Recognition of Phosphodegron Motifs in Human Cyclin E by the SCF<sub>Fbw7</sub> Ubiquitin Ligase*

Xin Ye<sup>a</sup>, Grzegorz Nalepa<sup>b,c,d</sup>, Markus Welcker<sup>*</sup>, Benedikt M. Kessler<sup>e,f</sup>, Eric Spooner<sup>a</sup>, Jun Qin<sup>a</sup>, Stephen J. Elledge<sup>g,h,i</sup>, Bruce E. Clurman<sup>*,a</sup>, and J. Wade Harper<sup>a</sup><sup,k</sup>

From the <sup>a</sup>Department of Pathology, <sup>b</sup>Center for Genetics and Genomics, <sup>c</sup>Department of Genetics, <sup>d</sup>Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115, the <sup>e</sup>Program in Cell and Molecular Biology and <sup>f</sup>Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030, and the <sup>i</sup>Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109

Turnover of cyclin E is controlled by SCF<sub>Fbw7</sub>. Three isoforms of Fbw7 are produced by alternative splicing. Whereas Fbw7α and -γ are nuclear and the -β isoform is cytoplasmic in 293T cells, all three isoforms induce cyclin E destruction in an <i>vivo</i> degradation assay. Cyclin E is phosphorylated on Thr<sup>62</sup>, Ser<sup>88</sup>, Ser<sup>372</sup>, Thr<sup>380</sup>, and Ser<sup>384</sup> <i>vivo</i>. To examine the roles of phosphorylation in cyclin E turnover, a series of alanine point mutations in each of these sites were analyzed for Fbw7-driven degradation. As expected, mutation of the previously characterized residue Thr<sup>380</sup> to alanine led to profound defects of cyclin E turnover, and largely abolished association with Fbw7. Mutation of Thr<sup>62</sup> to alanine led to a dramatic reduction in the extent of Thr<sup>380</sup> phosphorylation, suggesting an indirect effect of this mutation on cyclin E turnover. Nevertheless, phosphopeptides centered at Thr<sup>62</sup> associated with Fbw7, and residual binding of cyclin ET<sub>380A</sub> to Fbw7 was abolished upon mutation of Thr<sup>62</sup>, suggesting a minor role for this residue in direct association with Fbw7. Mutation of Ser<sup>384</sup> to alanine also renders cyclin E resistant to degradation by Fbw7, with the largest effects being observed with Fbw7β. Cyclin E<sub>S384A</sub> associated more weakly with Fbw7α and -β isoforms but was not defective in Thr<sup>380</sup> phosphorylation. Analysis of the localization of cyclin E mutant proteins indicated selective accumulation of cyclin E<sub>S384A</sub> in the nucleus, which may contribute to the inability of cytoplasmic Fbw7β to promote turnover of this cyclin E mutant protein.

Flux through signaling pathways is controlled in large part, by regulated protein destruction and reversible protein phosphorylation. During the last few years, it has become clear that, in many cases, protein destruction is initiated by site-specific phosphorylation of the target protein, which then facilitates the interaction of the target protein with the destruction machinery. Much of the ubiquitination that occurs in response to protein phosphorylation occurs via the SCF ubiquitin ligase pathway. Phosphorylated proteins are ubiquitinated by an E1-E2 thiol-ester cascade, wherein the E2 is brought to the phosphorylated substrate via an SCF E3 (1–3). SCF complexes are composed of a core ubiquitin ligase containing the scaffold Cul1, a ring finger protein called Rbx1/Roc1, and an adaptor protein called Skp1 (4–8). Rbx1 associates with and activates E2s including Cdc34 (7, 9), whereas Skp1 interacts simultaneously with Cul1 and with a member of the F-box family of proteins (10). F-box proteins constitute a large family (more than 70 members in humans) of specificity factors that link diverse substrates with ubiquitination machinery (3, 11–13). Many F-box proteins contain C-terminal protein-protein interaction domains including WD40 and leucine-rich repeats that allow for specific target recognition. Several F-box proteins, including Cdc4 and Grr1 in yeast and β-TRCP (β-tranducin-receptor containing protein), Skp2, and Fbw7 in humans, have been demonstrated to interact with target proteins in a phosphorylation-dependent manner (5, 14–19, 20–23). Structural and biochemical analysis of the cyclin-dependent kinase inhibitor Sic1, a target of the SCF<sub>Cdc4</sub> complex, has revealed a complex relationship between Sic1 phosphorylation events and recognition by WD40 repeats in Cdc4 (18, 24). In essence, a minimum of six phosphorylation events are required for recognition through a single phosphodegron binding site on Cdc4. Each of these phosphorylation events generates phosphodegrons that are, in isolation, suboptimal for tight binding with Cdc4 (18, 24). In contrast, structural analysis of the β-TRCP F-box protein responsible for destruction of IκBα and β-catenin indicates that two phosphorylation events are recognized as a unit by a specific network of basic residues (25). The extent to which other substrates of the Cdc4 class of F-box proteins require multiple phosphorylation events is unknown.

In this work, we have examined how phosphorylation is used to regulate the interaction of the human G1 cyclin, cyclin E, with the WD40 containing F-box protein, Fbw7. Fbw7 is most closely related to Cdc4 in budding yeast (17, 21, 26) and has

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<sup>1</sup>The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Cdk, cyclin-dependent kinase; MS, mass spectrometry.

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been implicated in the turnover of not only cyclin E but Notch (27), c-Myc (28–30), and c-Jun (31) as well. RNAi against Fbw7 in humans or its Drosophila ortholog Ago leads to increased levels of cyclin E in tissue culture cells (17, 21), and mutations in Ago affect cyclin E levels in the Drosophila (26). In addition, mice deficient in Fbw7 die during embryogenesis (~10.5 days postcoitum) and both the embryo and the placenta display stabilization of cyclin E and Notch (32). Finally, Fbw7 can ubiquitinate cyclin E in vitro (17, 21). Mammalian cyclin E functions to activate cyclin-dependent kinase 2 (Cdk2) at the G1/S transition. Previous work has demonstrated that phosphorylation of Thr380 in cyclin E, in part via an autophosphorylation mechanism, is required for its rapid turnover (33, 34). This residue can also be phosphorylated by GSK3β to promote turnover (35). Although mutation of Thr380 to alanine greatly stabilizes cyclin E, its turnover in vivo or ubiquitination in vitro is not completely eliminated (17, 21). Recent studies have also implicated phosphorylation of Thr382 and Ser384 in cyclin E turnover by Fbw7β (21, 35), but whether this reflects direct or indirect effects in Fbw7 recognition is not clear. Thr382 conforms to a Cdk2 consensus site but the identity of kinases involved in Thr382 phosphorylation are currently unknown (35). Available data indicates that phosphorylation of Thr382 is required for phosphorylation of Ser384 by GSK3β (35). Phosphorylation of Ser384 is dependent upon interaction of cyclin E with active Cdk2, and it has been proposed that Ser384 is directly phosphorylated by Cdk2 (35).

To understand in greater detail how Fbw7 recognizes cyclin E and promotes its turnover, we have used mass spectrometry to confirm and extend recent peptide mapping studies of cyclin E phosphorylation and have performed a series of biochemical and mutagenic experiments that examine the role of multiple phosphorylation events in recognition and turnover of cyclin E by three specific isoforms of Fbw7, α, β, and γ. The data indicate both direct and indirect roles for Thr382 in binding to Fbw7. Whereas synthetic peptides encompassing Thr382 bind Fbw7 in a phosphorylation-dependent manner, the major effect seen upon mutation of Thr382 to alanine is a dramatic reduction in the extent of Thr382 phosphorylation, suggesting an indirect effect of the T62A mutation on cyclin E degradation through the Thr380 degron. Nevertheless, residual association of cyclin EThr382A with Fbw7 is reduced upon mutation of Thr382 to alanine. Mutation of Ser384 to alanine reduced turnover of cyclin E by all three isoforms of Fbw7, although the effects seen with Fbw7β were much greater than that observed with α and γ. Cyclin EThr382A associated more weakly than wild-type cyclin E with both Fbw7α and Fbw7β. Unlike other cyclin E mutants examined, cyclin EThr382A appeared to be preferentially retained in the nucleus, partially explaining the reduced ability of cytoplasmic Fbw7β to promote cyclin E turnover.

MATERIALS AND METHODS

Plasmids, Transfections, and Immunofluorescence—The pcDNA-Flag-Myctagged cyclin E expression plasmid employed was from a previous study (33). Mutationss were generated using the Gene Editor System (Promega). Vectors for expression of FLAG-Fbw7α, β, and γ were created in pcMV-FLAG (Sigma). To create an Fbw7γ expression plasmid, PCR was used to amplify Fbw7γ-specific sequences from a brain cdNA library. The oligonucleotides used were as follows: forward, GATCAGACGCTGACCTGAAATACACACCCAGCAGCATGAGGCTG; reverse, TCTGATGACTGATGGTTTCTGAGCACCTG. The 570-bp PCR product was digested with HindIII and BspMI and ligated into the pcMV-FLAG-Fbw7 backbone previously digested with HindIII and BspMI, and the product was confirmed by DNA sequencing. Plasmids used for expression of untagged Fbw7 mutants were described previously (17). To generate an expression plasmid for dominant negative Cull, sequences encoding amino acids 1–453 were cloned into pcDNA3 (12). 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The indicated quantities of DNA were used in either calcium phosphate- or Fugene 6 (Roche Applied Science)-mediated transfections. Thirty-six hours after transfection, cells were either lysed in 25 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, 10 mM β-glycerophosphate, 10 mM NaF, and protease inhibitors (Roche Applied Science) or processed for immunofluorescence.

Microscopy and Image Analysis—For immunofluorescence, cells transfected with cyclin E or Fbw7αβγ plasmids were grown on ultrathin cover slides (Fisher) and fixed in methanol or 4% paraformaldehyde in phosphate-buffered saline followed by permeabilization with 0.2% Triton X-100 for 10 min. Cells were then blocked with 5% goat serum (Sigma) for 30 min, incubated with primary antibodies (anti-Myc or anti-FLAG at 1:100) for 1 h, washed three times with phosphate-buffered saline, incubated with fluorochrome-conjugated secondary antibody for 1 h, and generously washed. Nuclei were then counterstained with 4′,6-diamidino-2-phenylindole, and cells were mounted in SlowFade Light Antifade reagent (Molecular Probes, Inc., Eugene, OR). To visualize subcellular localization of Fbw7 isoforms, images were taken on a Nikon/DeltaVision deconvolution microscope (Applied Precision) as Radial as a concentration of 1 mg/ml immobilized peptides (7 μl) were incubated with the indicated in vitro translation products (5 μl) in a total volume of 120 μl of NETN (25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40). Complexes were washed three times with 1 ml of NETN prior to SDS-PAGE and autoradiography. In some cases, Thr62-containing peptides were employed as competitor. Immune complexes were subjected to SDS-PAGE, autoradiography, and quantitation using a PhosphoImager (Amersham Biosciences). To examine association of FLAG-Fbw7α with cyclin E post-transfection, extracts were subjected to immunoprecipitation with anti-FLAG antibodies, and immune complexes were immunoblotted with anti-Myc antibodies. To examine the extent of cyclin E phosphorylation at Thr380, antibodies were also used to separate cyclin E Thr380A and immunoblotted with anti-phospho-Thr380 antibodies (Santa Cruz Bio technology, Inc., Santa Cruz, CA), and the blot was subsequently stripped and reprobed with anti-Myc antibodies. The absence of reactivity of anti-phospho-Thr380 antibodies with cyclin E Thr380A points to the specificity of the antibodies employed. For peptide binding experiments, the indicated synthetic peptides were coupled to Affi-Gel-10 beads (Bio-Rad at a concentration of 1 mg/ml immobilized peptides (7 μl) were incubated with the indicated in vitro translation products (5 μl) in a total volume of 120 μl of NETN (25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40). Complexes were washed three times with 1 ml of NETN prior to SDS-PAGE and autoradiography. In some cases, Thr62-containing peptides were employed as competitor. Antibodies against a synthetic peptide containing Thr42 (SLIIPHPDKR) were made by Phospho-Solutions, Inc. (Aurora, ON). Mass Spectrometry—Mammalian cyclin E was produced in insect cells as a glutathione S-transferase fusion protein as described (17). To produce mammalian cell-derived cyclin E for mass spectrometry, pcDNA2-Myc-cyclin E was transfected into four 150-mm dishes using Fugene 6. Subsequently, Myc-cyclin E was isolated using immobilized 9E10 antibodies and purified by SDS-PAGE for mass spectral analysis. Approximately 500 μg of cyclin E was recovered for mass spectral analysis, degraded on, lysates (data not shown). Mass spectral analysis of phosphopeptides in cyclin E was performed using matrix-assisted laser desorption ionization mass spectrometry with delayed extraction (Voyager-DE; Perseptive Biosystems, Framingham, MA). Unless otherwise noted, an electrospray ion trap mass spectrometer (LCQ Finnigan, San Jose, CA) coupled on-line with a capillary high pressure liquid chromatograph (Magic 2002, Auburn, CA) was used for identification of phosphorylation sites. Cyclin EThr382A was sequenced by assisted laser desorption ionization mass spectrometry with delayed extraction (Magic 2002, Auburn, CA) was used for identification of phosphorylation sites. Cyclin EThr382A was sequenced by
liquid chromatography/mass spectrometry/mass spectrometry (liquid chromatography/MS/MS) using a Q-TOF micro (Micromass).

**RESULTS**

**Analysis of Cyclin E Phosphorylation in Vivo**—Previous studies of cyclin E have demonstrated that mutation of Thr380 or Thr62 to nonphosphorylatable alanine leads to partial stabilization of cyclin E in transfected cells, whereas simultaneous mutation of both leads to further stabilization (21). Additionally, these mutations lead to reduced ubiquitination efficiency in vitro (17, 21). Precisely how these phosphorylation events facilitate recognition by Fbw7 is unclear. In addition, it is possible that additional phosphorylation events in cyclin E are important for recognition by Fbw7. Recent studies have identified five cyclin E phosphorylation sites, Ser58, Ser75, Ser372, Ser88, and Thr62, by conventional peptide mapping (35). Using mass spectrometry, we confirmed and extended these results in cyclin E purified from mammalian cells after transfection with a pCMV-Myc-cyclin E expression plasmid and in 293T cells, we did not observe phosphopeptides containing either Ser58, which is known to be phosphorylated (35), or Thr62, possibly due to the large size and cysteine-rich character of the predicted tryptic peptide. Therefore, antibodies directed at phospho-Thr62 were generated using a synthetic peptide as antigen. Immunoblotting of affinity-purified Myc-tagged wild-type or T62A cyclin E with these antibodies revealed specific interaction with wild-type cyclin E (Fig. 1B). Moreover, treatment of Myc-cyclin E with λ-phosphatase resulted in loss of reactivity toward the phospho-Thr62 antibody, consistent with a specific phosphorylation-dependent interaction (Fig. 1D). These data indicate that cyclin E is phosphorylated on Thr62 in tissue culture cells. Overall, these data lead to the conclusion that cyclin E is phosphorylated on at least three sites in the C terminus and at least four sites near the N terminus (Fig. 1B).

**Contribution of Phosphorylation to Fbw7-mediated Cyclin E Turnover in Vivo**—Currently, three distinct isoforms of Fbw7 (α, β, and γ) have been described (17, 21, 36). These isoforms employ distinct 5′ exons encoding unique N termini fused with 10 common exons. We first asked where these proteins are localized in the cell. However, because anti-Fbw7 antibodies suitable for immunofluorescence are not available, we used transient transfection of Fbw7 expression vectors in which the N terminus was tagged with a FLAG epitope. In 293T cells, we found that both Fbw7α and Fbw7γ are localized primarily in the nucleus (Fig. 1, A and C). In contrast, Fbw7β is almost exclusively found in the cytoplasm (Fig. 1B). We previously reported that Fbw7β has an apparent transmembrane domain near the N terminus (17), and this may be involved in localizing Fbw7β to endoplasmic reticulum membranes. An in depth analysis of cis-acting signals important for proper localization will be reported elsewhere.3

To examine the contribution of multisite phosphorylation to cyclin E turnover, we employed an in vivo degradation assay wherein Myc-cyclin E and Cdk2 are transiently expressed in 293T cells in the presence or absence of Fbw7 isoforms (35). Expression of Myc-cyclin E and Cdk2 alone led to readily detectable Myc-cyclin E, as determined by immunoblotting of crude cell extracts (Fig. 2, A–C). Co-expression of increasing levels of FLAG-Fbw7α led to a dramatic decrease in the steady-state abundance of Myc-cyclin E. All three Fbw7 isoforms, when expressed at comparable levels, were capable of reducing the abundance of cyclin E but had no effect on Cdk2 abundance. These results extend previous results from multiple laboratories indicating that Fbw7β overexpression can drive cyclin E degradation when overexpressed (21, 35). As expected, cyclin E384A and cyclin E1797A/384A levels were largely unaffected by expression of all three isoforms of Fbw7. This is consistent with a major role for Thr380 phosphorylation in controlling cyclin E turnover, as determined by pulse-chase (Fig. 2D). We then examined the susceptibility of T62A, S88A, S372A, and S384A mutations to elimination by Fbw7 isoforms. We found that T62A was substantially defective in elimination by Fbw7β and -γ, but this defect was much less obvious with the Fbw7α isoform under these conditions (Fig. 2, A–C). However, using lower Fbw7α expression plasmids revealed clear defects in cyclin E1797A turnover (Fig. 2F). We also found that Fbw7β was profoundly defective in eliminating cyclin E384A (Fig. 2B), whereas turnover of cyclin E384A by Fbw7α and -γ was less affected (Fig. 2, A and C). At higher levels of cyclin E384A expression plasmid used for transfection, cyclin E384A was substantially more resistant to turnover by Fbw7α than was wild-type cyclin E (Fig. 2G). Cyclin E384A was efficiently degraded by all three Fbw7 isoforms (Fig. 2, A–C, and data not shown for cyclin E384A). Control experiments demonstrated comparable levels of all three Fbw7 isoforms in transient transfections (Fig. 2E).

**Phosphorylation of Thr380 in the C-terminal Phosphodegron of Cyclin E Is Sufficient for Interaction with Fbw7**—Given the multisite phosphorylation of cyclin E in residues flanking Thr380, we next examined the contribution of individual phosphorylation events to recognition by Fbw7 in the context of short peptide phosphodegrons. Previous studies have demonstrated that short phosphopeptides are specifically recognized by WD40-containing F-box proteins (17, 18, 22). Synthetic peptides spanning amino acids 361–388 of cyclin E (cyclin E361–388) were synthesized in various phosphorylated and unphosphorylated forms that mimic phosphorylation in vivo (Fig. 3A), coupled to agarose beads, and then tested for binding to in vitro

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3 B. E. Clurman and M. Welcker, unpublished data.
translated Fbw7 (Fig. 3B). As expected, unphosphorylated cyclin E361–388 did not associate with Fbw7 (Fig. 3B, lane 2). In addition, we found that phosphorylation of either Ser372 or Ser384 alone did not support Fbw7 binding (Fig. 3B, lanes 4 and 6). In contrast, cyclin E361–388 peptides containing phosphorylation at Thr380 alone (lane 4) or in combination with Ser372 and Ser384 (lanes 5 and 7) interacted efficiently with Fbw7. In the context of Thr380 phosphorylation, phosphorylation at either Ser372 or Ser384 did not enhance association with Fbw7 (Fig. 3B, lanes 5 and 7). Thus, within the context of immobilized peptides in vitro, phosphorylation of Thr380 appears to be the major determinant in Fbw7 binding to the C-terminal phosphodegron.

Interaction of Fbw7 with a Phosphodegron Centered at Thr62—Mutation of Thr62 to alanine leads to reduced cyclin E turnover upon expression of Fbw7 (Fig. 2) (21). A major question that emerges is whether cyclin E employs phosphorylated Thr62 as a phosphodegron or whether the effects on cyclin E abundance are indirect. The budding yeast Fbw7 isolog Cdc4 is known to preferentially interact with sequences having the consensus (I/L)(P/I/L)pTPP (where pT represents phosphotyrosine) (18). Whereas positions −2 and −1 in the potential Thr62 phosphodegron are compatible with this consensus, peptide array data would indicate that the potential Thr62 phosphodegron would be blocked from binding, due to the presence of lysine at +3 (18). In model peptides, those containing lysine or arginine at +3 were found to not interact with Cdc4. To directly examine whether the Thr62 region can act as a phosphodegron, we synthesized peptides encompassing Thr62 in phosphorylated and unphosphorylated forms and tested them for binding to Fbw7 as described above (Fig. 3, A and B). In addition, we also synthesized peptides that contained phosphoserine at position 58 (35). Fbw7 did not interact with unphosphorylated cyclin E50–69 or with cyclin E50–69 phosphorylated at Ser58 (Fig. 3A, lanes 8 and 10). However, Fbw7 did interact with Thr62-phosphorylated cyclin E50–69 (Fig. 3B, lane 9). The extent of interaction was similar to that seen with Thr380 phosphorylated cyclin E361–388 (Fig. 3B, lane 3). The interaction of Thr62-phosphorylated cyclin E50–69 was not enhanced by phosphorylation at Ser58 in the context of this synthetic phosphodegron (Fig. 3B, lane 1). Thus, phosphorylation of Thr62 could, in principle, serve to directly target cyclin E to Fbw7.

Distinct Phosphodegrons in Cyclin E Interact with an Overlapping Binding Site on Fbw7—The finding that Fbw7 can interact with two distinct phosphodegrons in cyclin E in the context of synthetic peptides raises the question of whether these distinct motifs interact with Fbw7 through the same recognition site. To examine this issue, two experiments were performed. One approach took advantage of a collection of point mutations in the WD40 propeller of Fbw7, which are known to reduce binding to phosphorylated cyclin E (12). In particular, three arginine residues (Arg385, Arg425, and Arg463, using the Fbw7β protein as reference) (Fig. 3E) have been shown to be important for interaction with human cyclin E (17), and the equivalent residues in Cdc4 have been shown to be important for binding to Sic1 and phosphodegrons derived from Sic1 (18) (Fig. 3F). We reasoned that if Thr62- and Thr380-derived peptides interact in a similar way with Fbw7, then mutations in these arginine residues would decrease binding to both phosphopeptides. To examine this, these three Fbw7 mutants were produced by in vitro translation and tested for interaction with phospho-Thr62- and phospho-Thr380-containing peptides. We found that the R385A mutant was strongly defective in association with both the Thr62- and the Thr380-based peptides, with the interaction being undetectable (Fig. 3C, lanes 6 and 10). Mutations in Arg425 and Arg463 displayed reduced interactions compared with wild-type controls, but interaction with both the Thr62- and Thr380-derived peptides were reduced to similar extents. These data are consistent with the idea that overlapping binding sites are used to bind both peptides.

If similar binding sites are used by both peptides, one would expect that the presence of one peptide in solution would compete with Fbw7 for binding to the second peptide immobilized to agarose. To establish this competition assay, Fbw7 was added to mixtures of immobilized phospho-Thr380 cyclin E361–388 peptide and increasing amounts of either phos-
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Fig. 2. In vivo degradation of cyclin E phosphorylation site mutants by Fbw7 isoforms. A–C, plasmids expressing the indicated FLAG-tagged Fbw7 isoforms were transfected into 293T cells (6-cm dish) together with vectors expressing Myc-tagged cyclin E mutants and Cdk2. After 36 h, cells were lysed, and immunoblots were probed with either anti-FLAG, anti-Cdk2, or anti-Myc antibodies. The quantities of plasmids employed were 1.5 and 3.0 μg for pCMV-FLAG-Fbw7, 0.3 μg for pCS2-Myc-cyclin E, and 1.5 μg of pCMV-Cdk2. D, pulse-chase analysis of Fbw7α-driven cyclin E turnover in 293T cells. Pulse-chase experiments were performed as described under "Materials and Methods." E, similar levels of Fbw7 proteins are expressed in cyclin E turnover assays. Extracts from co-transfection studies performed in Fig. 3, A–C (lanes 1–3), were subjected to immunoblotting using anti-FLAG antibodies to demonstrate comparable levels of Fbw7 proteins. F, analysis of cyclin E<sup>T62A</sup> turnover by Fbw7α at lower Fbw7α/cyclin E ratios. Transfections were performed in 10-cm dishes using 2 μg of pCS2-Myc-cyclin E, 4 μg of pCMV-Cdk2, and 0, 2, 4, and 8 μg of PCMV-Fbw7α expression plasmids. G, constant amounts of pCMV-Fbw7α (0.25 μg) and pCMV-Cdk2 (3 μg) expression plasmids were co-transfected with increasing amounts of pCMV-Myc-cyclin E or pCMV-Myc-cyclin E<sup>T380A</sup> plasmids (0.3, 0.6, 0.9, or 1.2 μg). After 48 h, extracts were subjected to immunoblotting with anti-cyclin E and anti-Cdk2 antibodies.

phorylated or unphosphorylated cyclin E<sup>50–69</sup> peptide in solution. After mixing, the extent of association of Fbw7 with immobilized cyclin E<sup>E50–388</sup> was determined by SDS-PAGE (Fig. 3D). Whereas unphosphorylated Thr<sup>62</sup> cyclin E<sup>50–69</sup> had no effect on the association of Fbw7 with cyclin E<sup>E50–388</sup>, phospho-Thr<sup>62</sup> cyclin E<sup>50–69</sup> dramatically decreased the efficiency of Fbw7 association with cyclin E<sup>E50–69</sup> (Fig. 3D). Taken together, these data indicate that Fbw7 is capable of interacting with phospho-Thr<sup>62</sup> and phospho-Thr<sup>380</sup> peptides through a single interaction site composed of a cluster of arginine residues.

Association of Cyclin E Phosphorylation Site Mutants with Fbw7 in Vivo—The data described above suggested the possibility that Thr<sup>62</sup>, in addition to Thr<sup>380</sup>, could be employed for Fbw7 association with cyclin E in vivo. To examine whether dual modes of interaction occur with intact cyclin E, binding experiments were performed with a series of cyclin E mutants and Fbw7α after transfection in 293T cells. The ability to accurately assess binding interactions requires that comparable levels of cyclin E mutants be expressed. However, mutation of Thr<sup>380</sup>, and to a lesser extent Thr<sup>62</sup>, leads to increased steady-state levels of cyclin E in the presence of FLAG-Fbw7 expression (data not shown). Therefore, to achieve approximately equal levels of cyclin E expression, we also co-transfected vectors expressing a dominant negative form of Cul1<sup>(P18)</sup>, which contains the Skp1 binding site but lacks the Rbx1 binding site. This form of Cul1 sequesters Skp1-F-box complexes and leads to stabilization of SCF targets (16, 37). As expected, expression of Cul1<sup>(P18)</sup> leads to equal accumulation of all cyclin E mutants examined, despite the presence of FLAG-Fbw7α (Fig. 4A, lane 1–5). Extracts from cells expressing mutant cyclin E proteins and FLAG-Fbw7α were then subjected to immunoprecipitation using anti-FLAG antibodies, and the levels of associated cyclin E were examined by immunoblotting. Cyclin E efficiently associated with Fbw7α (Fig. 4A, lane 1). Interestingly, cyclin E<sup>E<sup>T62A</sup></sup> was found to associate weakly with Fbw7α (lane 3), but mutation of Thr<sup>62</sup> to alanine in the context of the T380A mutant further reduced the interaction with Fbw7α (Fig. 4A, lane 4). Mutation of Thr<sup>62</sup> in cyclin E to alanine also led to a reduction in the extent of Fbw7α binding (Fig. 4B, lane 2).

The reduced association between Fbw7α and cyclin E<sup>E<sup>T62A</sup></sup> could reflect either a significant utilization of phosphorylated Thr<sup>62</sup> in binding to Fbw7α or could potentially reflect alterations in the phosphorylation of Thr<sup>380</sup>. To examine this issue, we tested the extent of phosphorylation of Thr<sup>380</sup> in the context of the T62A mutation under the same conditions employed in Fig. 4A. Lysates from transfected cells were immunoprecipitated with anti-Myc antibodies, and the levels of total cyclin E-
and Thr\textsuperscript{380}-phosphorylated cyclin E determined by immunoblotting (Fig. 4B). We found that replacement of T62 with alanine significantly reduced the extent of Thr\textsuperscript{380} phosphorylation. The extent of reduction was comparable with the reduction seen in the association of Fbw7\textsubscript{1}/H9251 with cyclin ET62A. Transfection of cyclin E mutants in the absence of Fbw7 and Cul1DN demonstrated that the effect on Thr\textsuperscript{380} phosphorylation was independent of these two components (data not shown). Taken together, these data indicate that mutation of Thr\textsuperscript{62} affects Thr\textsuperscript{380} phosphorylation, and the majority of its contribution to Fbw7-mediated turnover appears to be indirect.

Involvement of Ser\textsuperscript{384} in Recognition of Cyclin E by Fbw7—Results described above indicate that cyclin E\textsubscript{S384A} is partially defective in degradation by Fbw7 with turnover by Fbw7\(\beta\) being affected to the greatest extent. To examine whether this reflects recognition by Fbw7, we compared the ability of Fbw7\(\alpha\) and -\(\beta\) isoforms to immunoprecipitate cyclin E\textsubscript{S384A} in transfected 293T cells (Fig. 5). Interestingly, cyclin E\textsubscript{S384A} bound more weakly to both Fbw7 isoforms (lanes 3 and 6) than did wild-type cyclin E, suggesting a significant decrease in affinity in the context of full-length proteins in vivo. Association of cyclin E with Fbw7\(\beta\) was substantially lower than with the Fbw7\(\alpha\) isoform, possibly reflecting the fact that Fbw7\(\beta\) is largely cytoplasmic, whereas cyclin E is largely nuclear. Importantly, cyclin E\textsubscript{S384A} maintained wild-type levels of Thr\textsuperscript{380} phosphorylation, as determined by immunoblots of cyclin E immune complexes using anti-phospho-T380 antibodies (Fig. 4B).

Mutation of Ser\textsuperscript{384} Affects the Nuclear/Cytoplasmic Ratio of Cyclin E—As stated above, the Fbw7\(\beta\) isoform is profoundly defective in degradation of cyclin E\textsubscript{S384A} in transfection experiments, whereas the \(\alpha\) and \(\gamma\) isoforms were less defective. Because our previous experiment revealed that Fbw7\(\alpha\) and Fbw7\(\gamma\) are predominantly nuclear, whereas Fbw7\(\beta\) is localized to the cytoplasm, we examined whether the inability of Fbw7\(\beta\) to degrade cyclin E\textsubscript{S384A} could result from aberrant intracellular shuttling of this mutant. Previous studies have demonstrated that cyclin E shuttles between the nucleus and cytoplasm (38). To determine whether cyclin E\textsubscript{S384A} accumulates in the...
nucleus more efficiently than wild-type cyclin E or other cyclin E mutants that are prone to Fbw7/H9252-mediated degradation, we measured the nuclear/cytoplasmic ratios for cyclin E and the mutants used in this study (Fig. 6). The indicated plasmids were expressed in 293T cells and the levels of cytoplasmic and nuclear cyclin E were determined by immunofluorescence and quantitative image analysis (Fig. 6B). Wild-type cyclin E as well as the T62A, S88A, and T380A mutants displayed comparable nuclear/cytoplasmic ratios. In contrast, the nuclear/cytoplasmic ratio of cyclin E^S384A^ was 2-fold larger, indicating defects in nuclear export for this mutant protein (Fig. 6A). Thus, defects in turnover of cyclin E^S384A^ by Fbw7/β may reflect both decreased affinity and decreased accessibility due to nuclear accumulation of this cyclin E mutant.

**DISCUSSION**

WD40 repeats in F-box proteins serve as receptors for recognition of phosphodegrons (5, 24, 25). Two distinct WD40/phosphodegron complexes have been examined structurally. The *Saccharomyces cerevisiae* Cdc4 WD40 propeller uses multiple arginine residues to interact with a single phosphothreonine in phosphodegrons derived from Sic1 or cyclin E (24), whereas human β-TRCP employs a large basic surface to interact with dual phosphoserines in a β-catenin-derived phosphodegron (25). Interestingly, the ability of Cdc4 to interact with its sole essential G1 target, the Cdk inhibitor Sic1, depends upon the number of G1 Cdk-generated phosphodegrons present in Sic1 (18). In general, each of these phosphodegrons binds weakly to Cdc4 in isolation, but occupancy of six sites allows for facile Sic1 ubiquitination and turnover. The use of a large number of relatively weak interactions allows for ultrasensitivity in the response of Sic1 turnover to G1 Cdk activity (reviewed in Refs. 39 and 40). The extent to which other SCFCdc4 substrates use multiple phosphorylation events to control turnover is unclear.

In this report, we examined how phosphorylation regulates the interaction of cyclin E with its cognate E3, SCFFbw7. Fbw7, the closest homolog of Cdc4 in the human genome, exists as three distinct isoforms, α, β, and γ, which display tissue-specific expression patterns (17, 21, 26, 36). Differences in Fbw7 isoforms are concentrated at the N terminus and are not expected per se to affect association with substrates via the C-terminal WD40 repeats. The localization properties of endogenous Fbw7 isoforms are unknown due to the absence of antibodies suitable for immunofluorescence. Therefore, we employed transient transfection of epitope-tagged versions of Fbw7α, Fbw7β, and Fbw7γ to examine the localization properties of these proteins. Fbw7α and -β are found in the nucleus in 293T cells, whereas Fbw7γ appears to be exclusively cytoplasmic. The localization of Fbw7β in the cytoplasm probably reflects the presence of a transmembrane domain not present in the α and γ isoforms (17, 36). Co-expression of Fbw7β with a vector expressing an endoplasmic reticulum marker (GFP-ER) dem-
onstrated significant overlap of the fluorescence patterns, suggesting that Fbw7/H9252 is at least partially associated with endoplasmic reticulum membranes (data not shown).

Cyclin E has long been known to be phosphorylated on Thr380 through an autocatalytic function involving Cdk2 (33, 34), but recent experiments (35) also suggest a role for GSK3/H9252 in this process, functioning in an apparently redundant manner with Cdk2. Mutation of Thr380 to alanine leads to significant stabilization of cyclin E, implicating this phosphorylation event in cyclin E turnover. A role for this residue is also consistent with the finding that phosphopeptides encompassing Thr380 are capable of binding to Fbw7 (17). However, evidence of the involvement of Thr62 and Ser384 has also been presented (21, 35). Mutation of Thr62 partially stabilizes cyclin E in an Fbw7/H9252-overexpression (35) and enhances the stabilization of the cyclin E T380A mutant (21). However, Thr62 has not been demonstrated to be phosphorylated in vivo, and it is not clear whether it plays a direct or indirect role in cyclin E turnover. Likewise, mutation of Ser384 to alanine also leads to partial stabilization of cyclin E in an Fbw7β-driven turnover assay (35), again through an unknown mechanism. Although the finding that cytoplasmic Fbw7β overexpression can promote degradation of cyclin E (which is primarily nuclear) seems counterintuitive, we note that cyclin E is initially synthesized in the cytoplasm, where it assembles with Cdk2. In this context, newly synthesized cyclin E was generated by ectopic expression in the assays performed here and may be readily destroyed via an Fbw7β-mediated mechanism. Moreover, cyclin E is known to shuttle from the nucleus to the cytoplasm in a Crm1-independent pathway (38). Thus, elevated levels of Fbw7β, through the destruction of cytoplasmic cyclin E, could force the equilibrium in favor of nuclear export, thereby leading to depletion of the nuclear pool of cyclin E. Further studies are required to determine what Fbw7 isoforms are directly involved in cyclin E turnover in vivo.

In this paper, we systematically examined the role of various phosphorylation events in the interaction between cyclin E and Fbw7 as well as the ability of individual Fbw7 isoforms to promote turnover of various phosphorylation site mutants in cyclin E. We initially used mass spectrometry to establish

Fig. 6. Mutation of Ser384 to alanine affects subcellular shuttling of cyclin E. A, quantitative analysis of nuclear/cytoplasmic fluorescence intensity ratio in 293T cells subjected to indirect immunofluorescence 48 h post-transfection with five phosphorylation-deficient cyclin E point mutants (T62A, T380A, S88A, and S372A) and wild-type cyclin E reveals significantly enhanced nuclear accumulation of cyclin E T380A protein as compared with wild-type cyclin E and four other cyclin E mutants. The quantities of all plasmids utilized and the exposure times were constant (1.0 μg and 1 s, respectively). Results represent averaged and normalized (N/C N wt/C wt) values for 15 cells for each construct. B, representative cells expressing wild-type cyclin E as well as cyclin E phosphorylation-deficient mutants and respective immunofluorescence profiles are shown. N, nucleus; C, cytoplasm.
phosphorylation sites in cyclin E. Consistent with recent data using conventional peptide mapping (35), we found that cyclin E is phosphorylated on three sites near the C terminus (Ser372, Thr380, and Ser384). Whereas Thr380 is phosphorylated directly by Cdk2, Ser384 does not conform to a Cdk2 site, yet its phosphorylation is Cdk2-dependent (35). We also detected phosphorylation of Ser388, a candidate Cdk2 site that was not identified previously. As with other studies (35), we were unable to unambiguously identify Thr62-phosphorylated peptides by mass spectrometry, possibly due to the large size of this tryptic peptide. However, phosphospecific antibodies against Thr62 unequivocally demonstrated that Thr62 in cyclin E is phosphorylated in 293T cells. However, the stoichiometry of phosphorylation of this residue and how this process is regulated is currently unknown.

Two parallel series of experiments were performed to examine the consequences of phosphorylation at these sites. First, we examined the ability of Fbw7 isoforms to promote cyclin E degradation in vivo. Wild-type cyclin E was efficiently degraded by all three Fbw7 isoforms, as were cyclin E variants (32) phosphorylated on Ser388 and Ser384. As expected, we found that mutation of Thr380 and to a lesser extent Thr62, led to defects in cyclin E turnover. This defect was independent of Fbw7 isoform. Cyclin E is phosphorylated on Ser384 and Thr62, and phosphorylation of Thr380 affects Ser phosphorylation contribute to binding of Fbw7. Using quantitative imaging, we found that, with the exception of cyclin E phosphorylated at Thr62, the combination of these two phosphorylation sites in cyclin E. Phosphorylation of Thr62 contributes to binding of Fbw7. In this case, Thr58 phosphorylation depends absolutely on prior phosphorylation of Ser62. The phosphodegron in cyclin E is phosphorylated at Ser62 by GSK3β, and Ser384 is phosphorylated by a mitogen-activated protein kinase. In this case, Thr58 phosphorylation contributes to association of cyclin E recognition by Fbw7 independent of Thr62 phosphorylation (28). Taken together, our study reveals a complex interplay between phosphorylation of cyclin E and control of its degradation by Fbw7 and reveals that the stoichiometry of phosphorylation under these conditions examined here may be small relative to that of Thr380 phosphorylation, but this need not always be the case in vivo. Identification of the Thr62 kinase is required to address this issue in greater depth.

Our data as well as that of Welcker et al. (35) indicate that mutation of Ser384 to alanine blocks effective turnover of cyclin E by Fbw7. As assessed by immunoprecipitation in transfected cells, mutation of Ser384 to alanine substantially reduces the association of cyclin E with Fbw7, suggesting that Ser384 phosphorylation contributes to binding to Fbw7. Using quantitative imaging, we found that, with the exception of cyclin E phosphorylated at Thr62, all of the other cyclin E proteins tested displayed indistinguishable nuclear/cytoplasmic ratios when transiently expressed in 293T cells. However, cyclin E phosphorylated at Thr62 displayed a dramatic (more than 2-fold) increase in the nuclear/cytoplasmic ratio. Thus, the effects seen in degradation of cyclin E phosphorylated at Thr62 by Fbw7 may reflect, in part, the inaccessibility of the cyclin E phosphorylated mutant with the Fbw7 isoform. Interestingly, c-Myc has recently been used to demonstrate the ubiquitination of cyclin E phosphorylated at Thr62 by Fbw7 in a phosphorylation-dependent manner (28, 29). In this case, Thr58 is phosphorylated by GSK3β, and Ser384 is phosphorylated by a mitogen-activated protein kinase. In this case, Thr58 phosphorylation depends absolutely on prior phosphorylation of Ser384.

The phosphodegron in cyclin E is phosphorylated at Ser62 by GSK3β, and Ser384 is phosphorylated by a mitogen-activated protein kinase. In this case, Thr58 phosphorylation contributes to association of cyclin E recognition by Fbw7 independent of Thr62 phosphorylation (28). Taken together, our study reveals a complex interplay between phosphorylation of cyclin E and control of its degradation by Fbw7 and reveals that the stoichiometry of phosphorylation under these conditions examined here may be small relative to that of Thr380 phosphorylation, but this need not always be the case in vivo. Identification of the Thr62 kinase is required to address this issue in greater depth.

REFERENCES
Recognition of Phosphodegron Motifs by SCF\textsuperscript{Fbw7}
Recognition of Phosphodegron Motifs in Human Cyclin E by the SCF^Fbw7^ Ubiquitin Ligase
Xin Ye, Grzegorz Nalepa, Markus Welcker, Benedikt M. Kessler, Eric Spooner, Jun Qin,
Stephen J. Elledge, Bruce E. Clurman and J. Wade Harper

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