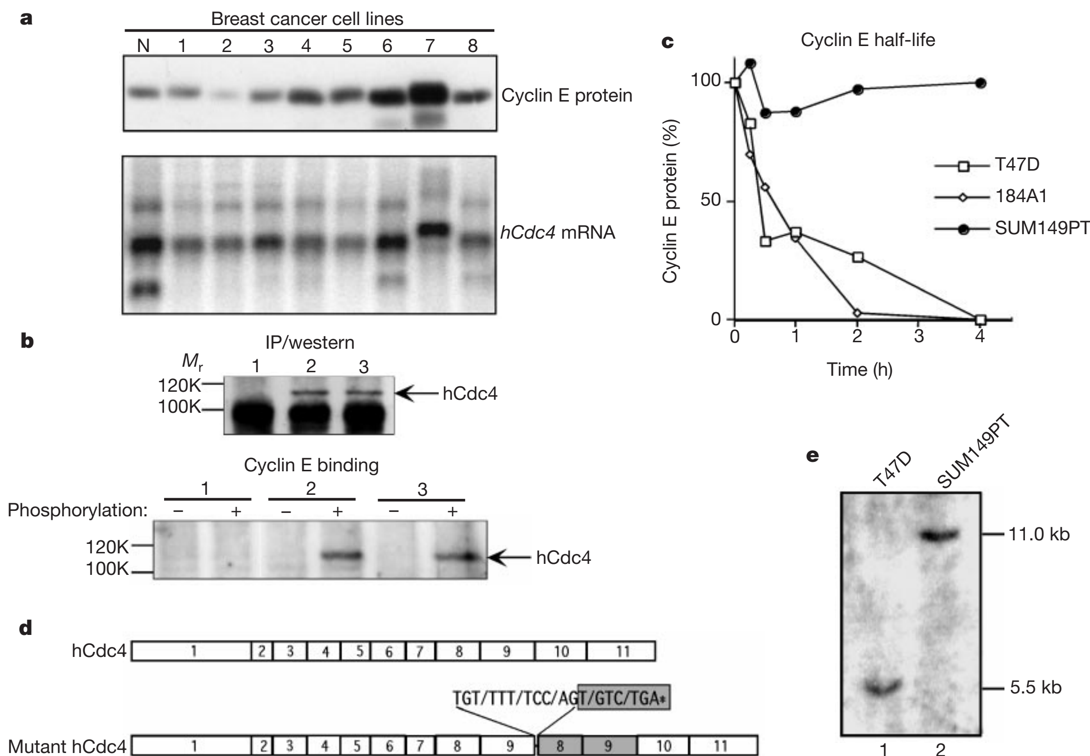

Figure 4 Aberrant hCdc4 mRNA, loss of hCdc4 protein and loss of heterozygosity in cell lines derived from breast cancer with high cyclin E expression. **a**, Eight randomly chosen breast-cancer-derived cell lines (lanes 1–8) and a breast epithelial cell line (184A1, lane N) were analysed for cyclin E 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.

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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-

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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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