

Multisite Phosphorylation by Cdk2 and GSK3 Controls Cyclin E Degradation

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Summary

Autophosphorylation-triggered ubiquitination has been proposed to be the major pathway regulating cyclin E protein abundance: phosphorylation of cyclin E on T380 by its associated CDK allows binding to the receptor subunit, Fbw7, of the SCF^{Fbw7} ubiquitin ligase. We have tested this model *in vivo* and found it to be an inadequate representation of the pathways that regulate cyclin E degradation. We show that assembly of cyclin E into cyclin E-Cdk2 complexes is required *in vivo* for turnover by the Fbw7 pathway; that Cdk2 activity is required for cyclin E turnover *in vivo* because it phosphorylates S384; that phosphorylation of T380 *in vivo* does not require Cdk2 and is mediated primarily by GSK3; and that two additional phosphorylation sites, T62 and S372, are also required for turnover. Thus, cyclin E turnover is controlled by multiple biological inputs and cannot be understood in terms of autophosphorylation alone.

Introduction

Cyclin E is an evolutionarily conserved protein whose function is to promote the G1 to S phase transition by binding to and activating a cyclin-dependent kinase, Cdk2 (Sherr, 1993). Cyclin E protein abundance oscillates during a normal cell cycle (Dulic et al., 1992; Koff et al., 1992). It attains a maximum level of expression in late G1 and a minimum in M phase and early G1. Normal cell cycle regulation requires stringent controls on the amount and timing of cyclin E-Cdk2 activity and hence on the amount and timing of cyclin E expression. Thus, ectopic overexpression of cyclin E in mammalian fibroblasts causes cyclin E-Cdk2 to be active both earlier in the cell cycle and to a higher level than it normally

is (Ohtsubo and Roberts, 1993). This causes a premature transition from G1 to S phase, a delayed progression through S phase, and genomic instability (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Spruck et al., 1999). Conversely, inhibition of cyclin E-Cdk2 complexes blocks the start of S phase (Ohtsubo et al., 1995; van den Heuvel and Harlow, 1993).

The impact of cyclin E levels on cell cycle regulation is also reflected in the fact that many types of human tumors express amounts of cyclin E protein that greatly exceed even the peak G1/S phase levels seen in normal cells (Donnellan and Chetty, 1999; Keyomarsi and Herliczek, 1997; Porter et al., 1997; Sherr, 1996). Moreover, in most cases, high cyclin E levels correlate with increased tumor aggression and increased patient mortality (Porter et al., 1997).

The abundance of cyclin E is controlled primarily at the levels of gene transcription and ubiquitin-dependent proteolysis. The cyclin E gene is a target of the E2F family of transcription factors, which indirectly places cyclin E transcription under the control of the Retinoblastoma (Rb) protein and its various upstream regulators (Botz et al., 1996; Duronio and O'Farrell, 1995; Geng et al., 1996; Ohtani et al., 1995). Inactivation of Rb, either as part of the normal program of cell cycle progression or abnormally by tumor-specific gene mutations or viral oncoproteins, leads to upregulation of cyclin E transcription (Weinberg, 1995).

Two pathways for ubiquitin-dependent proteolysis are critically important for setting the level of cyclin E protein in normal cells. One pathway uses Cul-3 to promote the ubiquitination of cyclin E that is not associated with Cdk2 (Clurman et al., 1996; Singer et al., 1999). When cyclin E is bound to Cdk2, it is protected from ubiquitination by the Cul-3 pathway, and instead its ubiquitination becomes dependent on phosphorylation of threonine 380 (Clurman et al., 1996; Won and Reed, 1996) and threonine 62 (Strohmaier et al., 2001). Phosphorylation of these amino acids allows cyclin E to be recognized by Fbw7 (hCdc4) (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). Fbw7 is one of the substrate recognition subunits in a family of related E3 ubiquitin ligases, the SCFs, which are specialized for the recognition and ubiquitination of phosphorylated proteins (Feldman et al., 1997; Skowyra et al., 1997). Mutations in Fbw7 have been reported in some human tumor cell lines that express increased amounts of the cyclin E protein (Moberg et al., 2001; Strohmaier et al., 2001). Indeed, Fbw7 is localized to chromosome region 4q32, which is deleted in over 30% of human tumors and may therefore function as a tumor suppressor (Spruck et al., 2002).

The current molecular model for cyclin E turnover holds that autophosphorylation of T380 by its associated Cdk2 subunit is the trigger for recognition by the SCF^{Fbw7} complex, although the direct evidence for this derives exclusively from *in vitro* studies using immunoprecipitated cyclin E-Cdk2 complexes (Won and Reed, 1996). By this model, ubiquitination and degradation of cyclin E would be an automatic and inevitable conse-

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quence of its assembly into an active kinase complex. We have now tested this model *in vivo* and found that it does not adequately represent the pathways that regulate cyclin E ubiquitination by the SCF^{Fbw7}. We show that cyclin E turnover is controlled by assembly of cyclin E-Cdk2 complexes, by at least two kinases (GSK3 and Cdk2), and by phosphorylation on at least four different sites: T62, S372, T380, and S384. Our results imply that degradation of cyclin E is subject to a much wider range of controls than had been expected and that it may be a nodal sensor for multiple signal transduction pathways.

Results

SCF^{Fbw7}-Dependent Turnover of Cyclin E Requires Assembly of the Cyclin E-Cdk2 Complex and Cdk2 Activity

Two independent lines of investigation have shown, first, that ubiquitination of cyclin E requires both Cdk2 activity and assembly of cyclin E-Cdk2 complexes, and, second, that an F box protein, Fbw7, mediates cyclin E ubiquitination. To understand the relationship between these pathways, we have asked whether turnover of cyclin E by Fbw7 requires Cdk2 activity and the assembly of cyclin E-Cdk2 complexes.

Our experimental strategy was to independently assess the contributions of Cdk2 activity and Cdk2 assembly on turnover of cyclin E by Fbw7. In the first set of experiments we used two different assays to determine whether downregulation of cyclin E by the SCF^{Fbw7 β} pathway required Cdk2 activity. We found that ectopic expression of Fbw7 β was able to decrease the steady-state abundance of cotransfected cyclin E (Figure 1A, lane 3). This reaction was specific to cyclin E because the abundance of the other Cdk2-associated cyclin, cyclin A, was unaffected by Fbw7 (Figure 1C). In contrast, Fbw7 had no effect on cyclin E if we simultaneously blocked or inactivated endogenous Cdk2 either by cotransfecting cyclin E with an excess of kinase-dead (dominant-negative) Cdk2 (Figure 1A) or by overexpressing the Cdk2 inhibitors p21 and p27 (Figure 1B). In the second assay we found that the rate of Fbw7-dependent degradation of cyclin E depended on binding of cyclin E to active or inactive Cdk2. Thus, cyclin E had a half-life of 1.5 hr when bound to active Cdk2 but was more than 8 hr when bound to kinase-dead Cdk2 (Figure 1D). In these experiments we used Fbw7 β , one of three alternatively spliced forms of Fbw7. The other forms of Fbw7, Fbw7 α and Fbw7 γ , also turned over cyclin E in this assay (data not shown).

We then tested the requirement for assembly of cyclin E-Cdk2 complexes by comparing Fbw7-mediated turnover of wild-type cyclin E with the turnover of two mutant versions of cyclin E that are incapable of binding to Cdk2 (Clurman et al., 1996). Both of these mutants have small changes within the cyclin:CDK interaction domain (M1 is R130A, and M2 is FDRYM[156–160]AAAAA). Although Fbw7 could promote the turnover of wild-type cyclin E (Figure 1A), it did not affect the abundance of either of the two nonbinding cyclin E mutants (Figure 1E). Thus, the ability of cyclin E to assemble into a complex with Cdk2 was required *in vivo* at some step in the Fbw7-dependent turnover pathway. Together, these

observations establish that Fbw7 mediates the degradation of cyclin E within catalytically active cyclin E-Cdk2 complexes and is not involved in the turnover of unbound or unphosphorylated cyclin E. The focus of the experiments described below is on understanding how Cdk2 activity contributes to the turnover of cyclin E by the Fbw7 pathway.

Phosphorylation of Cyclin E *In Vivo*

F box proteins bind to phosphorylated protein substrates, suggesting that phosphorylation of cyclin E by Cdk2 might explain the Cdk2 requirement for cyclin E turnover. To test this directly, we mapped the sites on which cyclin E was phosphorylated *in vivo* and determined the contribution of Cdk2 and other kinases to the phosphorylation of those sites.

We used two-dimensional phosphopeptide mapping to identify the sites in cyclin E that were phosphorylated *in vivo*. Human U2OS cells were transfected with wild-type cyclin E. After orthophosphate labeling cyclin E was immunoprecipitated and subjected to tryptic phosphopeptide mapping (for a description of all peptides and their migration patterns see Supplemental Figure S1 at <http://www.molecule.org/cgi/content/full/12/2/381/DC1>). Three major cyclin E phosphopeptides were observed which we named spots A, B, and C (Figure 2A). Phosphoamino acid analyses revealed that spot A contained equal amounts of phosphoserine (pSer) and phosphothreonine (pThr) while spot B contained only pSer and spot C only pThr (data not shown). Sequential Edman degradation of phosphopeptide A eluted the radiolabeled phosphoamino acids in cycles two and ten corresponding to phosphorylation at S372 and T380 (see below). Spots B and C were also analyzed by sequential Edman degradation and revealed phosphoamino acids in positions two (spot B) and ten (spot C) (data not shown). This suggested that both spots B and C corresponded to the single phosphorylation of S372 and T380, respectively, while spot A contains both phosphorylated residues on the same peptide. We confirmed the identity of these sites by site-directed mutagenesis. Mutation of T380 to serine resulted in spot A containing pSer only as did spot C (data not shown). Furthermore, mutation of T380 to alanine resulted in loss of spots A and C and increased abundance of spot B (Figure 2A). Alternatively, mutation of S372 to alanine eliminated spots A and B and increased the abundance of spot C (data not shown). Mutation of both T380 and S372 to alanine resulted in the loss of all major cyclin E phosphopeptides (Figure 2A). Together these experiments showed that T380 and S372 were two major sites of cyclin E phosphorylation *in vivo*.

Cotransfection of U2OS cells with cyclin E and Cdk2 caused two important changes in the *in vivo* phosphopeptide map of cyclin E. First, phosphopeptide C was significantly enhanced relative to phosphopeptide B, which showed that Cdk2 could contribute to T380 phosphorylation, at least when Cdk2 was overexpressed (Figure 2B). Second, an additional phosphopeptide (D) was detected. Spot D contained both pSer and pThr (data not shown), and sequential Edman degradation revealed phosphorylation of positions two, ten, and fourteen. These data suggested that spot D resembled peptide

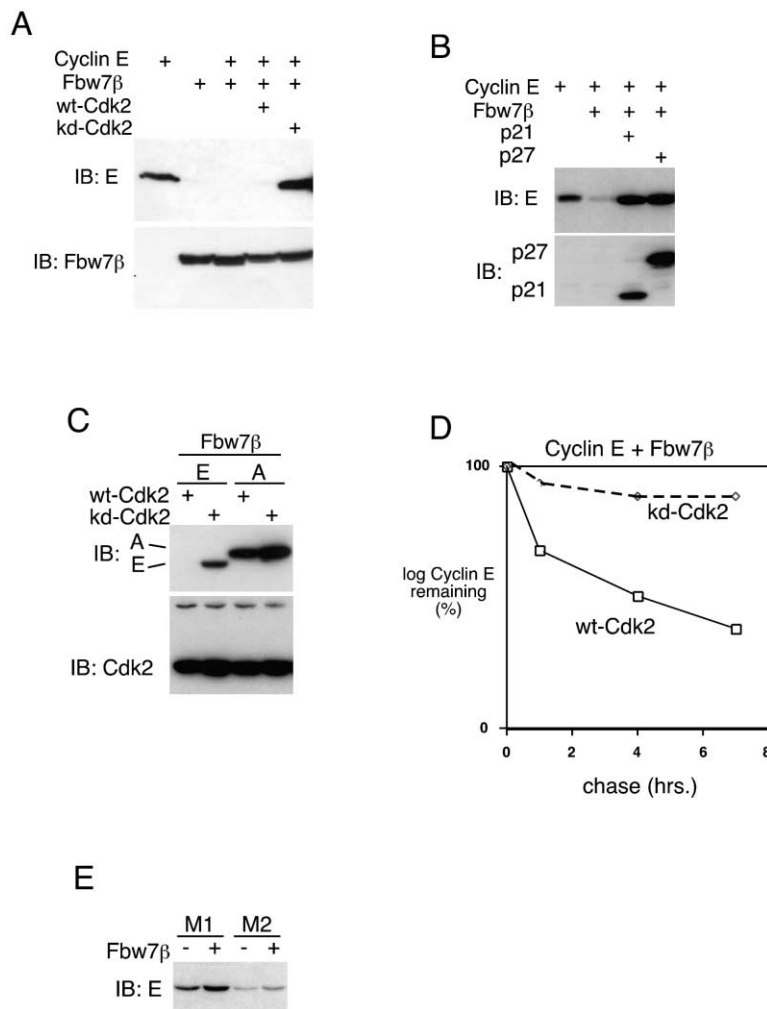


Figure 1. Fbw7β Degrades Cyclin E in a Cdk2 Activity- and Assembly-Dependent Manner

(A) Fbw7β eliminates cyclin E only in the presence of wild-type (wt), but not dominant-negative (kd, kinase-dead) Cdk2. 293 cells were transfected as indicated, and whole-cell extracts were subjected to immunoblot analysis for myc-tagged cyclin E and Flag-tagged Fbw7β.

(B) Endogenous Cdk activity is required for cyclin E turnover by Fbw7β. Assay is similar to that used in (A).

(C) Fbw7β specifically targets cyclin E. Cyclin E, but not A, is degraded by Fbw7β. Assay is similar to that used in (A).

(D) Active Cdk2 is required for cyclin E turnover by Fbw7. Pulse-chase analysis of Fbw7β-mediated cyclin E turnover in the presence of wt-Cdk2 or kd-Cdk2.

(E) Cyclin E mutants that cannot assemble into stable complexes with Cdk2 are resistant to Fbw7-mediated degradation. Two cyclin-box mutants of cyclin E (M1, R130A; M2, FDRYM[156-160]AAAAA) whose Cdk2 interaction is severely diminished (data not shown and see Clurman et al., 1996) were cotransfected with Fbw7β and cell lysates immunoblotted for M1 and M2 steady-state levels.

A (phosphorylation at S372 + T380) with an additional phosphorylation at position fourteen, which corresponded to S384. This assignment was confirmed by mutation of S384 to alanine (Figure 2B) or threonine (data not shown).

Although S384 phosphorylation was first detected in the context of Cdk2 overexpression, further analysis demonstrated that endogenous Cdk2 phosphorylated S384 and, moreover, that S384 phosphorylation was strictly dependent on endogenous Cdk2 activity. We repeated the phosphopeptide maps of cyclin E transfecting smaller amounts of cyclin E that were not in great excess over the amount of endogenous Cdk2. Under these more physiological conditions we observed that cyclin E was phosphorylated on S384 (Supplemental Figure S2). Moreover, phosphorylation of S384 strictly depended on endogenous Cdk2 activity, because it was abolished by cotransfection of cyclin E with either kinase-dead Cdk2 or the Cdk2 inhibitor p21. We noted that phosphorylation of T380 and S372 was not affected by Cdk2 inhibition. This is discussed further below.

Multiple Phosphorylation Sites Control Cyclin E's Activity and Turnover

The above analysis demonstrated three major sites of cyclin E phosphorylation in vivo: S372, T380, and S384.

We determined the role for each of these in Fbw7-dependent turnover of cyclin E. Wild-type cyclin E or various phosphosite mutants were transfected into 293 cells. Cotransfection of Fbw7β caused a substantial decrease in the expression of the wild-type cyclin E protein (Figure 3A). As expected, mutation of T380 to alanine prevented Fbw7β-mediated turnover of cyclin E. We also confirmed that mutation of T62 to alanine blocked turnover of cyclin E by Fbw7 (Strohmaier et al., 2001), although we have not yet been able to demonstrate directly that this represents a phosphorylation site. An unexpected result was that mutation of S372 to alanine also decreased Fbw7β-dependent degradation, though to somewhat lesser extent than the T62A or T380A mutations. Most interesting was that mutation of S384 to alanine prevented cyclin E turnover (Figure 3A), and this likely reflects a requirement for phosphorylation of S384, because mutation of this site to a phosphomimetic residue, glutamic acid, restored Fbw7β-mediated turnover (Figure 3B). Increased steady-state abundance of the S384A mutant correlated with enhanced protein stability (Figure 3C). Mutation of other sites of cyclin E phosphorylation (S58, S75; see below) had no effect on turnover of cyclin E by Fbw7 (Figure 3A).

The importance of S384 in cyclin E turnover was also reflected in the role that this residue plays in controlling

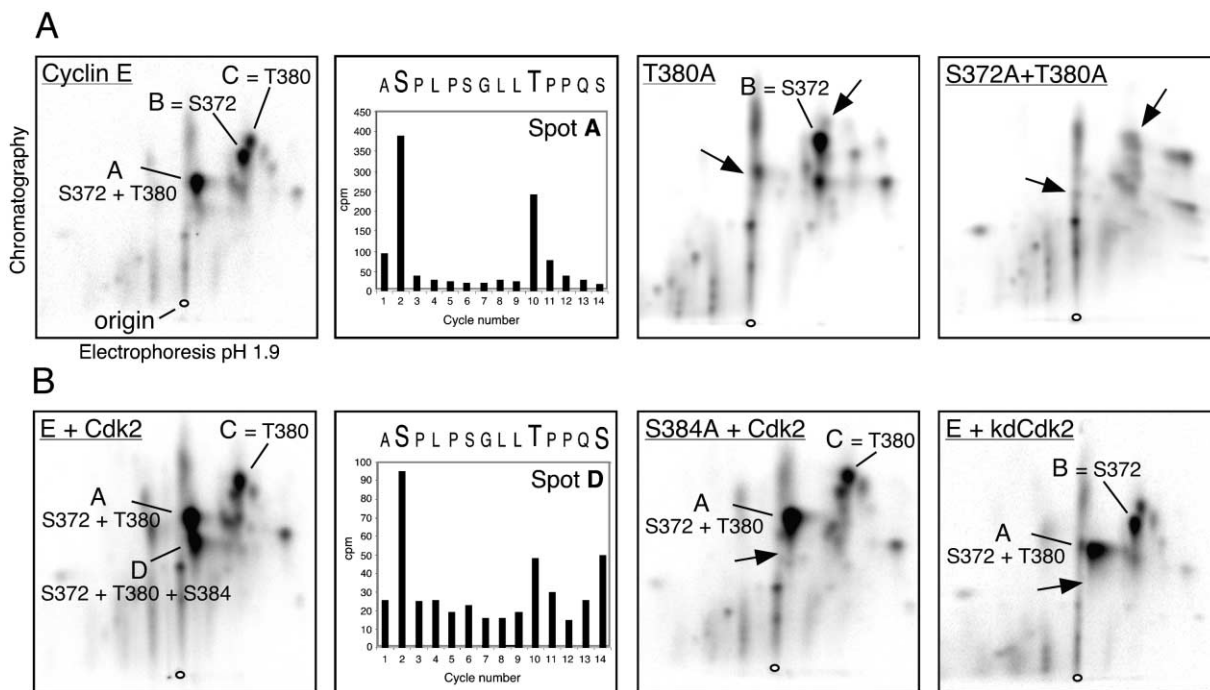


Figure 2. Phosphorylation of Cyclin E In Vivo

(A) Overexpressed cyclin E is phosphorylated on S372 and T380. Cyclin E (or mutants thereof) was transfected into U2OS cells, labeled with ^{32}P orthophosphate, and subjected to tryptic phosphopeptide mapping. Spots A, B, and C were subsequently analyzed for their phosphoamino acid content (data not shown) and for the positioning of the radiolabeled amino acid within the peptide by manual sequential Edman degradation. The identity of S372 and T380 phosphorylation was confirmed by mutational analysis of various alanine and Ser-to-Thr (or vice versa) exchanges. Only T380A and S372A/T380A mutants are shown.

(B) S384 phosphorylation is dependent on Cdk2 activity in vivo. Analysis of spot D as in (A). The unexpected migration of spot D is probably due to inefficient trypsin cleavage at K386 upon S384 phosphorylation providing an additional positive charge (K387) to the peptide (see also Figure 4 and Supplemental Figure S1 on the *Molecular Cell* website).

the biological activity of cyclin E. Cyclin E and the S384A mutant were transduced into U2OS (data not shown) and NIH 3T3 cells, and flow cytometry was used to compare their effects on the cell cycle. The S384A mutant had a significantly greater ability than wild-type cyclin E to cause cells to accumulate in S phase (Figure 3D). This was correlated with increased expression of the S384A mutant and its elevated associated kinase activity as compared to wild-type cyclin E (data not shown). Similar results were obtained with the T380A mutant (Minella et al., 2002). We concluded that S384 represents an important link between Cdk2 activity and cyclin E degradation, and that phosphorylation of cyclin E on S372, T380, S384, and T62 was important for turnover of cyclin E by Fbw7.

Phosphorylation of Cyclin E on T380 Does Not Require Cdk2

Phosphorylation of cyclin E on T380 is pivotal for cyclin E turnover, and previous results had shown that Cdk2 phosphorylated T380 within immunoprecipitated cyclin E-Cdk2 complexes (Won and Reed, 1996). Is Cdk2 required for cyclin E turnover because it phosphorylates both S384 and T380? To answer this, we reexamined the role of Cdk2 in T380 phosphorylation, both in vitro and in vivo. We first used two-dimensional phosphopeptide mapping to identify the sites that could be auto-

phosphorylated in vitro within purified cyclin E-Cdk2 complexes. Cyclin E-Cdk2 complexes were immunoprecipitated from lysates of transfected U2OS cells, treated with lambda phosphatase to remove phosphate groups that had been added in vivo, and then labeled with ^{32}P -ATP in vitro. No ^{32}P label was incorporated into cyclin E when inactive Cdk2 was expressed instead of wild-type Cdk2, which indicated that phosphorylation of cyclin E in this assay was mediated only by Cdk2 and not by another associated kinase (data not shown). This was further confirmed by showing that all phosphorylation detected in this assay was blocked by the Cdk inhibitory drug, roscovitine (Supplemental Figure S3). Three major phosphopeptides were found in autophosphorylated cyclin E: spots E (pSer), F (mainly pThr), and G (pSer) (Figure 4). Spot F corresponded to T380, as determined by sequential Edman degradation (pThr in position ten) and by mutation of T380 to either alanine or serine. Spot F is therefore identical to spot C in Figure 2. Similarly, spot G was identified as containing phospho-S384 by sequential Edman degradation (pSer in position fourteen) and by mutation of this residue to either alanine or threonine. Spot E contained phospho-S75, a site that was detected only in vitro (data not shown). Note also that mutation of S75 had no impact on cyclin E turnover by Fbw7 in cells (Figure 3A) or on the ability of cyclin E to modulate cell cycle progression (Figure 3D).

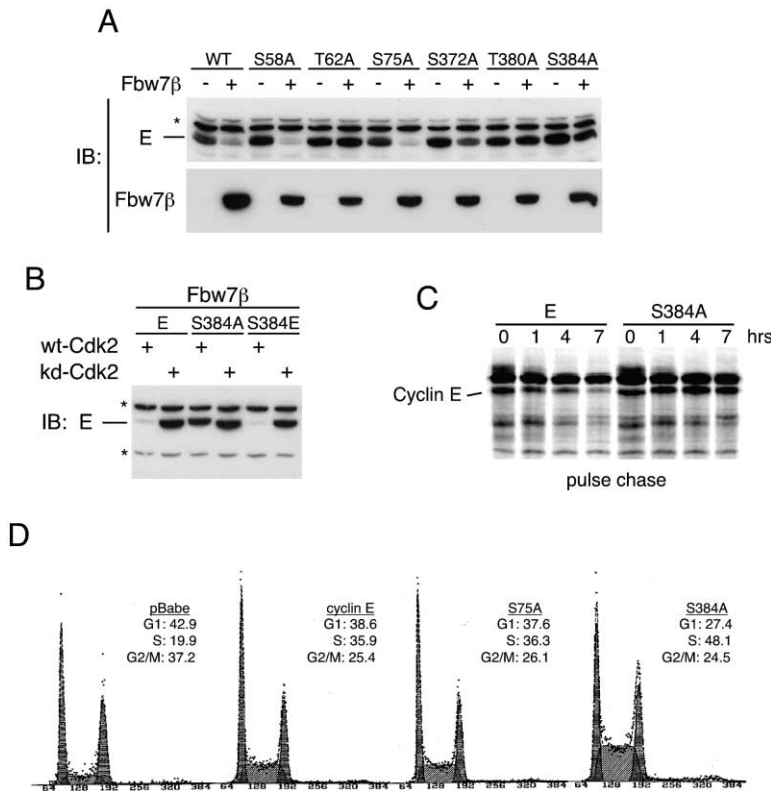


Figure 3. The S384A Phosphomutant Is Resistant to Fbw7 β -Mediated Degradation

(A) Several phosphosites are required for Fbw7 β -mediated destruction of cyclin E. 293 cells were transfected as indicated, and lysates were immunoblotted for cyclin E (anti-myc tag) and Fbw7 β as in Figure 1A. The band (*) above cyclin E is a crossreaction from the 12CA-5 antibody (anti-HA) and serves as loading control. We normally use 12CA-5 for detection of cotransfected Cdk2-HA. Although Cdk2 was omitted in this experiment, the data look identical if Cdk2 is coexpressed (data not shown).

(B) A phosphomimetic S384E mutant is degraded by Fbw7 β . Assay is similar to that used in (A), but with Cdk-2 coexpression.

(C) A nonphosphorylatable S384A mutant is stable. Pulse-chase autoradiography of 293 cells that were cotransfected with Cdk2, Fbw7 β , and either cyclin E or the S384A mutant. The band above cyclin E is a cross-reaction.

(D) S384A displays a higher percentage of cells in S phase as compared to wt-cyclin E or an S75A mutant. The cell cycle profiles of NIH 3T3 cells, which were transduced as indicated, are representatives of three independent replicates.

In sum, S75, T380, and S384 were all autophosphorylated by Cdk2 *in vitro*. Not only did this suggest a possible role for Cdk2 in T380 phosphorylation, but it also

showed that Cdk2 could directly phosphorylate S384. Neither S75 nor S384 conforms to the canonical proline-directed CDK consensus phosphorylation site. How-

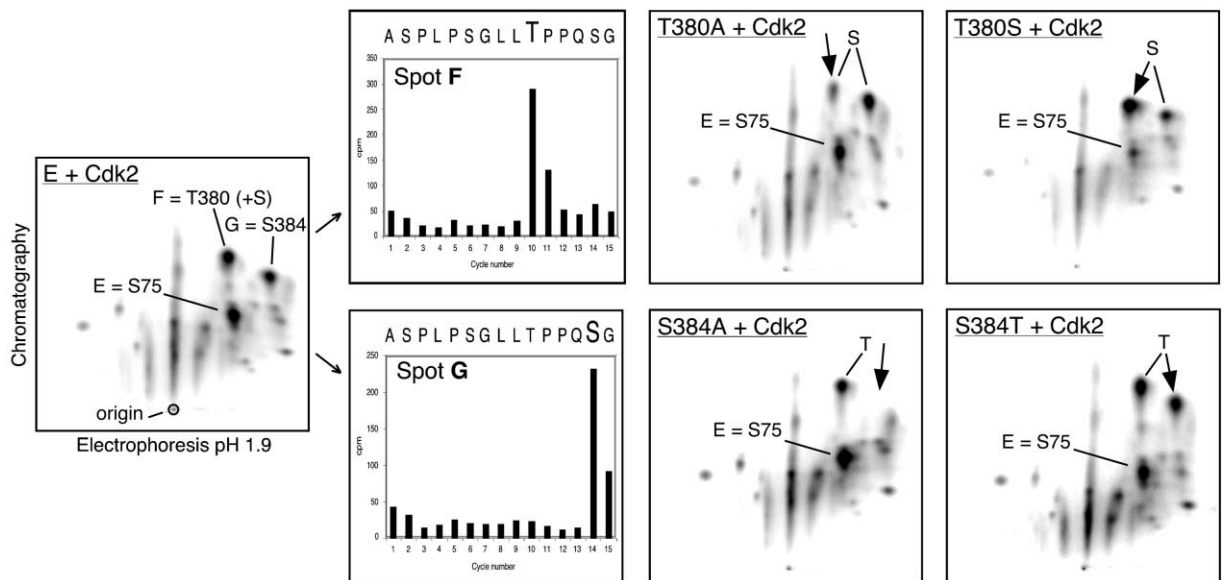


Figure 4. Phosphorylation of Cyclin E *In Vitro*

U2OS cells were cotransfected with myc-tagged cyclin E (or mutants thereof) and Cdk2. Cyclin E immunoprecipitates were phosphatase treated, subjected to *in vitro* kinase reactions, and analyzed by phosphopeptide mapping and manual Edman degradation as in Figure 2. Spot E contains phospho-S75 (analysis not shown). Spots F and G both represent monophosphorylations of the same peptide. Spot G (phospho S384) migrates with an additional positive charge in the +2 position, which most likely reflects inefficient trypsin cleavage at K386 upon S384 phosphorylation as seen with spot D in Figure 2 (see also Supplemental Figure S1 on the *Molecular Cell* website). Spot F (phospho T380) contains a trace amount of pSer (see T380A map) that results from some correct trypsin cleavage at K386 of peptide G. This is confirmed by the loss of the pSer portion in spot F with both the S384A and S384T mutants (data not shown).

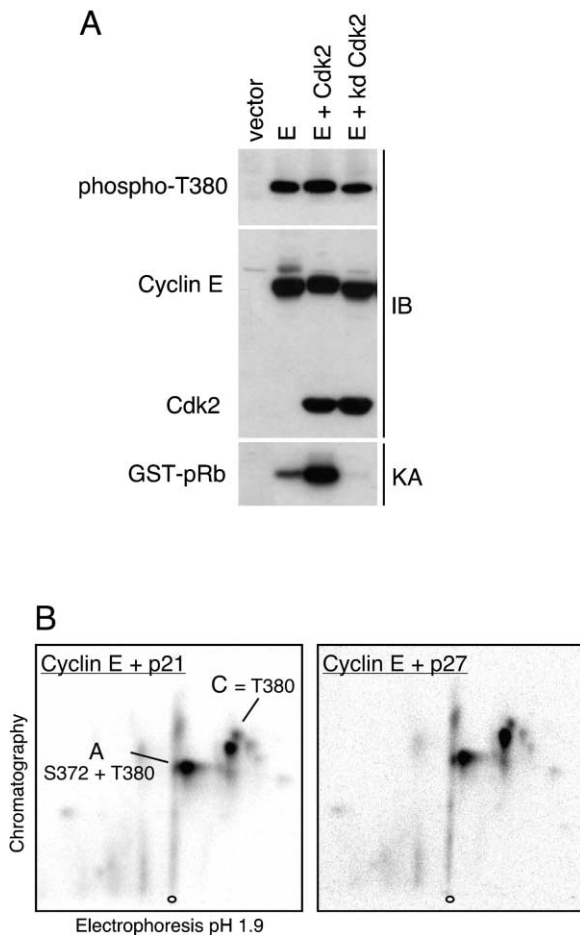


Figure 5. T380 Phosphorylation Is Not Cdk2 Dependent

(A) Expression of kd-Cdk2 eliminates cyclin E-associated kinase activity, but not T380 phosphorylation. 293 cells were transfected as indicated, and cyclin E immunoprecipitates were blotted for cyclin E with 9E-10 antibody specific to myc-tagged cyclin E or with a T380 phosphospecific antibody (see also Supplemental Figure S5 on the *Molecular Cell* website). The bottom panel demonstrates the absence of cyclin E-associated kinase activity in the presence of kd-Cdk2.

(B) Phosphorylation of T380 is independent of endogenous Cdk2 activity. Phosphopeptide maps of orthophosphate labeled U2OS cells transfected as indicated. Coexpression of p21 or p27 did not alter T380 phosphorylation significantly (compare to Figure 2).

ever, both of these sites contain a common motif, PxSxxK, which may represent a distinct class of CDK sites. The S384 motif is conserved among all cyclin E proteins.

Cdk2 phosphorylated T380 *in vitro*, and it could also stimulate phosphorylation of this site *in vivo*. Thus, as discussed above, overexpression of Cdk2 enhanced T380 phosphorylation on transfected cyclin E (Figure 2). However, unlike S384, T380 phosphorylation did not require Cdk2. We first used an anti-phospho T380 antibody to establish that T380 was phosphorylated even when kinase-inactive Cdk2 was highly overexpressed in human cells, a condition which is sufficient to inhibit all detectable Cdk2 activity (Figure 5A and Supplemental Figure S4). We further showed that inhibition of endogenous Cdk2 activity had only a minor impact on T380

phosphorylation as determined by phosphopeptide mapping. Thus, ectopic expression of kinase-dead Cdk2, p27, or p21 had little (if any) effect on the extent of T380 phosphorylation (Figures 2 and 5).

In conclusion, our results showed that Cdk2 inhibition had parallel effects on S384 phosphorylation and on Fbw7-dependent turnover of cyclin E. In contrast, there was no correlation between the effect of Cdk2 inhibition on cyclin E turnover and its effect on T380 phosphorylation—which was negligible. This suggested that Cdk2 affected cyclin E turnover primarily via S384.

Phosphorylation of Cyclin E by Glycogen Synthase Kinase

Although Cdk2 could directly phosphorylate T380 *in vitro*, we have demonstrated that T380 phosphorylation *in vivo* was not Cdk2 dependent. Hence, there must be another kinase capable of phosphorylating this residue. Glycogen synthase kinase 3 (α and β) (GSK3) was a good candidate because it is a proline-directed kinase that had previously been shown to be responsible for the phosphorylation-triggered turnover of another mammalian G1 cyclin, cyclin D1 (Diehl et al., 1998). Also, in addition to a proline in the +1 position there is a pSer at +4 (S384), the latter potentially representing a site for priming phosphorylation. Priming phosphorylation in the +4 position is a requirement in many, but not all, targets of GSK3 (Fiol et al., 1987; reviewed in Cohen and Frame, 2001).

The ability of GSK3 β to phosphorylate cyclin E was first tested *in vitro*. We used cyclin E bound to catalytically inactive Cdk2 as a substrate in order to prevent autophosphorylation by Cdk2. Cyclin E was well phosphorylated *in vitro* by GSK3 β (Figure 6A), and tryptic phosphopeptide mapping was used to identify the phosphorylated site(s) (Figures 6B–6D). One major pThr-containing peptide appeared in the same position as phosphopeptide A from the *in vivo* labeling of cyclin E-Cdk2 complexes, as described above. In addition, a much weaker pThr-containing peptide was detected in the same position as the *in vivo* peptide C. Phosphopeptides A and C both contained phosphorylated T380 (note that phosphopeptide A would also contain unlabeled phospho-S372 due to phosphorylation *in vivo*). This was confirmed by using a T380A mutant: neither peptide A nor C was labeled. Alternatively, a T380S mutant resulted in reappearance of spots A and C as pSer, demonstrating that T380 is a major GSK3 β phosphorylation site *in vitro*. As predicted, if the cyclin E (inactive)-Cdk2 complex was treated with lambda dba phosphatase prior to phosphorylation with GSK, then only the monophosphorylated spot C (T380) was observed (Figure 6C). These experiments also showed that phosphorylation of T380 by GSK was not dependent on a priming phosphorylation. We also showed that GSK3 β could phosphorylate cyclin E on T380 *in vivo*. Ectopic expression of enzymatically active, but not inactive, GSK3 β increased phosphorylation of cyclin E on T380 (see below).

In addition to phosphorylation at T380, there was a cluster of GSK-dependent phosphopeptides collectively indicated as “H” (Figure 6B). Each of these spots contained only pSer (data not shown), and mutational analyses demonstrated that they all corresponded to phos-

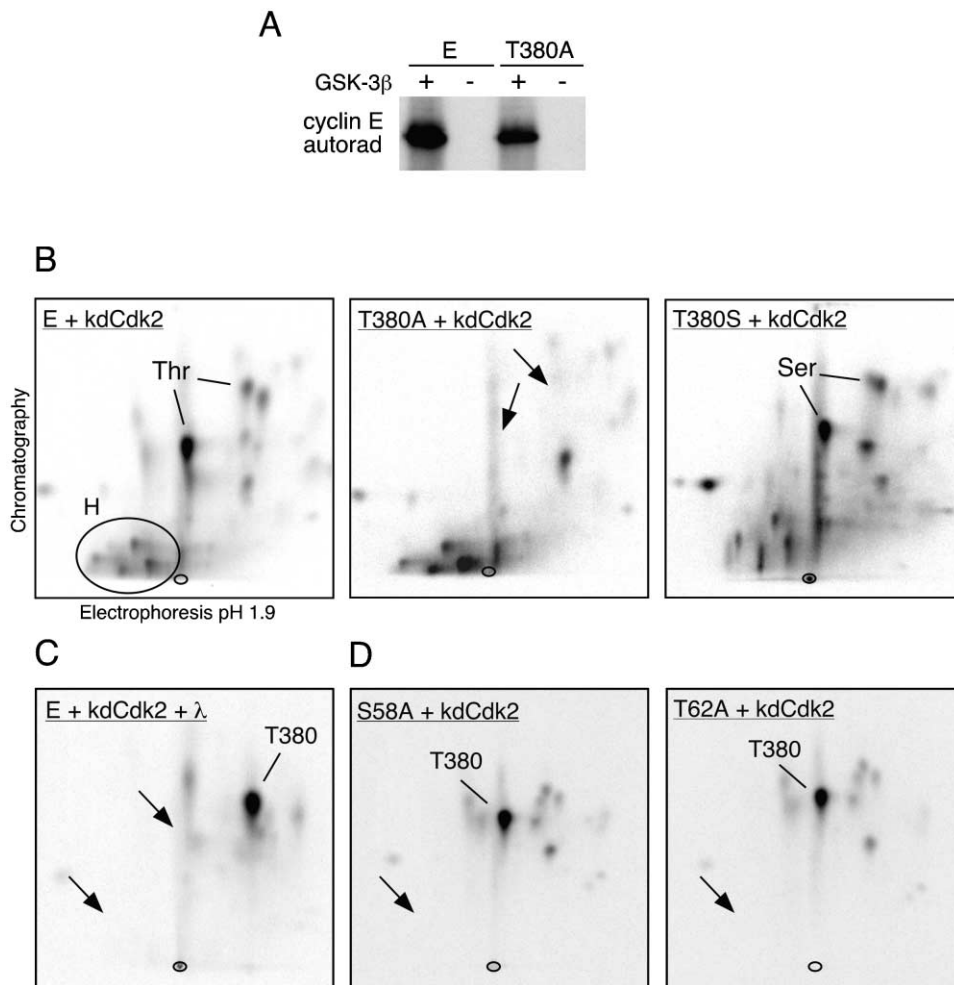


Figure 6. GSK3 Phosphorylates Cyclin E In Vitro

(A) Kinase assay using cyclin E or T380A (in complex with kd-Cdk2) as a substrate for recombinant GSK3 β . U2OS cells were transiently cotransfected with kd-Cdk2 and either cyclin E or the T380A mutant. Lysates were immunoprecipitated with 9E-10 antibody and immobilized cyclin-Cdk complexes were phosphorylated with recombinant GSK3 β .

(B) GSK3 β phosphorylates T380 (and cluster H). Tryptic phosphopeptide maps of identical reactions as in (A) plus an additional mutant, T380S. Phosphoamino acid analysis is not shown. The T380A map exposure is overenhanced to better demonstrate loss of T380 phosphorylation (this map also better demonstrates cluster H). The position of phospho-T380 at neutral charge results from nonlabeled in vivo phosphorylated S372 (data not shown and see [C], also compare to Figure 2, spot A).

(C) Pretreatment of the cyclin E-kd-Cdk2 complex with lambda phosphatase to strip off in vivo prephosphorylated sites results in T380 phosphorylation only by GSK3 β . The spot now migrates in the expected position for T380 monophosphorylated peptide (see also Figure 2, spot C). Cluster H is no longer detectable.

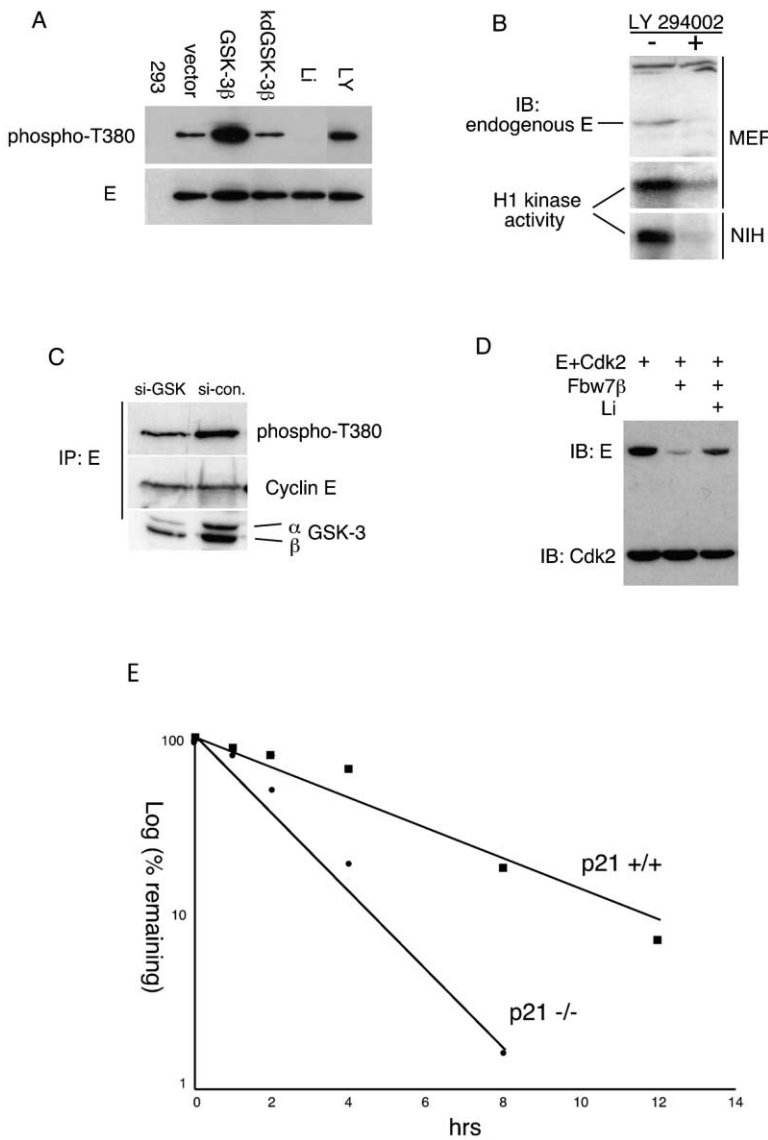
(D) GSK3 β phosphorylates S58 in a T62-dependent manner. Tryptic phosphopeptide maps of S58A and T62A mutants phosphorylated in vitro by GSK3 β as in (B). Cluster H consists usually of four to five serine-containing spots that all correspond to phospho-S58. This peptide most likely becomes degraded upon oxidization (see also Supplemental Figure S1 on the *Molecular Cell* website).

phorylation of S58 (Figure 6D). Phosphorylation of S58 was prevented by mutation of T62 to alanine, suggesting a priming function of phospho-T62 for S58 phosphorylation by GSK3 (Figure 6D). This was further supported by the observation that pretreatment of cyclin E with lambda phosphatase prevented phosphorylation of S58 (Figure 6C). T62 has previously been implicated in Fbw7-mediated turnover of cyclin E, which raised the possibility that this was indirectly due to its effect on S58 phosphorylation by GSK3. However, a S58A mutant was degraded like wild-type cyclin E, indicating that the function of T62 phosphorylation was not simply priming for S58 phosphorylation by GSK3 (Figure 3A).

Control of Endogenous Cyclin E by Endogenous Cdk2 and Endogenous GSK

Based on observations made with ectopically expressed proteins, our working model was that cyclin E turnover by Fbw7 was controlled both by Cdk2-dependent phosphorylation of S384 and GSK-dependent phosphorylation of T380. T62 and S372 probably are also important phosphorylation sites, but their respective kinases have not been identified. In support of this model, we verified that endogenous cellular Cdk2 and GSK regulate the cyclin E protein by similar mechanisms.

We first showed that endogenous cellular GSK3 phosphorylated cyclin E on T380. We treated cells with lith-



endogenous cyclin E. The elimination of Cdk inhibition by p21 results in diminished cyclin E protein levels (data not shown), increased specific cyclin E-associated kinase activity (data not shown), and reduced half-life of cyclin E.

ium, an inhibitor of GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), and found that this decreased phosphorylation of transfected cyclin E on T380 (Figure 7A). Conversely, we used the pharmacological agent LY294002 to activate GSK3 by inhibiting PI3-kinase. In this experiment, p21 was coexpressed to prevent any effect of endogenous Cdk2 on T380 and to prevent turnover of T380-phosphorylated cyclin E. This showed that activation of endogenous GSK increased the amount of T380 phosphorylation of transfected cyclin E (Figure 7A). To complement the pharmacologic approaches, we used RNA interference to simultaneously inhibit expression of both endogenous GSK3 α and GSK3 β , since both kinases can phosphorylate T380 (Figures 6 and 7 and data not shown). Reduced expression of both GSK enzymes caused a corresponding decrease in phosphorylation of cyclin E on T380 (Figure 7C). Data discussed above (Supplemental Figure S2) showed that inhibition

of endogenous Cdk2 blocked phosphorylation of cyclin E on S384. Thus, the endogenous GSK and Cdk2 kinases participated in cyclin E phosphorylation on T380 and S384, respectively.

Not only did endogenous GSK and Cdk2 phosphorylate cyclin E on the predicted sites, but activation of these kinases also had the expected effect of increasing endogenous cyclin E turnover. To address the role of endogenous GSK in cyclin E turnover, we used LY294002 to activate this enzyme. We treated mouse embryonic fibroblasts (MEFs) or NIH 3T3 cells to LY294002 for just 2 hr, which is less than one half-life of endogenous cyclin E (see below). This caused rapid degradation of all detectable endogenous cyclin E protein and disappearance of any measurable cyclin E-associated kinase activity (Figure 7B). Likewise, inhibition of endogenous GSK with lithium prevented, at least in part, the turnover of cyclin E by Fbw7, further demonstrating that endoge-

Figure 7. GSK3 Stimulates T380 Phosphorylation In Vivo

(A) Phosphorylation of T380 is enhanced upon overexpression of GSK or stimulation of endogenous GSK and is reduced upon inhibition of endogenous GSK. 293 cells were transfected with myc-tagged cyclin E, p21 (to inhibit endogenous Cdk2) and cotransfected/treated as indicated. Cell lysates were immunoprecipitated with 9E-10 antibody, and precipitates were immunoblotted with anti-phospho-T380 antibody. The same blot was subsequently probed for cyclin E with 9E-10 antibody. Lithium (20 mM) was added to cells right after washing off the precipitates for 24 hr until harvest. LY294002 (10 μ M) was added overnight before harvest. The assembled last lane (LY) originates from the very same blot and exposure of the same experiment.

(B) Stimulation of endogenous GSK results in loss of endogenous cyclin E expression and cyclin E-associated kinase activity in mouse cells. MEFs or NIH 3T3 cells were treated with 10 μ M LY294002 for 2 hr and lysates analyzed for endogenous cyclin E expression (upper panel) and cyclin E-associated kinase activity (lower panels).

(C) Reduction of endogenous GSK correlates with reduced T380 phosphorylation. U2OS cells were transduced with retroviruses encoding a small interfering hairpin RNA designed to target both GSK3 α and β at the same time or a mismatch siRNA as control. Transfected myc-tagged cyclin E was immunoprecipitated and blotted with 9E-10 antibody. The same filter was stripped and reprobed with the phospho-T380 antibody. GSK3 was probed with an antibody that recognizes both isoforms.

(D) Inhibition of endogenous GSK3 at least partially prevents the turnover of cyclin E by Fbw7 β . Assay is similar to that used in Figure 1A. Twenty millimolar lithium was added overnight before harvest.

(E) Endogenous Cdk2 activity participates in endogenous cyclin E turnover. Matching MEFs isolated from either wt or p21 $^{-/-}$ mice were subjected to pulse-chase analysis of endogenous cyclin E.

nous GSK participates in this pathway (Figure 7D). Last, we determined the effect of activating endogenous Cdk2 by comparing the stability of endogenous cyclin E in normal and p21 null MEFs (Figure 7E). In p21 null cells the specific activity of endogenous Cdk2 is significantly increased due to the loss of this CDK inhibitor (data not shown). We observed that the half-life of endogenous cyclin E was reduced from 8 hr in normal MEFs to just 4 hr in p21 null MEFs (Figure 7E). Thus, activation of either endogenous GSK or Cdk2 accelerates degradation of endogenous cyclin E.

Discussion

We have shown that turnover of cyclin E by the SCF^{Fbw7} complex requires the assembly of cyclin E into catalytically active complexes with Cdk2. Cdk2 activity is required primarily for phosphorylation of cyclin E on S384, which represents an important link between Cdk2 activity and cyclin E degradation. A second kinase, GSK3, is also important for cyclin E turnover because it phosphorylates cyclin E on T380. These kinases collaborate to control cyclin E abundance; activation of Cdk2 and GSK3 accelerate cyclin E degradation. Our observation that turnover of cyclin E by Fbw7 *in vivo* requires its assembly with Cdk2 complements the results of Strohmaier et al. (2001) who showed that once cyclin E is phosphorylated it can be ubiquitinated *in vitro* by the SCF^{Fbw7} independently of its binding to Cdk2. Therefore, the requirement for Cdk2 binding *in vivo* is likely to be at a step upstream of cyclin E's interaction with Fbw7. Two possibilities are that the binding of cyclin E to Cdk2 is necessary for its full phosphorylation or for its correct subcellular localization. Indeed, mutants of cyclin E that cannot bind to Cdk2 localize to the cytoplasm, whereas cyclin E-Cdk2 complexes are nuclear (our unpublished data).

Cyclin E Phosphorylation at Multiple Sites

At least four sites of phosphorylation contribute to optimal degradation of cyclin E by Fbw7: a cluster of C-terminal sites, S372, T380, and S384, and a lone N-terminal site, T62. The requirement for phosphorylation at multiple sites may be similar to the yeast Sic1 protein, whose ubiquitination by the SCF^{Cdc4} complex requires phosphorylation on six sites (Nash et al., 2001; Verma et al., 1997). In the case of Sic1, the six sites are all phosphorylated by the same kinase, Cln-Cdc28. This makes recognition of Sic1 by Cdc4 ultrasensitive to changes in Cln-Cdc28 activity and therefore causes degradation of Sic1 to occur in a switch-like fashion once a threshold of CDK activity has been reached (Nash et al., 2001). Similar logic may apply to the degradation of cyclin E because more than one of the phosphorylation sites can be a target of Cdk2 itself. However, phospho-T380 in cyclin E is a high-affinity binding site for Cdc4 and is sufficient, on its own (when introduced into Sic1), to promote efficient turnover of Sic1 by SCF^{Cdc4} (Nash et al., 2001). Therefore, unlike Sic1, phosphorylation of all sites in cyclin E may not be directly required for cyclin E to bind efficiently to Fbw7. For instance,

a subset of the cyclin E phosphorylation sites could promote the Fbw7 pathway by affecting the structure of the cyclin E-Cdk2 complex in a way that allows more effective access of Fbw7 to phospho-T380 or to lysine residues that are to be ubiquitinated. Another possibility is that phosphorylation of S384 may facilitate (prime) phosphorylation at T380 by GSK. Although the steady-state level of T380 phosphorylation did not depend on phosphorylation of S384, it remains to be determined whether phosphorylation of S384 stimulates the rate of T380 phosphorylation and thereby the rate of cyclin E turnover. In this respect, the S384E mutation could mimic a priming phosphate and therefore be efficiently degraded. Last, the fact that at least three different kinases are involved (Cdk2, GSK, and the kinase[s] that phosphorylates T62 and S372) suggests that cyclin E turnover may receive input from multiple sources, which together deliver information about mitogenic signaling, S phase entry, and other aspects of cell cycle progression.

Phosphorylation of Cyclin E by Cdk2

Activation of Cdk2-stimulated cyclin E degradation and inhibition of Cdk2 blocked cyclin E degradation by Fbw7. This was mediated by phosphorylation of cyclin E on S384. Thus, Cdk2 phosphorylated S384 *in vitro*, overexpression of Cdk2 increased S384 phosphorylation *in vivo*, and inhibition of Cdk2 prevented S384 phosphorylation *in vivo*. Although Cdk2 could also phosphorylate T380, it did not appear to be the major T380 kinase, because inhibition of Cdk2 had little effect on T380 phosphorylation. Hence, if Cdk2 had a role in T380 phosphorylation it was redundant with another kinase, GSK3.

Phosphorylation of Cyclin E by GSK3 β

Our experiments suggest that GSK3 is the kinase primarily responsible for phosphorylation of cyclin E on T380. Thus, both ectopic overexpression of GSK3 β in cultured cells and pharmacologic activation of endogenous GSK3 increased T380 phosphorylation. Conversely, inhibition of endogenous GSK3 with lithium or reduction in GSK3 abundance by siRNA decreased T380 phosphorylation. Moreover, activation of GSK3 accelerated cyclin E degradation, and inhibition of GSK3 inhibited cyclin E turnover by Fbw7. This was likely to represent direct phosphorylation of cyclin E by GSK3 because purified GSK3 β phosphorylated purified cyclin E-Cdk2 complexes on T380 *in vitro*. Three other proline-directed kinases, MAPK, cyclin A-Cdk2, and cyclin D1-Cdk4, did not phosphorylate T380 (data not shown).

GSK3 is a processive enzyme, and in some cases phosphorylation of the amino acid in the +4 position by a priming kinase stimulates subsequent phosphorylation by GSK3 at position 0, which could again function as a priming phosphate for phosphorylation in the -4 position and so on (Dajani et al., 2001; Frame et al., 2001; ter Haar et al., 2001; reviewed in Cohen and Frame, 2001). Phosphorylation of cyclin E on T380, however, was at least in part independent of priming for three reasons. First, mutation of the +4 residue (S384) had little detectable effect on T380 phosphorylation *in vivo*; second, phosphatase pretreatment of cyclin E did not diminish T380 phosphorylation by GSK3 *in vitro*; and third, phosphorylation of cyclin E by GSK3 *in vitro* was

not decreased when cyclin E was bound to inactive Cdk2 (S384 is not phosphorylated). In contrast, phosphorylation of cyclin E on S58 by GSK3 may depend on priming phosphorylation at T62. Thus, phosphorylation of S58 by GSK3 *in vitro* was prevented either by mutation of T62 to alanine or by prior phosphatase treatment of cyclin E. T62 phosphorylation appears to have a dual function: to prime for S58 phosphorylation by GSK3 β and independently to promote the Fbw7-mediated turnover of cyclin E. Thus far, we have not identified a role for S58.

The turnover of another mammalian G1 cyclin, D1, is also triggered by GSK3 β (Diehl et al., 1998). A signal transduction pathway containing Ras, PI3-kinase, and Akt negatively regulates GSK3 β (Cohen and Frame, 2001), and mitogenic signaling through this pathway has been shown to decrease GSK3 β phosphorylation of cyclin D1 on T286 (Diehl et al., 1998). Both CRM1-mediated nuclear export (Alt et al., 2000) and ubiquitination of cyclin D1 in the cytoplasm require phosphorylation on T286 by GSK3 β (A. Diehl, personal communication). Phosphorylation of cyclin E on T380 by GSK similarly promotes recognition by the SCF, but whether it also affects the subcellular location of cyclin E has not been determined.

Downregulation of GSK3 β by mitogens creates a window in the G1 phase of the cell cycle during which cyclin D1 accumulates due to its increased stability. In fact, mitogenic signaling regulates cyclin D1 not only at the level of protein turnover but also through its transcription, translation, nuclear import, and assembly with Cdk4 (Diehl et al., 1998). Therefore, cyclin D1 is considered to be the major link between mitogen signals and the cell cycle machinery.

In contrast, expression and activation of cyclin E is thought to be a downstream event, representing the transition of the cell cycle from a mitogen-dependent to a mitogen-independent state (Weinberg, 1995). For this reason, the finding that GSK3 β can phosphorylate cyclin E and regulate its turnover was unexpected. It would possibly place cyclin E directly under mitogen control and therefore change the picture of how mitogen signals are transduced to the cell cycle. This idea is at least consistent with the observations that cyclin E and cyclin D are required together for full inactivation of Rb (Harbour et al., 1999; Lundberg and Weinberg, 1998) and that cyclin E expression is not entirely E2F dependent (Duronio et al., 1995; Lukas et al., 1999). These results suggest that cyclin E may have a role prior to the restriction point (Ekholm et al., 2001).

In conclusion, we report that the pathways controlling cyclin E turnover are surprisingly elaborate and are comprised of both intrinsic (Cdk2) and extrinsic (GSK3) components. The association of cyclin E with Cdk2, the phosphorylation of cyclin E on four residues, the activities of at least three kinases, and the particular isoform of Fbw7 (our unpublished data) all contribute to cyclin E degradation. Given this complexity, there is a strong likelihood of tissue and cell type-specific regulation of cyclin E turnover. The observations also suggest multiple ways in which cyclin E turnover could be compromised during tumorigenesis.

Experimental Procedures

Cell Culture, Plasmids, Transient Transfection, and Retroviral Infection

NIH 3T3, 293, and U2OS cell lines were maintained in DMEM with 10% FCS. For transient gene expression, identical dishes (60 mm) were transfected with calcium phosphate precipitation overnight and harvested 24 hr after washing off the precipitates. For Fbw7-mediated turnover assays of cyclin E, 300 ng of cyclin plasmid was cotransfected with 3 μ g of pFLAG-Fbw7 plasmid with or without 3 μ g of pCMV-Cdk2HA or pCMV-dnCdk2HA. All transfections were normalized for equal amounts of total DNA concentration with empty vector. All phosphosite mutants of cyclin E have been generated by site-directed mutagenesis with the Quick Change method (Stratagene) with pCS2+6xMT-cyclin E as template (Clurman et al., 1996). All mutants were found to bind to and activate exogenous Cdk2 and to localize to the nucleus similar to wild-type cyclin E (data not shown). The two cyclin box mutants, M1 and M2, are severely restricted in their ability to bind Cdk2 and were constructed by annealing primers to single-stranded templates (Clurman et al., 1996; Sheaff et al., 1997; data not shown). Lithium was used at 20 mM for at least 16 hr; LY294002 (Calbiochem) at 10 μ M for various times. For retroviral infections, amphi- or ecotropic virus was made from 293/Phoenix cells by transient transfection of cyclin E or mutants cloned into pBabe. One milliliter of viral supernatant was mixed with 1 ml of fresh media to infect NIH or U2OS cells (60 mm dishes) overnight. Twenty-four hours after addition of fresh media, stably infected cells were selected with puromycin (10 μ g/ml for NIH cells, 1 μ g/ml for U2OS cells) for at least 2 days. For small interfering RNA experiments, we generated a pBabe-based retroviral vector expressing a hairpin RNA from the human H1 promoter cloned into the second LTR. The sequence ATCTTTGGAGCCACTGATT targets both GSK3 α and β . Our control siRNA corresponds to a mismatch in our GSK3 β specific sequence: TATGTCAAGTTGTATAGTTA.

Immunoblotting, Immunoprecipitation, and Kinase Assay

Cell extracts were made in Tween 20 buffer as in Matsushime et al. (1994), electrophoresed on 10% polyacrylamide gels, and transferred onto nitrocellulose membranes. The following primary antibodies were used: 9E-10 for myc-tagged cyclin E and derivatives thereof, HE-12 (immunoblotting, Santa Cruz) and HE-111 (immunoprecipitation, Santa Cruz) for endogenous cyclin E, 12CA-5 for HA-tagged Cdk2, anti-GSK3 α/β (#368662, Calbiochem), and M2 (Sigma) for Flag-tagged Fbw7 β . The phospho-T380 polyclonal antibody was generated by Zymed using a synthetic phosphopeptide (SGLLP TPPQSG). The antibodies were purified from rabbit serum in two rounds of chromatography; serum was passed through a column containing immobilized nonphosphorylated peptide. The flowthrough was passed through another column containing the phosphorylated peptide. Antibodies that were retained by this column were eluted and tested for specificity (see Supplemental Figure S5 on *Molecular Cell* website). For immunoprecipitation, cell lysates were incubated with primary antibody coupled to protein G Sepharose for 90 min at 4°C. For cyclin E autophosphorylation or phosphorylation by GSK3 β , washed immunoprecipitates were adapted to kinase buffer (Matsushime et al. 1994) and incubated in a 25 μ l kinase reaction containing 5 μ Ci 32 P- γ -ATP with or without 5 U of recombinant GSK3 β (Calbiochem) for 20 min at 30°C. To remove phosphates from cyclin E prior to autophosphorylation, immunoprecipitates were equilibrated to kinase buffer containing 5 mM DTT and 2 mM MnCl $_2$ without phosphatase inhibitors. Subsequently, beads were incubated with 100 U lambda phosphatase (Calbiochem) in a 25 μ l reaction for 10 min at 30°C, washed, and subjected to kinase reactions.

Pulse-Chase

Identically transfected 293 cells (60 mm dishes) were methionine-starved for 15 min in DMEM without methionine (ICN) containing 2% dialyzed FCS and subsequently labeled with 1 ml of trans- 35 S-label (100 μ Ci/ml, ICN) for 30 min. Dishes were washed and chased with normal complete DMEM. At the indicated time points, dishes were drained and collected at -80°C prior to lysis and immunoprecipitation.

Orthophosphate Labeling, Tryptic Phosphopeptide Mapping, Phosphoamino Acid Analysis, and Manual Edman Degradation Transfected U2OS cells (100 mm dishes) were washed with phosphate-free media (ICN) and labeled with ^{32}P -orthophosphate (1 mCi/ml) in 3 ml phosphate-free media containing 2% dialyzed FCS for 2 hr. After washing with 2×10 ml cold PBS, cells were lysed in 1 ml Tween 20 buffer with doubled amounts of phosphatase inhibitors. Immunoprecipitates were electrophoresed, blotted onto nitrocellulose membrane, and exposed to film. Filter pieces containing radio-labeled cyclin E were excised, trypsin-digested, oxidized, again trypsinized, and subjected to phosphopeptide mapping as in Hansen et al. (2001). In brief, peptides were separated on thin-layer chromatography (TLC) plates in two dimensions (electrophoresis, pH 1.9 buffer; chromatography, isobutyric buffer) and autoradiographically exposed. For phosphoamino acid analysis, phosphopeptides were eluted from the cellulose plate, acid hydrolyzed, separated by 2D electrophoresis, and analyzed as in Hansen et al. (2001).

Manual sequential Edman degradation was essentially performed as in Sullivan and Wong (1991). In brief, TLC-eluted phosphopeptides were coupled to Sequelon-AA membrane discs (Millipore) by use of carbodiimide coupling as described by the manufacturer. In each cycle, the N-terminal amino acid of the peptide was cleaved chemically with TFA (trifluoroacetic acid), collected, and scintillation counted.

Acknowledgments

We thank Wade Harper for the Fbw7 β cDNA, for communicating data, and for helpful discussions prior to publication. This work was supported by grants from The Leukemia & Lymphoma Society (M.W., #5204-02) and the NIH (B.E.C., RO1 #CA84069; K.R.L., K08-ES00382). B.E.C. is a W.M. Keck Distinguished Young Scholar in Medical Research. J.M.R. is an Investigator of the Howard Hughes Medical Institute.

Received: September 18, 2002

Revised: June 9, 2003

Accepted: June 9, 2003

Published: August 28, 2003

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