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Identification of Genes Regulated by Proteolysis

Substrate selection in ubiquitination reactions is achieved by ubiquitin ligases, which simultaneously bind both the target protein and a ubiquitin conjugating enzyme. We have developed a phosphopeptide based approach to facilitate the identification of ubiquitin ligases (e.g. F-box proteins) that recognize regulatory proteins in a phosphorylation-dependent manner. Thus far, we have used this approach to identify substrates of two F-box proteins, Fbw7 and beta-TRCP. Cyclin E associates with Fbw7 through a phosphodegron near its C-terminus. Experiments in vitro and in vivo have identified critical phosphorylation sites in this degron that are required for interaction with Fbw7. Interestingly, this motif is found in a number of other unstable oncogenic proteins, including c-myc, c-jun, and SREBP. In a second series of studies, we have identified the cell cycle regulatory protein Cdc25A as a target of the beta-TRCP protein. We have found that Chkl phosphorylates Cdc25A in response to DNA damage to generate a priming event that facilitates phosphorylation of Cdc25A on a motif that then binds to beta-TRCP. Using biochemical and genetic techniques, we demonstrate that this interaction is required for regulated proteolysis of Cdc25A.
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<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF 298</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5-7</td>
</tr>
<tr>
<td>Body</td>
<td>7-12</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>13</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>13-16</td>
</tr>
<tr>
<td>Conclusions</td>
<td>16</td>
</tr>
<tr>
<td>References</td>
<td>16-18</td>
</tr>
<tr>
<td>Appendices</td>
<td>19-54</td>
</tr>
</tbody>
</table>
Introduction

Protein ubiquitination requires three components: E1, E2, and E3 (Hershko and Ciechanover, 1998). In the first step, a ubiquitin-activating enzyme (E1) is charged with ubiquitin through a thiol-ester linkage. This ubiquitin is then transferred to one of a dozen or so ubiquitin conjugating enzymes (E2) also as a thiol-ester. The ubiquitin is finally transferred from the E2 to one or more lysine residues in the substrate with the aid of an E3 ubiquitin ligase. In essence, E3s function as substrate-specific adaptors by simultaneously binding substrate and the E2, although in some cases, E3s may also serve as an intermediate in the ubiquitin transfer process.

Much of our knowledge of E3s has come from genetic dissection of signaling pathways that involve one or more ubiquitin-dependent events (reviewed in Koepp et al., 1999; Patton et al., 1998). These studies have revealed 3 broad classes of E3s that are likely to be responsible for targeting the ubiquitination of hundreds of proteins: 1) the HECT domain class which includes E6-AP, 2) the ring finger class which include Cbl and MDM2, and 3) the cullin-based ubiquitin ligases which include SCF, VBC, and APC complexes. Given the size of these different protein families, it is clear that many aspects of the biology of these E3s are unexplored. There are at least 40 HECT domain proteins in the human genome and more than 250 ring-finger containing proteins that may be involved in ubiquitination.

The complexity of these systems is perhaps best exemplified by the cullin-based ligases of which the SCF complex is the best understood. These are multicomponent E3s that dock substrates with a core ubiquitin conjugating system via modular substrate specific adaptors (reviewed in Koepp et al., 1999). The core components include a member of the cullin family of proteins, which contains 6 members in mammals, a RING finger protein typified by Rbx1 and APC11, and an E2 (Cdc34 or Ubc4). These core complexes interact with distinct families of substrate specific adaptors to generate a large number of ubiquitin ligases with distinct functions. Our work has focused on the SCF sub-family of cullin-based ubiquitin ligases, which refers to the three major components (Skp1, Cul1, and a member of the F-box family of proteins) (See scheme on left). Through genetic and biochemical studies in budding yeast, we identified Skp1 as a component of the SCF that links Cul1 to F-box proteins (Bai et al., 1996; Skowyra et al., 1997). We also discovered the F-box motif as a Skp1 binding element that is found in a large number of proteins that can bind to particular ubiquitination substrates in a phosphorylation dependent manner (Bai et al., 1996; Skowyra et al., 1997). The timing of ubiquitination and destruction of many proteins are controlled by protein phosphorylation, including cyclin-dependent kinase inhibitors such as p27 and Sic1, and G1 cyclins (cyclin E and Cln proteins). Through biochemical reconstruction of the SCF mediated ubiquitination of Sic1 and Cln1, we were able to demonstrate that distinct F-box proteins recognize distinct targets in a phosphorylation dependent manner and allow ubiquitination via an Rbx1/Cul1 dependent pathway (Skowyra et al., 1997, 1999; Kamura et al., 1999). In addition to the F-box motif that mediates interaction with Skp1, F-box proteins frequently contain C-terminal protein-protein interaction domains (Bai et al., 1996). The most common are WD40 and leucine-rich repeat domains. In an effort to understand the...
complexity of mammalian F-box proteins, we have isolated a large number of cDNAs encoding F-box proteins (Winston et al., 1999a). In total, >68 distinct mammalian F-box proteins are now known to exist. We have shown that one of these, β-TRCP, is responsible for the ubiquitination of IkB, an inhibitor of the NFκB transcription factor complex required for the cytokine response as well as β-catenin, a transcription factor that functions as an oncogene when not properly destroyed by ubiquitin-mediated proteolysis (Winston et al., 1999b). The Cul2-based ubiquitin ligase has more than 20 known substrate adaptor proteins called SOCS-box proteins (Hilton et al., 1998), one of which is the von Hippel-Lindau tumor suppressor protein (Lisztwan et al., 1999). Although the functions of the vast majority of F-box and SOCS-box proteins are unknown, the finding that the limited number of F-box proteins that have been characterized all function to ubiquitinate multiple target proteins suggests that this family of E3s will be responsible for ubiquitination of possibly hundreds of proteins.

The challenge in the post-genome era will be: 1) to identify proteins whose abundance is regulated, 2) to determine what ubiquitin ligase pathways contribute to destruction of specific targets, and 3) to determine how the activities of particular ligases are controlled. Historically, the identification of ubiquitinated proteins has occurred on a case by case basis, and as such, we have a very limited view of the number and types of proteins in the cell that are controlled by this pathway. Moreover, we have little information that addresses how particular oncogenic events affect either the activities of different classes of ubiquitin ligases or the access of such ligases to their substrates. This is due, in part, to the fact that generally applicable methods are not available for identifying proteins that are destroyed in response to particular stimuli or in particular cellular contexts. In rare cases, it has been possible to use genetic screens in yeast to identify targets of ubiquitin ligases for which mutants were available. However, this approach is generally limited to yeast and even in cases where particular mutants in E3 components are available, substrates have been difficult to identify. In addition, approaches such as two-hybrid systems have not been particularly useful in identifying targets of ubiquitin ligase components such as F-box proteins. Given the large number of substrate adaptors that we and others have identified and that are likely to be identified in the future as a result of genome sequencing efforts, the identification of their important substrates will continue to be a major challenge.

Three complementary approaches are being undertaken to identify ubiquitination targets. In one approach, we are taking advantage of the facile genetics in budding yeast to identify targets of known ubiquitin ligase. Yeast has provided many of the important insights into ubiquitination pathways that have been shown to be general to all eukaryotes. Progress in this area was described in the previous progress report, including a publication in Science wherein we reported the identification of an F-box protein – Fbw7 - for human cyclin E. Human cyclin E is a prognostic marker for breast cancer and there is evidence that Fbw7 is mutated in human cancer, consistent with the possibility that it is a tumor suppressor. We have now performed a detailed examination of the biochemical mechanisms used in recognition of Cyclin E by Fbw7 and present these finding below, as well as a recent paper describing these results. Tasks 1 and 2 have been completed and were described in detail in the previous report. As part of task 3, we have designed and utilized a sectoring assay in budding yeast in the hope of identifying E3-substrate pairs. However, this approach has not yet been successful. In a second approach, we are using a collection of F-box proteins as biochemical and genetic reagents to identify substrates. Our progress on the identification of an E3 for Cdc25A - SCF-beta-TRCP - will be presented below. This work has recently been published. Finally, in a third approach, we have attempting to develop general methods to identify substrates in mammalian cells. For the reasons described below, we needed modified our initial objective (Aim 2) in this regard to take advantage of new technology that has emerged since this grant was submitted. Although the final system is still not complete, we think that the approach of RNAi libraries has a good chance of providing a general means by which to identify substrates of ubiquitin ligases.
Development of a library of F-box proteins

We previously reported the identification of 33 human F-box proteins. Through subsequent work, we have now expanded this to more than 68 family members in humans and 70 family members in the mouse. These F-box proteins fall into three classes: Fbws, which contain WD40 repeats, Fbls which contain leucine rich repeats, and Fbxs which contain either no recognized domain or have other classes of protein interaction domains. These C-terminal protein interaction domains are thought to mediate interaction with substrates.

Identification of an F-box protein important for degradation of the breast cancer oncoprotein Cdc25A. Previous work has demonstrated that Cdc25A is an oncogene and is overexpressed in breast cancer (Cangi et al., 2000; Evans, 2000). Cdc25A catalyzes the dephosphorylation of Cdk2, leading to its activation and thereby promoting S-phase entry. Cdc25A is regulated by multiple proteolytic mechanisms, including the anaphase promoting complex. Moreover, recent data indicate that Cdc25A is destroyed in response to DNA damage in a manner that depends upon the Chk1 kinase. Previous data suggested the involvement of an SCF complex in Cdc25A regulation but the identity of the F-box protein involved was not known.

β-TRCP Recognition Motif:

To address this, we first confirmed the previous finding that dominant negative inhibitors of Cul1 lead to stabilization of Cdc25A. Interestingly, this occurs independently of DNA damage. Moreover, we demonstrated that Cul1 associates with Cdc25A in vivo. To search for F-box proteins that interact with Cdc25A, we performed a series of transfection experiments using various F-box proteins and then performed immunoprecipitations using Cdc25A antibodies. We found one F-box protein – β-
TRCP - that consistently interacted with Cdc25A. We previously discovered β-TRCP as an F-box protein that interacts with destruction motifs in IkB and β-catenin. β-TRCP interacts with sequences containing the consensus: DSGIXS where both serine residues are phosphorylated. In the absence of phosphorylation, neither IkB nor β-catenin interact with β-TRCP. We scanned the sequence of Cdc25A for sequences that look like those found in IkB and found a sequence centered on Ser-81 that has similarities to the sequence in IkB. We demonstrated that Cdc25A binds to β-TRCP in a manner that depends upon phosphorylation of Ser-81 and this interaction requires the action of Chk1, a kinase that is activated by ATM/ATR in response to DNA damage. We demonstrated that RNAi against β-TRCP leads to dramatic stabilization of Cdc25A in response to DNA damage and this stabilization is accompanied by a defect in the inter S-phase DNA damage checkpoint. We reconstituted Chk1-dependent ubiquitination of Cdc25A by SCFβ-TRCP in vitro, demonstrating a requirement for phosphorylation of Ser76 by Chk1. Based on these and other data, we hypothesize that Chk1-mediated phosphorylation of Ser-76 in Cdc25A serves as a priming event for phosphorylation of Ser-81 by an as yet unidentified kinase. Future studies are aimed at identifying this kinase. All of this work is reported in the attached Genes and Development manuscript by Jin et al., 2003.

**Development of retroviral systems for identification of ubiquitination substrates in mammalian cells (Aim 2)**

The goal of aim 2 is to develop a retroviral based system for identifying unstable proteins in mammalian cells. Our original plan was to employ GFP-fused cDNA libraries to develop a flow-cytometry based screen that would allow us to identify candidate substrates of particular E3s. Our progress on this was described in the previous report. However, the recent development of RNAi strategies in mammalian cells has radically changed the types of experiments that we can perform. We are now incorporating loss of function type experiments into a new approach to identify substrates that has features of the previously proposed system but is more likely to provide important results. In this approach, we will generate libraries of mammalian cells expressing CFP-tagged cDNAs via integrating retroviral vectors wherein the CFP epitope splices into endogenous genes, creating protein fusions. The CFP-tagged cDNA will be linked via an IRES to GFP such that sub-libraries of cells with defined GFP/CFP ratios can be isolated by live cell sorting. Ablation of particular ubiquitin ligases by RNAi would be expected to lead to increased levels of target proteins, resulting in a shift in the ratio of GFP to CFP in individual cells where the target gene is CFP tagged. Cells displaying altered ratios will be isolated by cell sorting and the identities of recipient genes determined.

The strategy is built upon previous work employing retroviral based tagging of genes (29) using enhanced retroviral mutagen (ERM) vectors. This system has been used to identify dominant proliferative genes. The primary features of these vectors relative to this proposal is that the tagging epitope (CFP for example) under control of its own promoter (CMV for example) is physically linked with splice donor sequences and upon integration into a cellular gene, CFP coding sequences may be spliced into the recipient gene, creating a protein fusion between CFP and the coding sequence of the recipient. Hundreds of thousands of independent integration events occurring in individual cells can be selected by virtue of selectable markers on the retrovirus, allowing the creation of libraries of cells containing a particular gene fused with CFP. An added feature of this system is that even genes that are not normally expressed in the target cell line can come under control of the retroviral derived CMV promoter, thereby allowing expression of otherwise silent genes. We have constructed the tagging tagging vector that expresses two spectrally separated GFP coding sequences from the same CMV driven transcript using an intervening IRES (internal ribosomal entry sequence) (Figure 1). The first GFP is expressed as an
intact protein while the second variant GFP (CFP) lacks a stop codon but contains a splice-donor sequence. Upon integration into a recipient gene, GFP and the CFP fusion proteins should be produced at a constant ratio since they are derived from the same mRNA, although their individual stabilities may be different. Additional versions of this vector have been generated, including CD20 instead of GFP and vectors that splice in two other reading frames. CD20 is a cell surface protein that can be detected immunologically in live cells.

Because the ratio of GFP to CFP-fusion protein should be stable in each recipient cell, groups of cells with particular ratios can be isolated by fluorescence activated cell sorting and then expanded for experimental manipulation. We are currently in the process of generating MCF7 cells and related breast cancer cell lines that are stably transduced with this vector. Our initial goal is to generate pools of cells that each display a particular ratio of GFP (or CD20) to CFP-fusion protein. We will isolate 10 pools of cells that reflect GFP/CFP ratios ranging from 10 to 0.1. Each pool is expected to contain hundreds of thousands of different integration events. A substantial fraction of these integration events will lead to the production of a fusion protein between CFP and recipient coding sequences. The resulting CFP fusion proteins may represent full-length sequences or may represent fragments of the recipient gene, possibly containing sequences important for regulated turnover.

In principle, by making cells with vectors in all three reading frames prior to sorting all cells together, it should be possible to have a larger number of recipient genes represented in the collection of pools. This effort represents Aim 2, tasks 7 and 8.

A second part of this approach involves the development of RNAi for E3s. As part of a collaboration with Dr. Greg Hannon at Cold Spring Harbor, we have developed retroviral vectors expressing shRNA (short hairpin RNAs) for a large number of ubiquitin ligase components. These shRNAs are made against specific sequences in the E3 component and we have three different sequences per gene, thereby increasing the likelihood that knock-down can be achieved. Each of the shRNAs are under control of the U6 promoter. We are currently preparing DNA for shRNA vectors directed against ~50 F-box proteins which we have identified.

Depletion of a particular ubiquitin ligase by RNAi would be expected to lead to an increase in the abundance of requisite CFP-tagged substrates and this should be reflected in the alteration in the ratio of GFP to CFP. Thus, once pools of cells are established, RNAi will then be used to knock-down particular F-box proteins and cells that display increased levels of CFP-recipient fusion proteins relative to GFP isolated using FACS. Given the window of fluorescence ratios chosen for each pool, we anticipate to be able to identify cells in which the GFP/CFP ratio is decreased by as little as 3 fold. These cells contain tagged candidates for ubiquitination targets of particular F-box proteins. Based on our previous experience with RNAi against other ubiquitin ligases (Fbw7 for example) we easily see a 3-fold increase in cyclin E levels after RNAi (27), in the range we expect to be able to seen by FACS. If necessary, we will reconstruct the system using cyclin E as target to standardize parameters.

**Functional analysis of point mutations in Fbw7 found in human cancer**
In our previous report, we described our identification of Fbw7 as the F-box protein responsible for ubiquitination of cyclin E. There is now clear evidence of mutations in Fbw7 in human cancer, including ovarian, breast, and endometrial cancers (Moberg et al., 2000; Koepp et al., 2000; Spruck et al., 2002). Our work initially found defects in expression of Fbw7 in breast cancer cell lines relative to normal cell lines. Several point mutations were found in the WD40 repeats of Fbw7, including R385, R425, and R463. We thought that it may be worthwhile to follow up our studies by examining the consequence of these mutations on function. To determine the effect of these mutations on interaction with cyclin E, we introduced these mutations into Fbw7 and examined their interaction with cyclin E. Prior to performing these experiments, we determined the phosphorylation status of cyclin E in mammalian cells and identified several phosphorylation sites that we thought might be important to interaction with Fbw7. We identified phosphorylation at S372, T380, and S384. Peptides containing various phospho-forms of this region of cyclin E were synthesized and tested for binding to Fbw7. Also, the sequence around T62 is similar to the Fbw7 recognition sequence centered at T380, so various phosphopeptides in this region were made as well. Using binding reactions with in vitro translated Fbw7 and phosphopeptides immobilized on agarose, we found that phosphorylation of T380 is sufficient to interact with Fbw7. Phosphorylation of S384 or S372 in the context of T380 phosphorylation had no impact on binding. In addition, phosphorylation of T62 was sufficient to allow for binding but phosphorylation of S58 had no effect on this binding. We next tested the point mutants seen in Fbw7 in cancer for binding to both the T62 peptide and the T380 peptide. We found that cancer derived mutations either did not bind at all to these peptides (R385A) or bound poorly relative to WT Fbw7 (R425A, R463A). The ability of Fbw7 to interact with two different cyclin E derived phosphopeptides could reflect either the presence of two different binding sites or the presence of a single binding site, wherein binding is mutually exclusive. To examine this issue, we performed a competition experiment wherein association of Fbw7 with phospho-T380 peptide was competed with either T62 peptide or with phosphorylated T62 peptide. We found that phosphorylated T62 peptide (but not unphosphorylated peptide) competed for binding to phospho-T380. Therefore, it would appear that a single site is responsible for binding to both of the phosphopeptides. Therefore, phosphorylation of either of these sites would be expected to affect turnover of cyclin E by Fbw7. Experiments to examine this are underway, as are experiments that test the interaction between Fbw7 mutants and full-length cyclin E.

**Contribution of phosphorylation to Fbw7-mediated cyclin E turnover in vivo.**
Currently, three distinct isoforms of Fbw7 - α, β, and γ - have been described. 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 (Figure 3A, C). In contrast, Fbw7β is almost exclusively found in the cytoplasm (Figure 3B). We previously reported that Fbw7β has an apparent transmembrane domain near the N-terminus (Koepp et al., 2001) and this may be involved in localizing Fbw7β to ER membranes.

To examine the contribution of multi-site 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. Expression of Myc-cyclin E and Cdk2 alone led to readily detectable Myc-cyclin E, as determined by immunoblotting of crude cell extracts (Figure 2 in attached manuscript). 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 anti-pT62 had no effect on Cdk2 abundance. These results extend previous results from multiple labs indicating that Fbw7β overexpression can drive cyclin E degradation when overexpressed. As expected, cyclin E T380A and cyclin E T62A/T380A levels were largely unaffected by expression of all three isoforms of Fbw7. This is consistent with a major role for T380 phosphorylation in controlling cyclin E turnover, as determined by pulse chase (Figure 2D in attached manuscript). 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 (Figure 2A-C in attached manuscript). However, using lower Fbw7α expression plasmids revealed clear defects in cyclin E T62A turnover (Figure 2F in attached manuscript). We also found that Fbw7β was profoundly defective in eliminating cyclin E S384A (Figure 2B in attached manuscript), while turnover of cyclin E S384A by Fbw7α and γ was less affected (Figure 2A, C in attached manuscript). At higher levels of cyclin E S384A expression plasmid used for transfection, cyclin E S384A was substantially more resistant to turnover by Fbw7α than was wild-type cyclin E (Figure 2G in attached manuscript). Cyclin E S372A and cyclin E S88A were efficiently degraded by all three Fbw7 isoforms (Figure 2A-C in attached manuscript and data not shown for...
cyclin E\textsuperscript{S372A}). Control experiments demonstrated comparable levels of all three Fbw7 isoforms in transient transfections (Figure 2E in attached manuscript).

**Association of cyclin E phosphorylation site mutants with Fbw7 in vivo.**

The data described above suggested the possibility that T62, in addition to T380, 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 Fbw7\textalpha 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 T380, and to a lesser extent T62, 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 Cull (Cull\textsuperscript{DN}) which contains the Skp1 binding site but lacks the Rbx1 binding site. This form of Cull sequesters Skp1/F-box complexes and leads to stabilization of SCF targets. As expected, expression of Cull\textsuperscript{DN} leads to equal accumulation of all cyclin E mutants examined, despite the presence of Flag-Fbw7\textalpha (Figure 4A, lanes 1-5 in attached manuscript). Extracts from cells expressing mutant cyclin E proteins and Flag-Fbw7\textalpha were then subjected to immunoprecipitation using anti-Flag antibodies and the levels of associated cyclin E examined by immunoblotting. Cyclin E efficiently associated with Fbw7\textalpha (Figure 4A, lane 1 in attached manuscript). Interestingly, cyclin E\textsuperscript{T380A} was found to associate weakly with Fbw7\textalpha (lane 3) but mutation of T62 to alanine in the context of the T380A mutant further reduced the interaction with Fbw7\textalpha (Figure 4A, lane 4 in attached manuscript). Mutation of T62 in cyclin E to alanine also led to a reduction in the extent of Fbw7\textalpha binding (Figure 4B, lane 2 in attached manuscript).

The reduced association between Fbw7\textalpha and cyclin E\textsuperscript{T62A} could reflect either a significant utilization of phosphorylated T62 in binding to Fbw7\textalpha or could potentially reflect alterations in the phosphorylation of T380. To examine this issue, we tested the extent of phosphorylation of T380 in the context of the T62A mutation under the same conditions employed in Figure 4A in attached manuscript. Lysates from transfected cells were immunoprecipitated with anti-Myc antibodies and the levels of total cyclin E and T380 phosphorylated cyclin E determined by immunoblotting (Figure 4B in attached manuscript). We found that replacement of T62 with alanine significantly reduced the extent of T380 phosphorylation. The extent of reduction was comparable to the reduction seen in the association of Fbw7\textalpha with cyclin E\textsuperscript{T62A}. Transfection of cyclin E mutants in the absence of Flag-Fbw7 and Cull\textsuperscript{DN} demonstrated that the effect on T380 phosphorylation was independent of these two components (data not shown). Taken together, this data indicates that mutation of T62 affects T380 phosphorylation and the majority of its contribution to Fbw7-mediated turnover appears to be indirect.

**Involvement of S384 in recognition of cyclin E by Fbw7.** Results described above indicate that cyclin E\textsuperscript{S384A} is partially defective in degradation by Fbw7 with turnover by Fbw7\textbeta being affected to the greatest extent. To examine whether this reflects recognition by Fbw7, we compared the ability of Fbw7\textalpha and \textbeta isoforms to immunoprecipitate cyclin E\textsuperscript{S384A} in transfected 293T cells (Figure 5 in attached manuscript). Interestingly, cyclin E\textsuperscript{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\textbeta was substantially lower than with the \textalpha isofrom, possibly reflecting the fact that Fbw7\textbeta is largely cytoplasmic while cyclin E is largely nuclear. Importantly, cyclin E\textsuperscript{S384A} maintained wild-type levels of T380 phosphorylation, as determined by immunoblots of cyclin E immune complexes using anti-phospho-T380 antibodies (Figure 4B in attached manuscript). All of the work cited here is described in detail in the accompanying manuscript in Journal of Biological Chemistry by Ye et al.

Finally, using a fully recombinant system, we have established a method for cyclin E ubiquitination by an SCF-Fbw7 complex. This system employs all components made in insect cells
or in bacteria. We are currently employing this system to examine the possibility that SCF-Fbw7 function requires that it be a dimeric complex. This system is reported in the Methods in Enzymology chapter on the identification of SCF substrates which is in press.

**Key Research Accomplishments:**

**Year 1**

* Development of a yeast system for identification of proteins whose stability is regulated by a specific ubiquitin ligase

* The use of a related system to identify F-box proteins involved in cyclin E turnover

* Demonstration that Fbw7 in mammalian cells controls cyclin E levels

**Year 2**

* Completed an analysis of F-box proteins in the human and mouse genomes, leading to the identification of 68 human and 70 mouse F-box proteins

* Cloned and sequenced 50 human F-box proteins. Each F-box protein was placed into mammalian expression plasmids, facilitating the discovery of substrates

* Identified β-TRCP as a candidate ubiquitin ligase for the breast cancer oncoprotein Cdc25A using a panel of F-box proteins that we identified and cloned, and published the results.

* Developed new retroviral vectors that can be used in a dual fluorescence system to identify ubiquitination targets in combination with RNAi-dependent gene ablation

* Determined the requirements for interaction of cyclin E with Fbw7 and demonstrated that cancer-prone mutations in Fbw7 loose binding with cyclin E phospho-degrons.

**Year 3**

Performed a systematic analysis of F-box proteins encoded by the human and mouse genomes and reported a systematic nomenclature for this family of proteins. This result led to the identification of similar destruction motifs in c-myc, c-jun, and SREBP, work that we published in collaboration with other labs (see papers listed below).

Completed an analysis of the requirements for recognition of cyclin E by the Fbw7 F-box protein and published the results.

**Reportable outcomes.**

**Publications directly supported by this grant:**


Reviews and papers related to protein ubiquitination published by this lab during the last year:


Invited seminars to discuss the outcome of work funded by this grant:
Control of cell cycle transitions by the SCF ubiquitin ligase. The Cell Cycle, Cold Spring Harbor Laboratory, May 16, 2002

Protein ubiquitination and control of cell division. Division of Medical Oncology, University of Colorado Health Science Center, Denver, CO, July 15, 2002.

Control of cell division by protein phosphorylation and ubiquitin mediated proteolysis. Department of Pathology, Harvard Medical School, September 18, 2002.

Control of cell division by protein phosphorylation and ubiquitin mediated proteolysis. Fred Hutchison Cancer Research Center, November 12, 2002.


The SCF ubiquitin ligase pathway, University of Chicago, February 19, 2003

Substrate specificity of cullin-based ubiquitin ligases, Cold Spring Harbor Laboratory, The ubiquitin Family meeting, April 24, 2003

The ubiquitin-Proteasome Pathway, Karolinska Institute/Baylor College of Medicine Symposium, May 12, 2003

FASEB Symposium, *Ubiquitination and Cellular Regulation* June, 2004

Vallee Foundation Symposium, May 2004

AACR Symposium, *Cell Cycle Regulation* December, 2004

AACR National Meeting, Chair of the *Ubiquitin System in Cancer Symposium*, April 2005

Cold Spring Harbor, *The Ubiquitin Family* May, 2005

**Funding applied for using preliminary data from this grant:**


**Employment/Research Opportunities impacted by the grant:**

I was recruited to Harvard Medical School to join the Department of Pathology as the Bert and Natalie Vallee Professor of Molecular Pathology, a position I will assume on August 1, 2003. Our research funded by the grant contributed significantly to this recruitment.

**Conclusion**

The SCF pathway is widely used to control the levels of regulatory proteins. We are using multiple systems – both biochemical and genetic – to identify ubiquitin ligases for important cell cycle regulators as well as to identify substrates of particular ubiquitin ligases. The new system we are developing to identify substrates has the potential to help uncover numerous proteins whose levels are controlled by the ubiquitin pathway and may be involved in the genesis of breast cancer.

**References**


SCF$^{\beta-TRCP}$ links Chk1 signaling to degradation of the Cdc25A protein phosphatase

Jianping Jin, Takahiro Shirogane, Lai Xu, Grzegorz Nalepa, Jun Qin, Stephen J. Elledge, and J. Wade Harper

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Eukaryotic cells respond to DNA damage and stalled replication forks by activating protein kinase-mediated signaling pathways that promote cell cycle arrest and DNA repair. A central target of the cell cycle arrest program is the Cdc25A protein phosphatase. Cdc25A is required for S-phase entry and dephosphorylates tyrosine-15 phosphorylated Cdk1 (Cdc2) and Cdk2, positive regulators of cell division. Cdc25A is unstable during S-phase and is degraded through the ubiquitin–proteasome pathway, but its turnover is enhanced in response to DNA damage. Although basal and DNA-damage-induced turnover depends on the ATM-Chk2 and ATR-Chkl pathways, how these kinases engage the ubiquitin ligase machinery is unknown. Here, we demonstrate a requirement for SCF$^{\beta-TRCP}$ in Cdc25A turnover during an unperturbed cell cycle and in response to DNA damage. Depletion of $\beta$-TRCP stabilizes Cdc25A, leading to hyperactive Cdk2 activity. SCF$^{\beta-TRCP}$ promotes Chkl-dependent Cdc25A ubiquitination in vitro, and this involves serine 76, a known Chkl phosphorylation site. However, recognition of Cdc25A by $\beta$-TRCP occurs via a noncanonical phosphodegron in Cdc25A containing phosphoserine 79 and phosphoserine 82, sites that are not targeted by Chkl. These data indicate that Cdc25A turnover is more complex than previously appreciated and suggest roles for an additional kinase(s) in Chkl-dependent Cdc25A turnover.

[Keywords:]

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tion between checkpoint signaling and Cdc25 was established when it was found that the checkpoint kinase Chk1, and later Chk2, could phosphorylate Cdc25C on a site relevant to its checkpoint function in vivo (Peng et al. 1996; Sanchez et al. 1996, Furnari et al. 1997; Matsuoka et al. 1998). Furthermore, these kinases were shown to phosphorylate all three Cdc25 family members, suggesting they were general targets of DNA damage stress response pathways (Sanchez et al. 1996; Matsuoka et al. 1998). Analysis of Chk regulation of Cdc25 from several systems showed that phosphorylation of Cdc25C both inhibited kinase activity (Blasina et al. 1999, Furnari et al. 1999) and maintained Cdc25C in the cytoplasm, where it cannot access Cdk/cyclin complexes efficiently [Zeng et al. 1998, Kumagai and Dunphy 1999, Lopez-Girona et al. 1999; Zeng and Piwnica-Worms 1999]. However, mice lacking Cdc25C grow normally and have intact checkpoint responses (Chen et al. 2001), suggesting that other family members may play more prominent roles in Cdk regulation.

A second family member implicated in the damage response is Cdc25A. Cdc25A is capable of removing inhibitory tyrosine phosphorylation from both Cdk1 and Cdk2 kinases to promote entry into and progression through S phase and mitosis [Hoffmann et al. 1994, Vigo et al. 1999, for review, see Donzelli and Draetta 2003]. Cdc25A has also been shown to be a phosphorylation target of Chk kinases (Sanchez et al. 1996) and to be regulated by Chk kinases in response to DNA damage [for review, see Donzelli and Draetta 2003]. In contrast to regulation of Cdc25C, Cdc25A is destroyed in response to ionizing radiation (IR) and ultraviolet (UV) light through a process involving ubiquitin-mediated proteolysis. During G1, UV treatment leads to Chkl-dependent elimination of Cdc25A [Mailand et al. 2000] and persistent Cdk2 Y15 phosphorylation. During an unperturbed S phase, Cdc25A is unstable and this instability requires Cdc25A phosphorylation by Chkl [Falck et al. 2001; Sorensen et al. 2003]. IR during S phase leads to accelerated Cdc25A phosphorylation by Chkl with a concomitant increase in turnover. Defects in this intras-phase checkpoint lead to radio-resistant DNA synthesis [RDS; Falck et al. 2001; Xiao et al. 2003]. Whereas depletion of Chkl leads to an RDS phenotype, expression of a Cdk2 mutant that is resistant to inhibitory tyrosine phosphorylation overcomes IR-dependent S-phase arrest [Falck et al. 2001], implicating elimination of Cdc25A in the intra-S-phase checkpoint.

Recent studies indicate that Cdc25A turnover through the ubiquitin pathway involves at least two temporally distinct components [Donzelli et al. 2002, Donzelli and Draetta 2003]. During mitotic exit and early G1, Cdc25A stability is controlled by the anaphase-promoting complex in conjunction with Cdh1. During interphase, however, Cdc25A turnover is dependent on Cul1 [Donzelli et al. 2002], a central component of the SCF (Skp1/Cul1/F-box protein) ubiquitin ligase [Feldman et al. 1997; Skowyra et al. 1997]. Precisely how Cul1 promotes turnover of Cdc25A is unknown. In SCF complexes, Cul1 together with the RING-H2 finger protein Rbx1 forms the core ubiquitin ligase that binds ubiquitin-conjugating enzymes [Deshaies 1999, for review, see Koepp et al. 1999]. Specificity in these reactions is achieved by a substrate-binding module composed of Skp1 and a member of the F-box family of proteins. F-box proteins interact with Skp1 through the F-box motif [Bai et al. 1996] and with substrates through C-terminal protein interaction domains, including WD40 propellers [Skowyra et al. 1997; Gu et al. 2003, Orlicky et al. 2003]. Frequently, association of SCF targets with the requisite F-box protein requires that the substrate be modified, typically by phosphorylation to produce a short peptide motif displaying properties of a phosphodegron [Winston et al. 1999a; Koepp et al. 2001; Nash et al. 2001; Gu et al. 2003, Orlicky et al. 2003].

Here we report that constitutive and DNA-damage-induced turnover of Cdc25A occurs via the SCFß-TRCP ubiquitin ligase. Depletion of ß-TRCP by shRNA stabilizes Cdc25A, leading to inappropriately high levels of Cdk2 kinase activity characteristic of a checkpoint defect. Cdc25A ubiquitination by SCFß-TRCP in vitro involves Chkl-dependent phosphorylation principally at S76, consistent with the requirement for Chkl in vivo. However, Chkl-mediated phosphorylation of Cdc25A does not appear to be sufficient to generate the requisite phosphodegron for ß-TRCP recruitment. We find that residues 79–84 of Cdc25A constitute a phosphodegron for recognition by ß-TRCP and implicate S79 and S82 as phosphoacceptor sites in this motif. Indeed, S82 is in a sequence context (Asp–Ser–Gly–Phe) reminiscent of previously identified phosphodegrons in the ß-TRCP substrates IxBa and ß-catenin. We suggest that Chkl-mediated phosphorylation of S75 may promote Cdc25A turnover by facilitating the phosphorylation of the adjacent phosphodegron targeted by ß-TRCP.

Results

Involvement of the SCF pathway in DNA-damage-induced Cdc25A turnover

The precise pathways involved in interphase and DNA-damage-mediated turnover of Cdc25A and the role of phosphorylation in this process are unknown. To address these issues, we examined whether DNA-damage-dependent elimination of Cdc25A, like its interphase counterpart, is also involved in the SCF pathway. Cells were transiently transfected with a vector expressing a dominant-negative version of Cul1, Cul1ß-TRCP, which binds to Skp1 but does not associate with the essential ring-finger protein Rbx1 [Donzelli et al. 2002]. As expected, Cul1ß-TRCP expression resulted in accumulation of Cdc25A, as well as other SCF substrates including p27 and cyclin E, in the absence of DNA damage (Fig. 1A). In control transfected cells, mitosis, and ionizing radiation (IR) induced a time-dependent decrease in the abundance of Cdc25A (Fig. 1B, lanes 1–4). However, expression of Cul1ß-TRCP led to increased Cdc25A abundance throughout the time course (Fig. 1B, lanes 5–8), implicating an SCF-like complex in Cdc25A turnover in response to DNA damage, as well as during...
interphase. Flow cytometry [Fig. 1C] indicated that, under these conditions, expression of Cul1DN has only a minor influence on the cell cycle distribution in these cells. Thus, the dramatic stabilization of Cdc25A by Cul1DN does not appear to be caused by an indirect effect of cell cycle position.

We next examined association of Cdc25A with Cul1 in the presence and absence of DNA damage. Consistent with previous studies [Donzelli et al. 2002], endogenous Cdc25A was found to associate with endogenous Cul1 in the absence of DNA damage [Fig. 1D, lane 2]. Moreover, Cdc25A was also associated with Cul1 in the presence of IR [10 Gy], and quantification of the blot indicated an ~twofold increase in the quantity of Cdc25A-associated Cul1 [relative to Cdc25A levels] in the presence of DNA damage [Fig. 1D, lane 4]. Importantly, Cdc25A was associated exclusively with the neddylated, and therefore potentially activated, form of Cul1 [Fig. 1E].

The β-TRCP F-box protein specifically associates with Cdc25A

Substrate specificity in SCF-driven ubiquitination reactions is controlled by the identity of the F-box protein [Bai et al. 1996]. To search for F-box proteins involved in Cdc25A ubiquitination, we cloned and expressed 18 of the 70 known human F-box proteins [Winston et al. 1999b, J. Jin and J.W. Harper, unpubl.] as Myc-tagged protein fusions [Fig. 2]. These F-box protein expression plasmids were then individually cotransfected with vectors expressing either GST-Cdc25A or GST as a negative control. F-box proteins associating with GST-Cdc25A were identified by immunoblotting. Two WD40-containing F-box proteins—β-TRCP1 and β-TRCP2—were found to bind efficiently to GST-Cdc25A but not GST alone, and this binding occurred independently of addition of exogenous DNA damaging agents [Fig. 2, lanes 3,5]. β-TRCP proteins are derived from distinct genes, yet are closely related in sequence and appear to interact with identical phosphodegrons in several ubiquitinated targets, including β-catenin, IκBα, and Em1 [Hart et al. 1999, Latres et al. 1999, Winston et al. 1999a, Guardavaccaro et al. 2003]. Using previously characterized antibodies that react with both β-TRCP1 and β-TRCP2 [Spiegelman et al. 2000], we found that β-TRCP is present in Cdc25A immune complexes and that the association is enhanced ~twofold in the presence of ionizing irradiation [Fig. 1D, lanes 2,4]. These data suggest a pos-
sible regulatory connection between β-TRCP and Cdc25A turnover.

**Linkage of β-TRCP with elimination of Cdc25A in the presence and absence of DNA damage**

We next examined whether expression of β-TRCP can promote Cdc25A ubiquitination in tissue culture cells. 293T cells were transiently transfected with vectors expressing HA-Cdc25A, β-TRCP1, and/or His<sub>6</sub>-tagged ubiquitin. After 36 h, guanidine-denatured extracts were subjected to Ni-NTA purification, and bound proteins were immunoblotted with anti-HA antibodies. β-TRCP1 dramatically promoted the formation of high-molecular-weight Cdc25A/His<sub>6</sub>-Ub conjugates, when compared with those produced in the absence of exogenous β-TRCP (Fig. 3A, lanes 1,2). Immunoblotting for total Cdc25A in crude cell lysates indicated reduced levels of Cdc25A in cells expressing β-TRCP, when compared with control cell lysates (Fig. 3A, lower panels). Thus, the increased abundance of Cdc25A-ubiquitin conjugates is not a reflection of higher levels of total Cdc25A in this experiment. A similar dramatic reduction in the levels of Cdc25A was also seen when higher quantities of pCMV-β-TRCP were used (Fig. 2, lanes 3,5). None of the other 16 F-box proteins tested displayed an ability to reduce steady-state Cdc25A levels when coexpressed, indicating a high degree of specificity for β-TRCP in this regard.

To examine whether β-TRCP is required for Cdc25A turnover, we initially examined the effects of a dominant negative β-TRCP protein lacking the F-box motif. 293T cells were transfected with vectors expressing β-TRCP<sup>AF-box</sup> or Skp2<sup>AF-box</sup> as a negative control and then subjected to irradiation (Fig. 3B,C). In this experiment, a modified SDS-PAGE system was used to enhance the separation of phosphorylated and unphosphorylated forms of Cdc25A seen previously [Zhao et al. 2002; Materials and Methods]. Cdc25A was eliminated in cells expressing Skp2<sup>AF-box</sup> at a rate similar to that found with vector-only control cells (Fig. 3B, lanes 1–3, 7–9), while the levels of Cul1 used as a loading control were constant. In contrast, the levels of the more slowly migrating phosphorylated forms of Cdc25A were elevated in cells expressing β-TRCP<sup>AF-box</sup> both in the absence of irradiation and throughout the time course after DNA damage (Fig. 3B, lanes 4–6), suggesting that β-TRCP is important for turnover of Cdc25A in response to its phosphorylation. Rapid turnover of the more rapidly migrating Cdc25A isoforms in this experiment likely reflects a population of cells that did not receive the β-TRCP<sup>AF-box</sup> plasmid, as the efficiency of transfection under these conditions is <80% (data not shown). The levels of dominant-negative β-TRCP and Skp2 were maintained throughout the time course (Fig. 3C).

To extend these results, we used vectors expressing shRNA against β-TRCP proteins. The targeting sequence used represents a sequence fully conserved in both β-TRCP1 and β-TRCP2 and has been demonstrated to effectively knock down expression of both isoforms [Fong and Sun 2002], a result confirmed here in 293T and HCT116 cells (Fig. 3D). Expression of β-TRCP shRNA in both 293T and HCT116 cells resulted in accumulation of Cdc25A both in unirradiated cells (Fig. 3E, lane 2) and in cells exposed to IR (lane 4), when compared with control cells expressing shRNA against GFP [lanes 1,3]. These data are consistent with a role for β-TRCP in Cdc25A turnover.
Ubiquitin-mediated turnover of Cdc25A

Depletion of β-TRCP stabilizes Cdc25A and increases cyclin E/Cdk2 activity in the presence and absence of DNA damage

To examine Cdc25A turnover directly, we determined Cdc25A levels in the presence of cyclohexamide with or without depletion of β-TRCP by shRNA and in the presence or absence of DNA damage. Cdc25A was eliminated with an apparent half-life of ~90 min (Fig. 3A). As expected, DNA damage accelerated Cdc25A turnover. In contrast, Cdc25A was dramatically stabilized upon depletion of β-TRCP in both the presence and absence of DNA damage, with an apparent half-life of ~90 min [Fig. 3A, lanes 1–8; Fig. 4B]. In addition, the total abundance of Cdc25A was greatly elevated by depletion of β-TRCP, when compared with Cull used as a loading control [Fig. 3A, lanes 1–8 vs. lanes 9–16]. One explanation for altered Cdc25A turnover is that β-TRCP depletion leads to cell cycle arrest at a point in the cell cycle where Cdc25A is more stable. To examine this possibility, cells from the experiment shown in Figure 4A were processed for flow cytometry in parallel. As shown in Figure 4C, β-TRCP depletion had a negligible effect on the cell cycle distribution of 293T cells when compared with control cells expressing shRNA2GFP. Thus, β-TRCP is required for Cdc25A turnover in the presence and absence of DNA damage.

Previous studies have demonstrated that depletion of Chkl leads to increased abundance of Cdc25A with a concomitant increase in the activity of cyclin E/Cdk2, reflecting inappropriate dephosphorylation of Y15 by Cdc25A [Falck et al. 2001; Sorensen et al. 2003]. Consistent with this, we find that depletion of β-TRCP leads to increased levels of cyclin E associated kinase activity in both the presence and absence of DNA damage, while the total level of cyclin E-associated Cdk2 is unaffected [Fig. 4D]. These data are consistent with a role for both Chkl and β-TRCP in controlling Cdc25A and cyclin E/Cdk2 activity.
Figure 4. Stabilization of Cdc25A by depletion of β-TRCP in the presence or absence of DNA damage leads to deregulated cyclin E/Cdk2 activity. (A, B) Turnover of Cdc25A requires β-TRCP. 293T cells were transfected with pSUPER-shRNA-GFP or pSUPER-shRNA-β-TRCP using lipofectamine 48 h after dual transfection and then either left untreated or subjected to DNA damage (10 Gy). Translation was immediately blocked by addition of cyclohexamide, and cells lysed at the indicated time points prior to SDS-PAGE. (F) Blots were probed with anti-Cdc25A, stripped, and reprobed with anti-Cul1 antibodies. (C) Blots of comparable intensity for shRNA-GFP and shRNA-β-TRCP were quantified by densitometry. (C) Depletion of β-TRCP does not alter cell cycle progression. Cells from A were subjected to flow cytometry after staining with propidium iodide. (D) Increased cyclin E/Cdk2 kinase activity in cells depleted of β-TRCP. 293T cells were transfected with vectors expressing shRNA against GFP as control or β-TRCP using the dual-transfection protocol. After 48 h, cells were lysed and cyclin E immune complexes assayed for activity using histone H1 as a substrate. Cyclin E immune complexes were generated using a rabbit polyclonal antibody (C-19 from Santa Cruz Biotechnology). Parallel immunoblots were probed for Cdk2 and cyclin E to demonstrate equal loading. The cyclin E immunoblot was probed with a monoclonal antibody (HE12, Santa Cruz Biotechnology). Controls demonstrated a dramatic accumulation of Cdc25A in response to depletion of β-TRCP whereas Cul1 levels remained unchanged.

Chk1-dependent Cdc25A ubiquitination by SCFβ-TRCP in vitro

We next asked whether SCFβ-TRCP can function as a ubiquitin ligase for Cdc25A in vitro and whether this required phosphorylation of Cdc25A by Chk1. SCFβ-TRCP complexes were assembled by translation of β-TRCP in reticulocyte extracts. Such complexes have been shown to be proficient in ubiquitination of IkBα (Wu et al. 2003). SCFβ-TRCP complexes were then supplemented with E1, Ubc5, ubiquitin, ATP, and 35S-methionine-labeled Cdc25A added in the presence of wild-type or kinase-dead (KD) Chk1 (Fig. 5A). Cdc25A formed high-molecular-weight products in the presence of wild-type Chk1 (Fig. 5A, lane 2) but not in the presence of Chk1KD or samples lacking kinase (lanes 1, 3), and this correlated with a shift in the mobility of Cdc25A in the presence of Chk1 indicative of phosphorylation (lane 2). Although β-TRCP2 was also capable of promoting Chk1-dependent Cdc25 ubiquitination, three other WD40-containing F-box proteins present at equivalent levels (Fbw5, Fbw6, and Fbw7) failed to promote Cdc25A ubiquitination, demonstrating specificity for β-TRCP proteins [Fig. 5B]. Addition of the polyubiquitin chain terminator methyl ubiquitin led to the generation of shorter conjugates, demonstrating that the formation of high-molecular-weight Cdc25A species reflected ubiquitination [Fig. 5C]. Taken together, these data indicate Cdc25A can be ubiquitinated by SCFβ-TRCP and this process is dependent on Cdc25A phosphorylation by Chk1.

Role of Chk1-dependent phosphorylation in Cdc25A ubiquitination by SCFβ-TRCP

Given that Chk1 promotes Cdc25A turnover in response to DNA damage in vivo (Falck et al. 2001; Sorensen et al. 2003) and that Chk1 is required for Cdc25A ubiquitination by SCFβ-TRCP in vitro, we explored the role of Cdc25A phosphorylation in the ubiquitination process. Chk1 is known to phosphorylate Cdc25A on at least five
Ubiquitin-mediated turnover of Cdc25A

Degrons are considered to be minimal sequences that support recognition of cognate E3s and may possess relevant lysine side chains for ubiquitination. The finding that F-box proteins such as β-TRCP and Cdc4 frequently interact with short phosphopeptide motifs has led to the use of the term "phosphodegron" to refer to short sequences capable of interacting specifically with particular F-box proteins (Nash et al. 2001), although these sequences may not themselves contain relevant lysines for ubiquitination. The β-propeller of β-TRCP is known to interact with a phosphodegron containing the sequence DSGFCLDSP, residues 81-89) that displayed characteristics of the classical β-TRCP recognition motif and is located adjacent to S76 (Fig. 6A). In particular, the first four residues of this motif conform to a portion of the known β-TRCP recognition motif or phosphodegron (Fig. 6A).

To examine the possible involvement of this region, we first determined the effect of mutation of S82 to alanine. This residue corresponds to phospho-S83 in β-catenin, which interacts with a network of hydrogen bonds involving R285 and S325 in β-TRCP (Fig. 6D). Interestingly, Cdc25A S82A was not ubiquitinated by SCFβ-TRCP in vitro, suggesting the involvement of this motif in Cdc25A ubiquitination (Fig. 6B, lane 4). We next considered the possibility that S88 may potentially be involved in recognition, in which case, β-TRCP would need to accommodate alternative spacing between phospho-serine residues in the phosphodegron. However, we found that Cdc25A S88A was ubiquitinated with an efficiency similar to that found with the wild-type protein.

Identification of a novel phosphodegron in Cdc25A

Degrons are considered to be minimal sequences that support recognition of cognate E3s and may possess relevant lysine side chains for ubiquitination. The finding that F-box proteins such as β-TRCP and Cdc4 frequently interact with short phosphopeptide motifs has led to the use of the term "phosphodegron" to refer to short sequences capable of interacting specifically with particular F-box proteins (Nash et al. 2001), although these sequences may not themselves contain relevant lysines for ubiquitination. The β-propeller of β-TRCP is known to interact with a phosphodegron containing the sequence DSGFCLDSP, residues 81-89) that displayed characteristics of the classical β-TRCP recognition motif and is located adjacent to S76 (Fig. 6A). In particular, the first four residues of this motif conform to a portion of the known β-TRCP recognition motif or phosphodegron (Fig. 6A).
Figure 6. Chk1 phosphorylation sites in Cdc25A are required for SCFβ-TRCP-mediated ubiquitination but do not appear to constitute the major Cdc25A phosphodegron. (A) Schematic representation of Chk1 phosphorylation sites in Cdc25A and comparison of a putative phosphodegron in Cdc25A with the IkBα, β-catenin, and Emil phosphodegron recognized by β-TRCP. The Cdc25A residues are designated based on GenBank accession number AAH18642. (B) Identification of residues in Cdc25A important for Chk1-dependent ubiquitination. The indicated Cdc25A mutants were used in SCFβ-TRCP-driven ubiquitination reactions in the presence of Chk1. (C) Arg 474 in β-TRCP1 is required for Chk1-dependent Cdc25A ubiquitination. Ubiquitination of Chkl-phosphorylated Cdc25A was performed in the presence of β-TRCP1 or an R474A mutant. An aliquot of each β-TRCP synthesis reaction was supplemented with 35S-methionine to demonstrate equal expression of β-TRCP proteins [lower panel]. (D) Three-dimensional structure of β-TRCP bound to the phosphodegron of β-catenin depicting the interaction of D32 and phosphoserine-33 [pS33] in the phosphodegron with R474 and R285 in β-TRCP. Graphics were generated using Pymol.

We also noted that Cdc25A contains an additional serine residue at residue 79, which could potentially be part of the phosphodegron. We found that mutation of S79 to alanine abolished Cdc25A ubiquitination by SCFβ-TRCP [Fig. 6B, lane 3]. Additional mutagenesis experiments indicate the direct involvement of a phosphodegron containing phospho-S82 in Cdc25A recognition by β-TRCP. Mutation of D81 in Cdc25A abolished ubiquitination by β-TRCP in vitro [Fig. 6B, lane 8]. D81 corresponds to D32 in β-catenin, which is frequently mutated in stabilized alleles of β-catenin [see Wu et al. 2003]. In the β-catenin/β-TRCP crystal structure, D32 [which is invariant in β-TRCP substrates] is buried in the β-propeller and forms hydrogen bonds with R474 and Y488 [Fig. 6D]. Previous studies indicate that replacement of R474 with alanine blocks IkBα ubiquitination by SCFβ-TRCP, revealing that this interaction is crucial for recognition of canonical β-TRCP targets [Wu et al. 2003]. Likewise, we found that R474 in β-TRCP is required for Chk1-dependent Cdc25A ubiquitination [Fig. 6C, lane 4], consistent with the proposed interaction with D80 in Cdc25A.

Synthetic phosphopeptides have been useful in defining phosphodegrons in cyclin E, β-catenin, IkBα, and Sic1 [Winston et al. 1999a; Koepp et al. 2000; Nash et al. 2001]. Therefore, we directly examined whether sequences containing the DSG motif in Cdc25 could function as a phosphodegron. Immobilized peptides spanning this candidate phosphodegron were found to interact efficiently with β-TRCP when S82 or both S79 and S82 were phosphorylated, whereas the phospho-S79 peptide bound slightly better than the unphosphorylated control peptide [Fig. 7A]. In contrast, a peptide containing phospho-S76, the site of Chk1 phosphorylation, displayed no detectable interaction with β-TRCP in this assay [Fig. 7A], consistent with the idea that Chk1-mediated phos-
Table 1. Mass spectral analysis of Cdc25A phosphorylation by Chk1

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#These phosphopeptides were sequenced with LC/MS/MS.

To more accurately determine the relative affinities of various Cdc25A-derived phosphopeptides for β-TRCP, we developed a competition assay using β-TRCP-driven Cdc25A ubiquitination (Fig. 7C). In principle, this approach avoids the concentration and steric effects sometimes seen with immobilized peptides. Neither the unphosphorylated peptide nor the singly phosphorylated peptides containing phospho-S76 was capable of blocking Cdc25A ubiquitination in this assay (Fig. 7C, lanes 3–6, 23–26). In contrast, a doubly phosphorylated S79/S82 peptide, and to a lesser extent a singly phosphorylated S79 peptide, were capable of inhibiting Cdc25A ubiquitination by SCFβ-TRCP as judged by both the lower levels of ubiquitin conjugates and the increased abundance of unmodified Cdc25A (lanes 9–12). The peptide containing phospho-S82 reduced the extent of polyubiquitination (lanes 13–16). Interestingly, the extent of inhibition by the phospho-S79/S82 peptide was comparable to that observed with the phosphodegron from IκBα (Winston et al. 1999a) when examined in the same concentration range (Fig. 7D). Taken together, these data indicate that S79 and S82 are central components of the Cdc25A phosphodegron recognized by β-TRCP and indi-
cate that Chk1 phosphorylation alone is not sufficient to create the requisite epitopes for β-TRCP recognition. The data also indicate that Cdc25A and IkBa use at least partially overlapping binding sites in β-TRCP.

**Recognition of β-TRCP by Cdc25A in vivo requires a phosphodegron anchored by S82**

To verify the involvement of residues 76 to 82 in β-TRCP recognition in vivo, GST-Cdc25AΔC containing the N-terminal regulatory domain [Fig. 7E] and mutant versions in which S76, S79, D81, and S82 were individually replaced with alanine were tested for binding after expression in 293T cells [Fig. 6D]. Mutation of S76 reduced binding by ~10-fold (lane 4 vs. lane 2), whereas mutation of S79, D81, and S82 bound even less efficiently [lanes 6, 8, 10 vs. lane 2]. Taken together, these in vitro and in vivo data strongly implicate a novel phosphodegron in Cdc25A centered at S82 as being important for its association with the β-TRCP ubiquitin ligase.

**Discussion**

β-TRCP is involved in a large number of seemingly unrelated processes. β-TRCP controls phosphorylation-mediated destruction or processing of several transcriptional regulators, including IkB, β-catenin, ATF-4, and p105NFκB [Lattes et al. 1999; Spencer et al. 1999; Winston et al. 1999a; Fong and Sun 2002]. Moreover, β-TRCP controls multiple cell cycle-related processes, including centrosome duplication in Drosophila and destruction of the mitotic regulator Emi1 during mitosis [Wojcik et al. 2000; Guardavaccaro et al. 2003]. Our data now implicate β-TRCP1 and β-TRCP2 in control of Cdc25A turnover during both a normal cell cycle and in response to DNA damage. We have shown that endogenous Cdc25A forms complexes with SCF^β-TRCP^ in the presence or absence of DNA damage. Moreover, depletion of β-TRCP by siRNA stabilizes Cdc25A in the presence or absence of DNA damage, and SCF^β-TRCP^ can ubiquitinate Cdc25A in a Chk1-dependent manner in vitro. Several lines of biochemical evidence suggest the involvement of a novel phosphodegron in Cdc25A containing phosphorylated S76 and S82 in β-TRCP recognition. Chk1 appears to be required, but not sufficient, to generate this novel phosphodegron. Previous studies indicate that Cdc25A is phosphorylated by Chk1 during a normal cell cycle, probably during S phase, leading to its instability during S and G2 phases [Sorensen et al. 2003]. This process is accelerated in response to DNA damage [Sorensen et al. 2003]. Chk1 is essential for cell proliferation in mammals [Liu et al. 2000], and maintaining low levels of Cdc25A during S and G2 phases could represent a component of its essential functions. Inappropriately high levels of Cdc25A during DNA replication could influence the kinetics of S-phase progression and thereby affect the fidelity of DNA synthesis.

Our biochemical experiments indicate that Cdc25A turnover is more complex than previously appreciated. Early models suggested that phosphorylation of Cdc25A by Chk1 at multiple sites might be required for Cdc25A turnover. Our reconstitution studies indicate that of all the Chk1 sites in Cdc25A, only S76 phosphorylation plays a prominent role in facilitating SCF^β-TRCP^ dependent Cdc25A ubiquitination in vitro, consistent with recent work on the role of this residue in Cdc25A turnover in vivo [Goloudina et al. 2003; Hassepass et al. 2003]. Other known Chk1 sites in Cdc25A (S124, S179, S279, and S293) are not required for ubiquitination in vitro but could, nevertheless, be important for turnover in vivo. One possibility is that phosphorylation affects the site(s) of ubiquitination, which has recently been shown to affect the kinetics of proteasome-mediated destruction of an SCF substrate Sic1 [Pokorna and Deshaies 2003]. Moreover, we also note that we consistently see the formation of shorter ubiquitin conjugates with the Cdc25A mutant lacking these four Chk1 sites, which could affect the efficiency of recruitment to the proteasome.

Although Chk1-mediated phosphorylation is required for Cdc25A turnover in vivo and ubiquitination by SCF^β-TRCP^ in vitro, it appears that Chk1 activity is not sufficient for this process. First, Chk1 kinases are known to preferentially phosphorylate R-X-X-S/T motifs [O'Neill et al. 2002]. Although all of the known Chk1 sites in Cdc25A conform to this consensus sequence, none is expected to generate phosphodegrons of the type known to interact with β-TRCP. Consistent with this, peptides containing phospho-S76 failed to interact with β-TRCP in a direct binding assay. Moreover, these peptides did not efficiently inhibit Cdc25A ubiquitination in vitro. Second, mass spectral analysis of bacterial Cdc25A phosphorylated in vitro by recombinant Chk1 revealed strong phosphorylation of S76 and S124 (>90%), but no phosphorylation at S79 or S82 was detected, indicating that Chk1 cannot directly phosphorylate these two sites. Finally, bacterial Cdc25A that was previously phosphorylated by Chk1 was not a substrate for ubiquitination by purified SCF^β-TRCP^ complexes in vitro that are competent for IkBa ubiquitination [data not shown].

We propose that β-TRCP recognizes a novel phosphodegron in Cdc25A. This sequence contains a DSG motif characteristic of all previously identified β-TRCP targets [Emi1, β-catenin, IkBa, p105NFκB]. Several lines of evidence implicate this degron in β-TRCP recognition. First, point mutations in Cdc25A that remove S79 or S82 abolish ubiquitination by SCF^β-TRCP^ in vitro and association with β-TRCP in transfected cells. Second, point mutations in the aspartate of the DSG motif, or in its complementary ligand in the WD40 propeller of β-TRCP, R474, abolish ubiquitination by SCF^β-TRCP^. Finally, synthetic Cdc25A peptides containing phospho-S79 and phospho-S82 alone can associate with β-TRCP in vitro and can block Cdc25A ubiquitination by SCF^β-TRCP^ in a competition assay, as can a canonical phosphodegron from IkBa.

The simplest explanation for these results is that generation of the phosphodegron in Cdc25A involves two critical steps [Fig. 7F]. In the first step, Chk1 phosphorylates S76. In the second step, S76-phosphorylated
Cdc25A is then phosphorylated by one or more kinases on S82 and S79 to generate the phosphodegron recognized by β-TRCP. In the case of our in vitro Cdc25A ubiquitination assay, where S76 and Chk1 are required, this second kinase activity appears to be provided by one or more kinases in the reticulocyte extract. According to this model, Chk1 may perform a priming function that facilitates modification of S79 and/or S82. Other β-TRCP substrates also use analogous priming reactions. For example, β-catenin is phosphorylated by casein kinase I on T41 and T45, and this facilitates phosphorylation of S33 and S77 by GSK3β in a sequential mechanism (Liu et al. 2002). At present, we cannot exclude the possibility that Cdc25A is constitutively phosphorylated on S82/S79 and that Chk1 in a second step induces a conformational change in the adjacent S79/S82 phosphodegron that allows it to be recognized by β-TRCP or protects these residues from rapid dephosphorylation. The finding that Cdc25A[S76A] displays detectable, albeit greatly reduced, association with β-TRCP in vivo, whereas Cdc25A[S82A] mutants display no detectable binding, suggests that S76 phosphorylation is not an absolute requirement for S79/S82 phosphorylation. A determination of the mechanism by which Chk1 promotes formation of the degron awaits identification of relevant kinases. In this regard, one candidate kinase is casein kinase Iα. However, addition of two specific casein kinase Iα inhibitors [CKI-7, US Biologicals; IC261, Calbiochem] continuously throughout Cdc25A translation, Chk1 phosphorylation, and in vitro ubiquitination assay failed to block Cdc25A ubiquitination [data not shown]. It is also possible that additional cofactors may be involved in Cdc25A turnover independent of the kinases involved. For example, Cks1 is required for p27 ubiquitination by SCFSkp2 (see review, see Harper 2001).

The discovery of a role for β-TRCP in Cdc25A turnover now sets the stage for the elucidation of the interplay between regulatory mechanisms responsible for directing the destruction of Cdc25A and control of cell cycle transitions. Although Chk1 is a critical player in this regard, our studies reveal a previously unanticipated level of complexity in the targeting of Cdc25A for ubiquitination involving other participants. These new players may themselves be targets of regulation intended to control cell cycle progression. In addition to targeting Cdc25A for degradation, positive feedback mechanisms involving Cdkl phosphorylation have been implicated in stabilization of Cdc25A to promote mitotic entry (Mailand et al. 2002). How these competing regulatory pathways interface with the Chk1 pathway to control Cdc25A stability remains to be determined.

Materials and methods

Plasmids

F-box proteins were generated by PCR and cloned into pUNI50 (Liu et al. 1998) prior to sequence analysis. Details of these constructs will be published elsewhere. For expression, pUNI50 constructs were recombined with pCMV-Myc-lox-RS (pJP150) using Cre recombinase, placing N-terminally Myc-tagged F-box proteins under control of the CMV promoter. The pUNI backbone was then removed by RS-mediated recombination. Vectors for expression of Cdc25A and mutants were generated using pENTR vectors and recombined into the indicated bacterial or mammalian expression construct using clonase (EPICENTRE). To generate retroviral vectors expressing shRNA-S^PP and shRNA^β-TRCP^ double-stranded oligonucleotides were cloned into a modified version of pSuper-retro (Oligoengine, Inc.) wherein the puromycin marker was replaced with an expression cassette for enhanced cyan fluorescence protein (ECFP). The sequences used were: humanized Renilla GFP, GGACTTC CCGCAGTACCATGATTTGTTGTAAGGTCCG; β-TRCP, GTGGAATTCTGGAGCACAATGATGTGCT CACAAACCTCCAG (gene-specific sequences are in caps, and hairpin sequences are underlined). Point mutations in Cdc25A were generated using a QuickChange mutagenesis kit (Stratagene). Cdc25A^29d encodes amino acids 1-100 and was generated by replacing the destruction of Cdc25A and control of cell cycle transitions. Although Chk1 is a critical player in this regard, our studies reveal a previously unanticipated level of complexity in the targeting of Cdc25A for ubiquitination involving other participants. These new players may themselves be targets of regulation intended to control cell cycle progression. In addition to targeting Cdc25A for degradation, positive feedback mechanisms involving Cdkl phosphorylation have been implicated in stabilization of Cdc25A to promote mitotic entry (Mailand et al. 2002). How these competing regulatory pathways interface with the Chk1 pathway to control Cdc25A stability remains to be determined.

Ubiquitin-mediated turnover of Cdc25A

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analysis was performed using propidium iodide essentially as described [Ye et al. 2003]. Histone H1 kinase assays were performed as described [Ye et al. 2003]. With the exception of Figure 3A, all SDS-PAGE separations were performed using 8%-12% gradient minigels (Invitrogen). Figure 3A used a 15-cm 10% polyacrylamide gel, which gave higher resolution of Cdc25A isoforms observed previously (Zhao et al. 2002).

In vitro ubiquitination

Cdc25A ubiquitination was performed using in vitro 38S-me-thionine-labeled Cdc25A (2.5 µL) in the presence of insect-cell-derived Chk1 or Chk1S326 (100 ng), E1 ubiquitin-activating enzyme (50 ng), UbC5 (200 ng), ubiquitin (1 mg/mL), 1 µM ubiquitin aldehyde, 2.3 µL of in vitro translated F-box protein, and 4 mM ATP in a total volume of 10 µL (30 min, 30°C). In some experiments, methyl ubiquitin (Boston Biochemicals) was used as a chain terminator or synthetic peptides derived from the IkBα phosphodegron (Winston et al. 1999a) or Cdc25A used as a competitive inhibitor. Phosphorylated or unphosphorylated peptides encompassing the Cdc25A phosphodegron and containing a N-terminal cysteine were synthesized by Tufts Medical School Protein Core Facility or by Invitrogen and coupled to Sulfo-link agarose (Pierce) or Affigel-10 (Biorad) at 1 mg/mL. Binding reactions were performed with 10 µL of peptide agarose beads in combination with 5 µL of in vitro translated 38S-methionine-labeled β-TrCP1 in a total of 100 µL of extraction buffer. Beads were washed three times with extraction buffer prior to SDS-PAGE and autoradiography.

Phosphorylation analysis

Mass spectral analysis of bacterial Cdc25A previously phosphorylated with Chk1 was performed using established procedures. Mass spectral analysis of phosphopeptides was performed using Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/TOF) with delayed extraction (Voyager-DE, Perseptive Biosystems) as described [Zhang et al. 1998]. An electrospay ion trap mass spectrometer (LCQ, Finnigam) coupled on-line with a capillary high-pressure liquid chromatograph (Magic 2002) was used for identification of phosphorylation sites.

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References


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

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Turnover of cyclin E is controlled by SCF<sup>Fbw7</sup>. Three isoforms of Fbw7 are produced by alternative splicing. Whereas Fbw7α and -γ are nuclear and the β-isofrom is cytoplasmic in 293T cells, all three isoforms induce cyclin E destruction in an <i>in vivo</i> degradation assay. Cyclin E is phosphorylated on Thr<sup>62</sup>, Ser<sup>68</sup>, Ser<sup>272</sup>, Thr<sup>380</sup>, and Ser<sup>384</sup> <i>in 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 E<sup>Thr<sup>62A</sup></sup> to Fbw7 was abolished upon mutation of Thr<sup>62</sup>, suggesting a minor role for this residue in direct association with Fbw7. Mutations of Ser<sup>384</sup> to alanine also rendered cyclin E resistant to degradation by Fbw7, with the largest effects being observed with Fbw7β. Cyclin E<sup>Thr<sup>62A</sup></sup> 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<sup>Thr<sup>62A</sup></sup> 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-E21 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/Rocl, and an adaptor protein called Skp1 (4–8). Rbx1 associates with and activates E2s including Cdc43 (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-transducin-repeats-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<sup>Cdc4</sup> 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 IkBα 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 G<sub>1</sub> cyclins, 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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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Cdk, cyclin-dependent kinase; MS, mass spectrometry.

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 (Cd2k) at the G1/S transition. Previous work has demonstrated that phosphorylation of Thr^308 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 Thr^{308} to alanine greatly stabilizes cyclin E, its turnover in vitro or ubiquitination in vitro is not completely eliminated (17, 21). Recent studies have also implicated phosphorylation of Thr^{62} and Ser^{284} in cyclin E turnover by Fbw7 (21, 35), but whether this reflects direct or indirect effects in Fbw7 recognition is not clear. Thr^{62} conforms to a Cd2k consensus site but the identity of kinases involved in Thr^{62} phosphorylation are currently unknown (35). Available data indicates that phosphorylation of Thr^{62} is required for phosphorylation of Ser^{284} by GSK3β (35). Phosphorylation of Ser^{284} is dependent upon interaction of cyclin E with active Cd2k, and it has been proposed that Ser^{284} is directly phosphorylated by Cd2k (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 Thr^{62} in binding to Fbw7. Whereas synthetic peptides encompassing Thr^{62} bind Fbw7 in a phosphorylation-dependent manner, the major effect seen upon mutation of Thr^{62} to alanine is a dramatic reduction in the extent of Thr^{308} phosphorylation, suggesting an indirect effect of the T62A mutation on cyclin E degradation through the Csk2 pathway. Nevertheless, residual association of cyclin E^Thr62A with Fbw7 is reduced upon mutation of Thr^{62} to alanine. Mutation of Ser^{284} to alanine reduced turnover of cyclin E by all three isoforms of Fbw7, although the effects seen with Fbw7 were much greater than that observed with α and γ. Cyclin E^Ser284A associated more weakly than wild-type cyclin E with both Fbw7α and Fbw7β. Unlike other cyclin E mutants examined, cyclin E^Ser284A 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 pCS2-Myc-cyclin E expression plasmid employed was from a previous study (35). Mutations were generated using the Gene Editor System (Promega). Vectors for expression of FLAG-Fbw7β, -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, GATCAAG-CTTATGTCAAAACCGGGAAAACCTACTC; reverse, TGTCTGAGCTG-CTTCTGAGCTGACTCTATCAAG. The 576-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 Fbw7γ mutants were described previously (17). To generate an expression plasmid for dominant negative G11, sequences encoding amino acids 1–453 were cloned into pDNA3 (Invitrogen); 1295F 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-glycerol phosphate, 10 mM NaF, and protease inhibitors (Roche Applied Science) or processed for immunofluorescence.

**Microscopy and Image Analysis**—For immunofluorescence, cells were transfected with cyclin E or Fbw7β/γ 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 five 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 Slow-Fade Light Antifade reagent ( Molecular Probes, Inc., Eugene, OR). To determine subcellular localization of 2Fbw7 isoforms, images were taken on a Nikon/DeltaVision deconvolution microscope (Applied Precision) as a series of 0.2-μm-thick Z-sections and processed with a Softworx image work station. Each image represents single transcellular Z-section.

For fluorescence signal quantification, cells were fixed and immunostained as described above 48 h post-transfection with the indicated antibodies. Immunoreactive values were normalized against internal background controls for each cell. Statistical significance was confirmed by analysis of variance.

**Immunoprecipitation and Protein Interactions**—To examine cyclin E degradation, lysates from transfected cells were subjected to immunoprecipitation using anti-Myc (9E10) antibodies (Covance). To examine cyclin E turnover, cells were transfected with the indicated plasmids, and 36 h later, cells were cultured for 1 h in methionine-free medium prior to pulse labeling with [35S]methionine (0.1 μCi/ml). Twenty minutes later, [35S]methionine-containing medium was replaced with fresh medium containing 10 μM cold methionine. Extracts made at the indicated times were used for immunoprecipitation with anti-Myc antibodies. 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 antibody for 1 h, washed five times with 1 ml of NETN prior to SDS-PAGE and autoradiography. In some cases, Thr^{308}-containing peptides were coupled to Affi-Gel 10 beads (Bio-Rad) at a concentration of 1 mg/ml. Immobilized peptides (1 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 7.5, 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, Thr^{308}-containing peptides were employed as competitor. Antibodies against a synthetic peptide containing Thr^{308} (SLHP[T]PDK) were made by Phospho-Solutions, Inc. (Aurora, OH).

**Mass Spectrometry**—Recombinant 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, pCS2-Myc-cyclin E was transfected into four 150-mm dishes with Fugene 6. Subsequently, Myc-cyclin E was isolated using immobilized 9E10 antibodies and purified by SDS-PAGE for mass spectral analysis. Approximate quantities of cyclin E or cyclin E^Thr62A were digested with trypsin and analyzed by matrix-assisted laser desorption ionization mass spectrometry with delayed extraction (Voyager-DE; Perseptive Bionsystems, Framingham, MA). Unless otherwise noted, an electrospray ion trap mass spectrometer (Mariner) coupled on line with an internal background corrected pressure liquid chromatograph (Magic 2002, Aurora, CA) was used for identification of phosphorylation sites. Cyclin E^Thr62A 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 Thr<sup>380</sup> or Thr<sup>62</sup> 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, Ser<sup>38</sup>, Ser<sup>62</sup>, Ser<sup>373</sup>, Thr<sup>380</sup>, and Ser<sup>384</sup>, 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 cyclin E expressed in insect cells with Cdk2 (Table I). In brief, various forms of cyclin E<sup>361-388</sup> were found to contain either two or three phosphates, as determined by treatment with calf intestinal phosphatase. Peptide sequencing by liquid chromatography/MS/MS identified Ser<sup>38</sup>, Thr<sup>380</sup>, and Ser<sup>384</sup> as sites of phosphorylation in the triply phosphorylated peptide (Table I). Peptide sequencing of the cyclin E<sup>381-398</sup> peptide by liquid chromatography/MS/MS demonstrated phosphorylation on Ser<sup>38</sup> (Table I and data not shown). Ser<sup>38</sup> in cyclin E has not been previously shown to be phosphorylated but does conform to a minimal Cdk2 consensus site. In these experiments, we did not observe phosphopeptides containing either Ser<sup>58</sup>, which is known to be phosphorylated (35), or Thr<sup>62</sup>, possibly due to the large size and cysteine-rich character of the predicted tryptic peptide. Therefore, antibodies directed against phospho-Thr<sup>62</sup> 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 but not T62A cyclin E (Fig. 1C). Moreover, treatment of Myc-cyclin E with λ-phosphatase resulted in loss of reactivity toward the phospho-Thr<sup>62</sup> antibody, consistent with a specific phosphorylation-dependent interaction (Fig. 1D). These data indicate that cyclin E is phosphorylated on Thr<sup>62</sup> 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.

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). Coexpression 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 Fbw7 overexpression can drive cyclin E degradation when overexpressed (21, 35). As expected, cyclin E<sup>T62A</sup> and cyclin E<sup>S384A</sup> levels were largely unaffected by expression of all three isoforms of Fbw7. This is consistent with a major role for Thr<sup>380</sup> 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 Fbw7α 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 E<sup>T62A</sup> turnover (Fig. 2F). We also found that Fbw7β was profoundly defective in eliminating cyclin E<sup>F384A</sup> (Fig. 2D), whereas turnover of cyclin E<sup>384A</sup> by Fbw7α and -γ was less affected (Fig. 2, A and C). At higher levels of cyclin E<sup>F384A</sup> expression plasmid used for transfection, cyclin E<sup>F384A</sup> was substantially more resistant to turnover by Fbw7α than was wild-type cyclin E (Fig. 2G). Cyclin E<sup>S372A</sup> and cyclin E<sup>S38A</sup> were efficiently degraded by all three Fbw7 isoforms (Fig. 2, A–C, and data not shown for cyclin E<sup>S372A</sup>). Control experiments demonstrated comparable levels of all three Fbw7 isoforms in transient transfections (Fig. 2E).

Phosphorylation of Thr<sup>380</sup> in the C-terminal Phosphodegron of Cyclin E Is Sufficient for Interaction with Fbw7—Given the multisite phosphorylation of cyclin E in residues flanking Thr<sup>380</sup>, 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 E<sup>361-388</sup>) 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

<table>
<thead>
<tr>
<th>Tryptic fragment</th>
<th>Calculated mass</th>
<th>293T</th>
<th>Insect cells</th>
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<tbody>
<tr>
<td>F14-15 (aa 81–98)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1934.3</td>
<td>2014.2 (1P)</td>
<td>2014.5&lt;sup&gt;b&lt;/sup&gt; (1P)</td>
</tr>
<tr>
<td>F41-43 (aa 362–386)</td>
<td>2608.0</td>
<td>2848.2 (3P), 2768.5 (2P), 2688.1 (1P)</td>
<td>2848.3&lt;sup&gt;b&lt;/sup&gt; (3P), 2768.2 (2P), 2688.2 (1P)</td>
</tr>
<tr>
<td>F42-43 (aa 363–386)</td>
<td>2479.8</td>
<td></td>
<td>2640.6&lt;sup&gt;b&lt;/sup&gt; (2P), 2560.0 (1P)</td>
</tr>
</tbody>
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<sup>a</sup> aa, amino acids.
<sup>b</sup> Phosphopeptides sequenced by liquid chromatography/MS/MS.

<sup>3</sup> B. E. Clurman and M. Welcker, unpublished data.
translated Fbw7 isoforms. Expression plasmids encoding the indicated N-terminally FLAG-tagged Fbw7 isoforms were transiently transfected into 293T cells using Fugene 6. After 36 h, cells were subjected to indirect immunofluorescence. Anti-FLAG immunofluorescence is shown in red. Nuclei, stained with 4',6-diamidino-2-phenylindole, are shown in blue. B, schematic representation of phosphorylation sites found in cyclin E (see Table 1). C and D, antibodies directed at phospho-Thr62 of cyclin E selectively interact with wild-type cyclin E purified from 293T cells after transient transfection but do not react with cyclin E° or with cyclin E treated previously with α-phosphatase. C, 293T cells were transfected with pCMV-Myc-cyclin E DNA, and after 48 h, cell lysates were immunoprecipitated with anti-Myc antibodies prior to immunoblotting with either anti-Myc antibodies or anti-phospho-Thr62 antibodies. In D, Myc-cyclin E immune complexes were incubated in the presence or absence of α-phosphatase prior to immunoblotting with anti-phospho-Thr62 antibodies.

![Figure 1](image_url)

**A**

DAP/Fbw7α

Fbw7α

**B**

Cyclin E

anti-Myc

anti-pT62

**C**

wild-type

T2A

wild-type

T2A

D

λ phosphatase

cyclin E

anti-pT62

**D**

Δ phosphatase

cyclin E

anti-pT62

Fig. 1. A, differential localization of Fbw7 isoforms. Expression plasmids encoding the indicated N-terminally FLAG-tagged Fbw7 isoforms were transiently transfected into 293T cells using Fugene 6. After 36 h, cells were subjected to indirect immunofluorescence. Anti-FLAG immunofluorescence is shown in red. Nuclei, stained with 4',6-diamidino-2-phenylindole, are shown in blue. B, schematic representation of phosphorylation sites found in cyclin E (see Table 1). C and D, antibodies directed at phospho-Thr62 of cyclin E selectively interact with wild-type cyclin E purified from 293T cells after transient transfection but do not react with cyclin E° or with cyclin E treated previously with α-phosphatase. C, 293T cells were transfected with pCMV-Myc-cyclin E DNA, and after 48 h, cell lysates were immunoprecipitated with anti-Myc antibodies prior to immunoblotting with either anti-Myc antibodies or anti-phospho-Thr62 antibodies. In D, Myc-cyclin E immune complexes were incubated in the presence or absence of α-phosphatase prior to immunoblotting with anti-phospho-Thr62 antibodies.

translated Fbw7 (Fig. 3B). As expected, unphosphorylated cyclin E°-esp did not associate with Fbw7 (Fig. 3B, lanes 2). In addition, we found that phosphorylation of either Ser1^72 or Ser3^78 alone did not support Fbw7 binding (Fig. 3B, lanes 4 and 6). In contrast, cyclin E°-esp peptides containing phosphorylation at Thr3^80 alone (lane 4) or in combination with Ser1^72 and Ser3^78 (lanes 5 and 7) interacted efficiently with Fbw7. In the context of Thr3^80 phosphorylation, phosphorylation at either Ser1^72 or Ser3^78 did not enhance association with Fbw7 (Fig. 3B, lanes 5 and 7). Thus, within the context of immobilized peptides in vitro, phosphorylation of Thr3^80 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 isolg Cdc4 is known to preferentially interact with sequences having the 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. In particular, three arginine residues (Arg5^86, Arg2^66, and Arg6^63, 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. 3E). We reasoned that if Thr62- and Thr2^66-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-Thr2^66-containing peptides. We found that the R385A mutant was strongly defective in association with both the Thr62- and the Thr2^66-based peptides, with the interaction being undetectable (Fig. 3C, lanes 6 and 10). Mutations in Arg2^66 and Arg6^63 displayed reduced interactions compared with wild-type controls, but interaction with both the Thr62- and Thr2^66-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-Thr2^66 cyclin E°-esp peptide and increasing amounts of either phos-
Recognition of Phosphodegron Motifs by SCF<sup>Fbw7</sup>

**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 immunoblotting was 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, 0.5 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 experiments were 1.5 and 3.0 μg of cyclin E614 peptide in (CUlDN), which contains the Skp1 binding site but lacks the Cull-DN 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 (Cul1<sup>DN</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>DN</sup> leads to equal accumulation of all cyclin E mutants examined, despite the presence of FLAG-Fbw7α (Fig. 4A, lanes 1–3). 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>Fbw7α</sup> was found to associate weakly with Fbw7α (lane 2), 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>Fbw7α</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<sup>380</sup>-phosphorylated cyclin E determined by immunoblotting (Fig. 4B). We found that replacement of T62 with alanine significantly reduced the extent of Thr<sup>380</sup> phosphorylation. The extent of reduction was comparable with the reduction seen in the association of Fbw7α with cyclin E<sup>E<sub>384A</sub></sup>. Transfection of cyclin E mutants in the absence of Fbw7 and Cul1<sup>DN</sup> demonstrated that the effect on Thr<sup>380</sup> phosphorylation was independent of these two components (data not shown). Taken together, these data indicate that mutation of Thr<sup>380</sup> affects Thr<sup>380</sup> phosphorylation, and the majority of its contribution to Fbw7-mediated turnover appears to be indirect.

**Involvement of Ser<sup>384</sup> in Recognition of Cyclin E by Fbw7**—Results described above indicate that cyclin E<sup>E<sub>384A</sub></sup> is partially defective in degradation by Fbw7 with turnover by Fbw7β being affected to the greatest extent. To examine whether this reflects recognition by Fbw7, we compared the ability of Fbw7α and -β isoforms to immunoprecipitate cyclin E<sup>E<sub>384A</sub></sup> in transfected 293T cells (Fig. 5). Interestingly, cyclin E<sup>E<sub>384A</sub></sup> 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 Fbw7β was substantially lower than with the α isoform, possibly reflecting the fact that Fbw7β is largely cytoplasmic, whereas cyclin E is largely nuclear. Importantly, cyclin E<sup>E<sub>384A</sub></sup> maintained wild-type levels of Thr<sup>380</sup> phosphorylation, as determined by immunoblots of cyclin E immune complexes using anti-phospho-Thr<sup>380</sup> antibodies (Fig. 4B).

**Mutation of Ser<sup>384</sup> Affects the Nuclear/Cytoplasmic Ratio of Cyclin E**—As stated above, the Fbw7β isoform was profoundly defective in degradation of cyclin E<sup>E<sub>384A</sub></sup> in transfection experiments, whereas the α and γ isoforms were less defective. Because our previous experiment revealed that Fbw7α and Fbw7γ are predominantly nuclear, whereas Fbw7β is localized to the cytoplasm, we examined whether the inability of Fbw7β to degrade cyclin E<sup>E<sub>384A</sub></sup> could result from aberrant intracellular shuttling of this mutant. Previous studies have demonstrated that cyclin E shuttles from the nucleus to the cytoplasm (38). To determine whether cyclin E<sup>E<sub>384A</sub></sup> accumulates in the
Recognition of Phosphodegron Motifs by SCFPbw7

**Fig. 4. Role of Thr<sup>48</sup> and Ser<sup>86</sup> phosphorylation in assembly with Fbw7α in vivo.** Plasmids expressing the indicated Myc-tagged cyclin E proteins were co-transfected with plasmids expressing FLAG-Fbw7α in the presence of pCMV-Cul<sup>DN</sup>. After 36 h, lysates were either directly immunoblotted for cyclin E or subjected to immunoprecipitation using anti-FLAG antibodies prior to immunoblotting. B, anti-cyclin E immune complexes were subjected to immunoblotting with anti-phospho-Thr<sup>5</sup> antibodies, and the blot was stripped and reprobed with anti-cyclin E antibodies. Corresponding cell extracts were probed with anti-Myc antibodies to detect cyclin E and anti-FLAG antibodies to detect Fbw7α.

**Fig. 5. Mutation of Ser<sup>86</sup> in cyclin E reduces association with Fbw7α and β isoforms.** Vectors expressing wild-type cyclin E and the indicated cyclin E mutants were cotransfected with vectors expressing Cul<sup>DN</sup> and either FLAG-Fbw7α or FLAG-Fbw7β. After 36 h, lysates were either directly immunoblotted for cyclin E or subjected to immunoprecipitation using anti-FLAG antibodies prior to immunoblotting. 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 G<sub>1</sub> target, the Cdk inhibitor Sic1, depends upon the number of G<sub>1</sub> 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. Use of a large number of relatively weak interactions allows for ultrasensitivity in the response of Sic1 turnover to Cdk activity (reviewed in Refs. 39 and 40). The extent to which other SCFP<sup>Cdc4</sup> 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, SCFPbw7. 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, -β, and -γ to examine the localization properties of these proteins. Fbw7α and -γ are found in the nucleus in 293T cells, whereas Fbw7β 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-
The text is about the recognition of phosphodegron motifs by SCF<sup>Fbw7</sup>. It discusses the results of an experiment involving the mutation of specific serine residues in cyclin <i>E</i>. The mutation of Ser<sup>384</sup> to alanine affects subcellular shuttling of cyclin <i>E</i>. 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 <i>E</i> point mutants (T62A, T380A, S88A, and S372A) and wild-type cyclin <i>E</i> reveals significantly enhanced nuclear accumulation of cyclin <i>E</i><sup>S88A</sup> protein as compared with wild-type cyclin <i>E</i> and four other cyclin <i>E</i> 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 (<i>N</i><sub>wt</sub> - <i>N</i><sub>mut</sub>) values for 15 cells for each construct. A, representative cells expressing wild-type cyclin <i>E</i> as well as cyclin <i>E</i> phosphorylation-deficient mutants and respective immunofluorescence profiles are shown. N, nucleus; C, cytoplasm.

Cyclin <i>E</i> has long been known to be phosphorylated on Thr<sup>380</sup> through an autocatalytic function involving Cdk2 (33, 34), but recent experiments (35) also suggest a role for GSK3β in this process, functioning in an apparently redundant manner with Cdk2. Mutation of Thr<sup>380</sup> to alanine leads to significant stabilization of cyclin <i>E</i> in an Fbw7/3-driven turnover assay. A role for this residue is also consistent with the finding that phosphopeptides encompassing Thr<sup>380</sup> are capable of binding to Fbw7 (17). However, evidence of the involvement of Thr<sup>62</sup> and Ser<sup>384</sup> has also been presented (21, 35). Mutation of Thr<sup>62</sup> partially stabilizes cyclin <i>E</i> to Fbw7β overexpression (35) and enhances the stabilization of the cyclin E<sup>T380A</sup> mutant (21). However, Thr<sup>62</sup> has not been demonstrated to be phosphorylated in vivo, and it is not clear whether it plays a direct or indirect role in cyclin <i>E</i> turnover. Likewise, mutation of Ser<sup>384</sup> to alanine also leads to partial stabilization of cyclin <i>E</i> in an Fbw7β-driven turnover assay (35), again through an unknown mechanism. Although the finding that cytoplasmic Fbw7β overexpression can promote degradation of cyclin <i>E</i> (which is primarily nuclear) seems counterintuitive, we note that cyclin <i>E</i> is initially synthesized in the cytoplasm, where it assembles with Cdk2. In this context, newly synthesized cyclin <i>E</i> was generated by ectopic expression in the assays performed here and may be readily destroyed via an Fbw7/3-mediated mechanism. Moreover, cyclin <i>E</i> 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 <i>E</i>, could force the equilibrium in favor of nuclear export, thereby leading to depletion of the nuclear pool of cyclin <i>E</i>. Further studies are required to determine what Fbw7 isoforms are directly involved in cyclin <i>E</i> turnover in vivo.

In this paper, we systematically examined the role of various phosphorylation events in the interaction between cyclin <i>E</i> and Fbw7 as well as the ability of individual Fbw7 isoforms to promote turnover of various phosphorylation site mutants in cyclin <i>E</i>. We initially used mass spectrometry to establish
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 Ser384, a candidate Cdk2 site that was not identified previously. As with other studies (35), we were unable to unequivocally 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 E380A and cyclin E501-72A. 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 E501-72A also displayed defects in turnover, but degradation by Fbw7β was affected to the greatest extent. We note that high levels of cyclin E380A are also substantially resistant to degradation induced by Fbw7α. The stoichiometric relationship between cyclin E and Fbw7 isoforms in vivo is not known, and further studies need to be performed to understand what Fbw7 isoforms are most important for cyclin E turnover in vivo.

We then went on to examine how various phosphorylation events in cyclin E promote the interaction with Fbw7. Initially, we used phosphopeptides centered at Thr62 and Thr62 and tested these for binding to Fbw7 in vitro. Phosphorylation of Thr380 is sufficient to allow for interaction of the C-terminal phosphodegron with Fbw7. Peptides phosphorylated at Ser372 or Ser384 alone were not capable of interacting with Fbw7, and phosphorylation at these sites did not enhance interaction of phospho-Thr380 phosphopeptides with Fbw7 in the context of the in vitro binding assay. Consistent with this, cyclin E380A demonstrated a greatly reduced ability to interact with Fbw7α in transfected cells. These data are consistent with previous studies indicating that a single phosphorylation event in a related cyclin E-derived peptide is sufficient for interaction with budding yeast Cdc4 in vitro (18). Interestingly, we found that peptides containing phospho-Thr62 were also capable of interacting efficiently with Fbw7, whereas phosphorylation of Ser62 did not support interaction with cyclin E. The interaction of phospho-Thr62-containing peptides with Fbw7 required the same Fbw7 binding site as used by Thr380 peptides. Thus, in principle, this Thr62 phosphorylation could be involved directly in cyclin E recognition by Fbw7 independent of Thr380 phosphorylation. To examine this issue further, we determined the effects of replacement of Thr62 by alanine on the interaction of cyclin E with Fbw7 in vivo. The major effect found with cyclin E521A is a dramatic reduction in the extent of Thr380 phosphorylation, as measured using a phosphospecific antibody against Thr380, indicating at least a partially indirect effect of this mutation on interaction with and turnover by Fbw7α. However, residual association of cyclin E521A with Fbw7α was reduced further upon mutation of Thr62, indicating a direct, albeit small, contribution of Thr62 to Fbw7α recognition. The mechanism by which mutation of Thr62 to alanine affects phosphorylation of Thr380 is unknown. Although the direct contribution of Thr62 to cyclin E turnover appears to be small under the conditions examined here, we cannot exclude the possibility that Thr62 phosphorylation may be used in particular circumstances and be a major determinant of cyclin E degradation. For example, the stoichiometry of phosphorylation under the 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 E521A, all of the other cyclin E proteins tested displayed indistinguishable nuclear/cytoplasmic ratios when transiently expressed in 293T cells. However, cyclin E380A displayed a dramatic (more than 2-fold) increase in the nuclear/cytoplasmic ratio. Thus, the defects seen in degradation of cyclin E380A by Fbw7 may reflect, in part, the inaccessibility of the cyclin E380A mutant with the Fbw7β isoform. Interestingly, c-Myc has recently been demonstrated to be ubiquitinated by SCFFbw7 in a phosphorylation-dependent manner (28, 29). In this case, Thr58 is phosphorylated by GSK3β, and Ser62 is phosphorylated by a mitogen-activated protein kinase. In this case, Thr58 phosphorylation depends absolutely on prior phosphorylation of Ser62. The phosphodegron in c-Myc (LPpTPPLpSP) is quite similar to the phosphodegron with Fbw7. Peptides phosphorylated at Thr380 were able to promote the interaction with Fbw7, but in the case of synthetic peptides, the phosphorylation depended absolutely on prior phosphorylation of Ser384. The phosphodegron in c-Myc (LPpTPPLpSP) is quite similar to that found in cyclin E (LPpTPpQpsG). Because of the dependence of Ser384 phosphorylation on Thr58 phosphorylation, it has not been possible to examine whether Ser58 phosphorylation contributes to association of c-Myc with Fbw7, but in the case of synthetic peptides, the Thr58 phosphate bond to Fbw7 independently of Ser62 phosphorylation (28). Taken together, our studies reveal a complex interplay between phosphorylation of cyclin E and control of its degradation by Fbw7 and reveal that mutational analysis of cyclin E can in some cases lead to apparent indirect effects in its turnover by altering phosphorylation and/or localization.

REFERENCES

Recognition of Phosphodegron Motifs by SCFβTr7

quencing reactions with purified PCR products were performed by using Big Dye Terminator chemistry and analyzed on an automated capillary sequencer. Reactions were automatically analyzed by using Phred/Phrap and Polyphred [47, 48]. For SNPs identified through this analysis, PCR invader assays [Third Wave Technologies, Madison, WI] were designed and tested on 90 samples from the Polymorphism Discovery Resource panel (PDx90) [49]. Successful assays were subsequently used to analyze samples from our study. Genotypes were assigned automatically by cluster analysis (N. Olivier et al., in preparation). Differences among genotypes were analyzed by one-way ANOVA using STATVIEW 4.1 software (Abacus Concepts, Inc., Berkeley, CA). SNPs 1 to 4 are available in dbSNP under accession numbers ss3199913, ss3199914, ss3199915, and ss3199916, respectively.

35. Subjects were a combined subset of 501 healthy, non-diseased persons tested by a combination of controls and a subset of 300 patients with ischemic heart disease. G. G. I. Dubchak et al., Science 288, 136 (2000).

36. Fasting blood samples were obtained from subjects exiting their usual diet and after 4 to 6 weeks of consuming diets containing high fat (35 to 46% energy) and low fat (20 to 24% energy) [50, 51]. Plasma lipid and lipoprotein measurements were performed as previously described [50, 51]. In addition, on the high- and low-fat diets, total lipoprotein mass was measured by analytic ultracentrifugation [50, 51].

37. Genotypes were assigned automatically by cluster analysis (N. Olivier et al., in preparation). Differences among genotypes were analyzed by one-way ANOVA using STATVIEW 4.1 software (Abacus Concepts, Inc., Berkeley, CA). SNPs 1 to 4 are available in dbSNP under accession numbers ss3199913, ss3199914, ss3199915, and ss3199916, respectively.

38. To genotype the C/T SNP polymorphisms upstream of apoAV probes in ULTRAhyb buffer (Ambion). (E.M.R.), through the U.S. Department of Energy under contract no. DE-AC03-76SF00098 (E.M.R.), the NIH-NHLBI grant HL-18574 (R.M.K., E.M.R.), the NIH-NHLBI Program for Genomic Applications Grant HL66681 (E.M.R.), through the U.S. Department of Energy under contract no. DE-AC03-76SF00098 (E.M.R.), HL-53917 (J.C.C.), and an appointment to the Alexander Hollaender Distinguished Postdoctoral Fellowship Program sponsored by the U.S. Department of Energy, Office of Biological and Environmental Research, and administered by the Oak Ridge Institute for Science and Education (L.A.P.).

39. The 510 individuals in the original study, 388 were successfully genotyped by PCR amplification for the St1 polymorphism as previously described [16, 28].

40. To genotype the C/T SNP polymorphisms upstream of apoAV, oligonucleotides 5'CTGACTGATAAGGAGCACTG-3' and 5'AGGAACTGGAGCGAAA-3' were used to assemble a 197-bp fragment from genomic DNA. The penultimate base of Mse I (New England Biolabs) at 37°C for 3 hours. The PCR reactions were performed in 20 μl volumes containing 50 nmol/ml dCTP, 10 mmol/liter Tris (pH 8.3), 1.5 mmol/liter MgCl2, 0.2 mmol/liter of each primer. DNA was amplified under the following conditions: initial denaturation of 96°C for 2 min, followed by 32 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 3 min. PCR product (20 μl) was digested with 10 U of Mse I (New England Biolabs) at 37°C for 3 hours. The PCR products were size-fractionated on 3% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator.


49. Animals were killed, and tissues were harvested for either total RNA isolation by using the RNAasy-midi protocol (QIAGEN) or for poly(A)+ mRNA isolation by using the FastTrack 2.0 system (Invitrogen, Carlsbad, CA). About 10 μg of total RNA or 2 μg of poly(A)+ mRNA were separated in 10% agarose gels by gel electrophoresis and the RNA was transferred to a charged nylon membrane (Ambion, Austin, TX). The RNA blots were hybridized with [α-32P]-UTP-random primed apoAV probes in ULTRAhyb buffer (Ambion). Probe templates were generated by PCR amplification of liver cDNA with degenerate primers degApaAV-5'-CCCCTGTTGCGGGCAAAGACA-3' and degApaAV-5'-TCCGGAGCTGTCCGTACCT-3'. Filters were washed in 2× saline sodium citrate at room temperature for 20 min. This was supported by the National Dairy Promotion and Research Board in cooperation with the National Dairy Council and NIH-NHLBI grant HL-18574 (R.M.K., E.M.R.), the NIH-NHLBI Program for Genomic Applications Grant HL66681 (E.M.R.), through the U.S. Department of Energy under contract no. DE-AC03-76SF00098 (E.M.R.), HL-53917 (J.C.C.), and an appointment to the Alexander Hollaender Distinguished Postdoctoral Fellowship Program sponsored by the U.S. Department of Energy, Office of Biological and Environmental Research, and administered by the Oak Ridge Institute for Science and Education (L.A.P.).

### Reports

#### Phosphorylation-Dependent Ubiquitination of Cyclin E by the SCF<sup>Fbw7</sup> Ubiquitin Ligase

Deanna M. Koepp, Laura K. Schaefter, Xiaoye Xu, Khandan Keyomarsi, Claire Chu, J. Wade Harper, Stephen J. Elledge

Cyclin E binds and activates the cyclin-dependent kinase Cdk2 and catalyzes the transition from the G1 phase to the S phase of the cell cycle. The amount of cyclin E protein present in the cell is tightly controlled by ubiquitin-mediated proteolysis. Here we identify the ubiquitin ligase responsible for cyclin E ubiquitination as SCF<sup>Fbw7</sup> and demonstrate that it is functionally conserved in yeast, flies, and mammals. Fbw7 associates specifically with phosphorylated cyclin E, and SCF<sup>Fbw7</sup> catalyzes cyclin E ubiquitination in vitro. Depletion of Fbw7 leads to accumulation and stabilization of cyclin E in vivo and in mammalian cells. Multiple F-box proteins contribute to cyclin E stability in yeast, suggesting that overlap in SCF E3 ligase specificity allows combinatorial control of cyclin E degradation.

Passage through the cell cycle is controlled by the activity of cyclin-dependent kinases (CDKs) (1). Cyclin E is the regulatory subunit of Cdk2 and controls the G1 to S phase transition, which is rate-limiting for proliferation. Cyclin E is tightly regulated by ubiquitin-mediated proteolysis, which requires phosphorylation on Thr<sup>308</sup> and Cdk2 activation (2–4). Failure to properly regulate cyclin E accumulation can lead to accelerated S phase entry (5), genetic instability (6), and tumorigenesis (7). Elucidating the mechanism(s) that control cyclin E destruction has important implications for understanding control of cell proliferation during development and its subversion by tumorogenesis.

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

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

Cyclin E is a direct substrate of the SCF complex, which is a protein complex composed of a Skp1, Cull, and an F-box protein. The F-box protein is responsible for recognizing its substrate, in this case, cyclin E. This recognition is mediated by a WD40 repeat and/or LRR (leucine-rich repeat) repeats. The F-box protein, when bound to its substrate, facilitates the ubiquitination and subsequent degradation of the substrate by the SCF complex.

Several observations suggest that accumulation of cyclin E might be controlled through the SCF pathway. Cyclin E, like many other SCF substrates, requires phosphorylation for degradation. In yeast F-box protein mutant strains, cyclin E is stabilized in a similar manner to that seen with core SCF mutants and was also stabilized in yor080 mutants (Fig. 1A). Cyclin E was stabilized in cdc4-1 strains to an extent similar to that seen with core SCF mutants. Furthermore, the degradation of cyclin E was prevented when catalytically inactive cyclin E was overexpressed in yeast F-box protein mutant strains.

To further explore the contribution of SCF to cyclin E ubiquitination, we used a pulse-chase assay to perform a pulse-chase analysis of cyclin E protein in wild-type and cdc4-1, cdc34-2, or cdc53-1 mutants. To prevent cell cycle position effects, we arrested cells in G1 phase by addition of 200 mM hydroxyurea throughout the experiment. Cyclin E was unstable in wild-type cells but stabilized in SCF mutant cells (Fig. 1A). We examined cyclin E stability in yeast F-box protein mutant strains cdc4-1, gat1, ydr219, ypl149, yml088ufa1, ynl230b/ela1, ynl311, and yor080dna2. Cyclin E was stabilized in cdc4-1 strains to an extent similar to that seen with core SCF mutants and was also stabilized in yor080 mutants (Fig. 1A). Cdc4 and Yor80 contain WD40 and LRR motifs, respectively. We incubated recombinant SCF(Cdc4) and SCF(Yor80) complexes with recombinant cyclin E-Cdk2, E1, Cdc34 (E2), Ub, and adenosine triphosphate (ATP) (Fig. 1B). Ubiquitination of cyclin E was detected with both complexes in an F-box- and ubiquitin-dependent manner (Fig. 1B). Ubiquitination was also stimulated by phosphorylated cyclin E as it was largely prevented when catalytically inactive cyclin E-Cdk2 complexes were used as substrate (Fig. 1B).

To find the mammalian F-box protein that recognizes cyclin E, we surveyed previously identified F-box protein (Fb) for those that bound cyclin E after coexpression in insect cells or in vitro using 35S-methionine-labeled translation products and immobilized glutathione S-transferase (GST)-cyclin E-Cdk2 complexes. Seventeen F-box proteins were tested, including 16 that contained either WD40 or LRR motifs (18). Of these, only the WD40-containing Fbw7 (19) bound specifically to GST-cyclin E-Cdk2 but not to GST alone (Fig. 1C) (20). This interaction was specific for cyclin E, as it was not detected by Coomassie staining. The positions of endogenous insect cell GST protein (GST) and recombinant GST (rGST) are indicated.

Fig. 1. Interaction between cyclin E and SCF components in yeast and mammalian cells. (A) Stabilization of cyclin E in skp1-11, cdc34-2, cdc53-1, and yor080 mutants (12, 32). Strains of the indicated genotypes were grown in medium containing raffinose; cyclin E expression was induced 6 h later by galactose addition and at time = 0 was repressed by addition of glucose. Cells were harvested at the indicated times, and the abundance of cyclin E was determined by immunoblotting. Extracts from uninduced cells are shown in lane 1. WT, wild type. (B) Cyclin E is ubiquitinated in vitro by SCF complexes. SCF(Cdc4) or SCF(Yor80) complexes were purified from insect cells (13) and supplemented with ubiquitin (Ub), E1, Cdc34 (E2), and ATP, as indicated, before addition of His6-cyclin E-Cdk2 purified from insect cells (13). (C) GST-cyclin E-Cdk2 binds Fbw7. Immunoblotting of GST-cyclin E-Cdk2 (lane 3) or GST (lane 2) was incubated with in vitro-translated F-box proteins (31, 32). Lane 1 contains in vitro translation (IVT) product (33% of input). The bottom panel shows GST-cyclin E-Cdk2 and GST as detected by Coomassie staining. The positions of endogenous insect cell GST protein (eGST) and recombinant GST (rGST) are indicated. (D) Fbw7 preferentially binds cyclin E-Cdk2. The indicated Cdk complexes (lanes 2 to 6) were purified from insect cells and used for in vitro binding with Fbw7 as above. Cyclins were fused to GST for affinity purification, except for cyclin D1 where GST-Cdk4 is used. (E) Phosphorylation-dependent association of Fbw7 with cyclin E-Cdk2. Immobilized GST-cyclin E-Cdk2 was treated with 3-phosphatase in the presence (lane 1) or absence (lane 2) of phosphatase inhibitors (P) before in vitro binding to His6-Fbw7. Untreated GST-cyclin E-Cdk2 (lane 3) and GST (lane 4) were used as controls. Binding reactions were performed as in (C). (F) Immobilized cyclin E or cyclin D-derived peptides with or without phosphorylation were incubated with Fbw7, Fbw1 (B-TRCP), Fbw2, and Fbw6 IVT products as in (C). The peptide sequence and sites of phosphorylation (P) are indicated. (33)
for cyclin E as Fbw7 did not interact tightly with other cyclin-Cdk complexes (Fig. 1D). The interaction between Fbw7 and cyclin E was phosphorylation-dependent (Fig. 1E). Furthermore, Fbw7 bound specifically to a phosphopeptide containing the region of cyclin E required genetically for ubiquitination (Fig. 1F). Thus, the properties of Fbw7 are consistent with the predicted properties of a cyclin E ubiquitin ligase.

The mouse and human Fbw7 cDNA encode a protein of 627 amino acids containing seven WD40 repeats (Fig. 2, A and B). The presence of stop codons in all three reading frames of the 5′ untranslated region (UTR) indicates that the encoded open reading frame (ORF) is full-length. Database searches revealed substantial sequence similarity with Caenorhabditis elegans sel-10, which is involved in the presenilin (sel-12) and Notch/lin-12 pathways (21), and the predicted protein encoded by Drosophila melanogaster CG15010 (DmFbw7). Among S. cerevisiae F-box proteins, Fbw7 is 28% identical to Cdc4 (Fig. 2A). The relationship between sel-10 and a partial cDNA containing two COOH-terminal WD40 repeats from Fbw7 was noted previously (21). The extreme NH2-terminus of Fbw7 contains a 23-residue stretch (residues 7 to 29) of highly hydrophobic amino acids recognized by the SMART protein analysis program as a transmembrane domain (22).

Fig. 2. Characterization of the WD40-repeat-containing F-box protein, Fbw7. (A) Conservation between human (Hs) Fbw7 and C. elegans (Ce) sel-10, S. cerevisiae (Sc) Cdc4, and D. melanogaster (Dm) Fbw7 (33). Identical residues are shaded black and similarities are shaded gray. Asterisks indicate conserved arginine residues required for cyclin E binding. (B) Domain structures of Fbw7 homologs, F-box: W, WD40 repeat. (C) Three surface arginines on Fbw7 are required for binding cyclin E. Wild-type (WT) and mutant Fbw7 IVT products were used for binding with GST–cyclin E–Cdk2 (lanes 6 to 9) or GST (lane 5). One-third of the input is shown (lanes 1 to 4). (D) Model of the β-propeller structure of human Fbw7 displaying Arg417, Arg457, and Arg465 in red. The model was generated with Swissmodel with β-transducin as template. (E) Expression of Fbw7 in adult human tissues. Northern blots containing the indicated mRNAs were probed with the Fbw7 cDNA. Pn, pancreas; K, kidney; S, skeletal muscle; L, liver; Lu, lung; PL, placenta; Br, brain; and He, heart. (F) Fbw7 assembles into an SCF complex. Vectors expressing Cul1HA and Skp1HA were transfected into 293T cells in the presence of pCMV-Fbw7 (lane 1) or empty vector (lane 2) (37). After 48 hours, extracts were immunoprecipitated with antibodies to Myc and immunoblotted with antibodies to HA (top panel) or antibodies to Myc (bottom panel). The asterisks indicate the positions of immunoglobulin G heavy and light chains.

To examine the importance of the WD40 motifs in cyclin E recognition, we searched for basic residues located on the surface of the β-propeller structure that are conserved in Hs-Fbw7, Cdc4, Sel-10, and DmFbw7 but not in other Fbw proteins. Such residues would be candidates for phosphorylation-dependent interaction with ubiquitination targets. Arg417, Arg457, and Arg465, located in WD40 repeats 3, 4, and 5, met these criteria (Fig. 2, A and D). These residues were independently replaced with alanine, and the resulting proteins were tested for binding to GST–cyclin E in vitro. Mutation of Arg417 or Arg465 abolished binding to cyclin E, whereas mutation of Arg457 reduced binding (Fig. 2C).
Fbw7 mRNA is abundant in adult brain, heart, and skeletal muscle, tissues with a high percentage of terminally differentiated cells (Fig. 2E). Cotransfection of vectors encoding Myc-tagged Fbw7 with hemagglutinin (HA)-tagged Cul1 and HA-tagged Skp1 in 293T cells allowed detection of Fbw7 in SCF complexes, consistent with involvement of Fbw7 in ubiquitination (Fig. 2F).

We tested cyclin E ubiquitination in reticulocyte lysates in which either Fbw7 or Fbw2 had been translated. Ubiquitinated forms of cyclin E were observed in the presence of Fbw7 but not Fbw2 (Fig. 3A). Fbw7-dependent ubiquitination of cyclin E was also achieved in more purified systems. His6-Fbw7 was affinity-purified on immobilized GST–cyclin E–Cdk2 (Fig. 3, B and C) or antibodies to His6 (Fig. 3D) and used in ubiquitination reactions. Cyclin E ubiquitination was dependent on Fbw7 (Fig. 3, B and C) and was stimulated by Cul1-Rbx1 (Fig. 3, B to D). A small fraction of Fbw7 was associated with endogenous Cul1 in reticulocyte lysates (20). The pattern of conjugates was distinctly different when a form of ubiquitin that cannot undergo polyubiquitination (GST-UbR) was used in place of ubiquitin. (D) Cyclin E phosphorylation enhances ubiquitination of cyclin E. Reaction mixtures were immunoblotted with antibodies to cyclin E. No lysate was added to lanes 1 or 4. (B and C) Ubiquitination of GST–cyclin E by prebound His6–Fbw7. (B) Immobilized GST–cyclin E–Cdk2 was incubated with reticulocyte extracts in the presence or absence of Fbw7. Beads were supplemented with E1, Cdc34 (E2), and either ubiquitin (Ub; 100 µg/ml) or GST–UbR (100 µg/ml). Where indicated, 50 ng of a purified Cul1-Rbx1 complex was added. (B) The pattern of conjugates was distinctly different when a form of ubiquitin that cannot undergo polyubiquitination (GST–UbR) was included in the reaction mixture (Fig. 3C), indicating that the larger forms of cyclin E are ubiquitin conjugates. The ubiquitination reaction was also stimulated by phosphorylation of cyclin E (Fig. 3D) and was reduced when the cyclin E Thr380 → Ala (T380A) mutant was used as substrate (Fig. 3E).

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

Conversely, inhibition of Fbw7 should lead to increased accumulation of cyclin E. To test this, we used the small interfering RNA (siRNA) technique to reduce expression of Fbw7 in HeLa cells (23). Cells transfected with a double-stranded RNA (dsRNA) oligo corresponding to Fbw7 showed increased accumulation of cyclin E when compared with cells transfected with a control dsRNA oligo (Fig. 4B). Amounts of Cdk2 and bulk Cdk2 activity remained unaffected (Fig. 4B). The amount of p27 was similar in both Fbw7- and green fluorescent protein (GFP)–inhibited cells at the 48-hour time point, indicating that the accumulation of cyclin E in Fbw7-inhibited cells was not substantially influenced by p27 (20). To assess the effect of Fbw7 on cyclin E stability, we used the siRNA-inhibited cells for a pulse-chase analysis of cyclin E (2). Cells were labeled in vivo with 35S-methionine, samples were taken at the indicated times after replacement with medium containing unlabeled methionine, and cyclin E was immunoprecipitated (Fig. 4C). In the GFP siRNA cells, cyclin E was unstable, whereas in Fbw7-inhibited cells, cyclin E remains stable for the course of the experiment. Immunoblotting of the immunoprecipitates indicated that cyclin E amounts remained constant throughout the experiment. We also used the RNA interference (RNAi) technique to ablate Fbw7 in D. melanogaster (S2) cells (24). Transfection of S2 cells with dsRNAs corresponding to various portions of the DmFbw7 gene reduced amounts of DmFbw7 mRNA (Fig. 4D) and increased accumulation of cyclin E protein but not that of a control protein, Mle1 (Fig. 4D). In contrast, amounts of cyclin E mRNA were unaltered or slightly reduced, indicating that DmFbw7 regulates cyclin E through a posttranscriptional mechanism. Control dsRNAs had no effect on DmFbw7 or cyclin E (Fig. 4E). RNAi with the COOH-terminal fragment of Fbw7 was less efficient in destabilizing Fbw7 mRNA; thus, smaller decreases in cyclin E accumulation were observed.

In this report, we show that SCF/Fbw7-related ligases control the stability of cyclin E in a manner conserved through evolution. The finding that different E3s can control cyclin E levels in yeast may have implications for control of cell proliferation in mammals. Such a role would allow multiple signals to be independently integrated through different E3s to control cyclin E levels and cell proliferation. This could allow tissues to exert combinatorial control of proliferation and differentiation, consistent with the tissue-specific expression of...
Fbw7. Cells lacking the F-box protein Skp2 also accumulate cyclin E (25). However, this effect may be an indirect result of the accumulation of the Skp2 substrate, p27 (26, 27). Individual E3s often control the ubiquitination of cyclin E (25, 26, 27). Medium containing unlabeled methionine was added at time = 0. Samples were also immunoblotted with polyclonal antibodies against cyclin E (bottom panel). Arrows indicate the two major forms of cyclin E. (D and E) Accumulation of DmCycE in response to ablation of DmFbw7 by RNA interference. S2 cells were transfected with dsRNA corresponding to the N-terminal (N-term), COOH-terminal (C-term), or F-box region of DmFbw7 or against β-galactosidase (β-gal) as a control (34). At the indicated times, cells were harvested and cell lysates were generated. Samples were immunoblotted with antibodies to cyclin E or Cdk2. (C) Cyclin E is stable in Fbw7-inhibited cells. Cells were transfected as in (B), and pulse-chase analysis was performed as described (2). In whole-cell extracts, cyclin E was detected, and therefore, controlling accumulation of cyclin E through expression of a particular E3 may limit the function of other signaling pathways as a consequence. Thus, using different E3s to control cyclin E might lead to regulation of different constellations of signaling pathways in a tissue-specific manner. It is likely that Fbw7 controls the ubiquitination of other proteins in addition to cyclin E. Putative substrates include Noct and Presenilin proteins, as the C. elegans homolog sel-10 has been implicated in the control of both Noct and Presenilin signaling (21, 28). As a negative regulator of cyclin E, Fbw7 is a potential tumor suppressor. Consistent with this, we have observed that amounts of Fbw7 mRNA are decreased in breast tumor lines that have increased amounts of cyclin E (see supplemental Web figure 1 on Science Online at www.sciencemag.org/cgi/content/full/294/5540/173/DC1). Thus far, we have not identified mutations in the Fbw7 gene in these or other tumors. However, Fbw7 maps to 4q22, a site of loss of heterozygosity in a number of cancers (29). Additional studies will be required to resolve Fbw7's role in tumorigenesis.

References and Notes
18. F-box proteins tested for cyclin E binding include Fbw5, Fbw6, Fbw7, Fbw8, Fbw9, Fbw10, Fbw12, Fbw11, and Fbw13. The Fbw6 construct for in vitro translation was generated from an expressed sequence tag (EST) previously named Fbx29 (Genbank accession number AF176977) (11).
19. We previously identified the F-box motif in EST clone AI936668 as Fbx50 (11). Further sequence analysis of a Fbw7 cDNA (Genbank accession number AY033553) revealed WD40 repeats, and it was re-named Fbw7 according to convention (10, 11).
23. RNA interference was performed as described (24) except that Effectene (QIAGEN) was used for transfection. dsRNAs corresponded to nucleotides 1 to 505 (5′-terminus), 2678 to 3159 (F-box), and 3469 to 3981 (C-terminal) of the Fbw7 coding region. The siRNA oligo corresponded to nucleotides 713 to 3197 of the human Fbw7 coding region.
24. We thank C. Lehrer, M. Kuroda, T. Orr-Weaver, R. Duronio, M. Tyers, J. Roberts, L. Greenwald, A. New- man, H. Zheng, and S. Reed for gifts of reagents, plasmids, and helpful discussions and D. Lu for technical assistance. D.M.K. is a Howard Hughes Medical Institute. 8 August 2001; accepted 13 August 2001. Published online 30 August 2001; 10.1126/science.1065203. Include this information when citing this paper.
Much of the targeted protein ubiquitylation that occurs in eukaryotes is performed by cullin-based E3 ubiquitin ligases, which form a superfamily of modular E3s. The best understood cullin-based E3 is the SCF ubiquitin ligase (Feldman et al. 1997; Skowrya et al. 1997), which is composed of a modular E3 core containing CUL1 and RBX1 (also called ROC1), and a substrate specificity module composed of SKP1 and a member of the F-box family of proteins (Cardozo and Pagano 2004). The CUL1/RBX1 complex functions as a scaffold to assemble the E2 ubiquitin conjugating enzyme with the substrate specificity module (Zheng et al. 2002). CUL1 interacts with RBX1 through its C terminus and with SKP1 through its N terminus. The interaction of F-box proteins with SKP1 occurs through the F-box motif, an ~40-amino acid motif first identified in budding yeast Cdc4p and human cyclin F, the latter giving the name to the entire family [Bai et al. 1996]. F-box proteins contain additional protein interaction domains that bind ubiquitylation targets. The overall architecture of SCF complexes is conserved in the superfamily of SCF-like ubiquitin ligases that use cullin proteins as a scaffold. All cullins characterized to date (CUL1–5) are known to interact with RBX1 or RBX2 but use distinct specificity modules, which generally display structural and functional similarities with the SKP1/F-box protein module. For example, CUL2 and CUL5 are known to interact with the SKP1-like protein elongin C, which, in turn, interacts with F-box protein-like specificity factors called BC/SCOS-box proteins (Deshaies 1999; Guardavaccaro and Pagano 2003). In addition, CUL3 interacts with the BTB/POZ family of proteins, which appear to merge the functions of SKP1 and the F-box protein into a single polypeptide [Furukawa et al. 2003; Geyer et al. 2003; Pintard et al. 2003; Xu et al. 2003], with the BTB domain displaying structural relationships with SKP1 (Schulman et al. 2000; Xu et al. 2003). Cul4 forms a complex wherein DDB1/DDB2 and CSA proteins appear to function as substrate specificity modules [Groisman et al. 2003]. Thus, the current expectation is that all cullin-containing ligases will share the modular nature of the original SCF family of ligases.

A major strategy employed by the SCF is the use of extended protein families as specificity factors. In 1999, we reported the identification of 47 F-box proteins in mammals [Cenciarelli et al. 1999; Winston et al. 1999]. These proteins fell into three major classes, depending on the types of substrate interaction domains identified in addition to the F-box motif. The two largest classes of interaction domains are WD40 repeats [Smith et al. 1999] and leucine-rich repeats (LRRs) [Kobe and Kajava 2001]. A third generic class of F-box proteins contained various other types of protein interaction domains or no recognizable domains. These classes of F-box proteins were designated FBWs, FBLs, and FBXs, respectively, followed by a numerical identifier [Cenciarelli et al. 1999; Winston et al. 1999]. Paralogous genes in the same species used the same number followed by a letter (a, b, ...) representing the individual genes in the paralogous group. The Human Genome Organization (HUGO) Gene Nomenclature Committee adopted a related four-letter gene nomenclature: FBXW, FBXL, and FBXO, respectively, where "O" in FBXO refers to "other" domains. Since this initial work, subsequent efforts, particularly cDNA and genomic sequencing projects, have facilitated the further identification of F-box protein-coding genes. However, the inconsistent use of nomenclature standards has greatly limited the utility of the sequence database. This inconsistency is due in part to the rapid pace of research in this area that has precluded coordination of gene names. A survey of F-box proteins in GenBank revealed several issues: (1) several different F-box protein...
## Table 1. Mammalian F-box proteins

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coding genes have been given the same gene name; (2) multiple individual F-box genes have been given several different names; (3) the nomenclature used for clearly orthologous mouse and human genes is inconsistent; (4) several genes present in GenBank encode F-box proteins but are not annotated as such; (5) mRNA sequence revisions and refinement of algorithms for detection of F-box motifs have led to the removal of some genes from the F-box category; and (6) improvements in structural domain identification suggest that genes previously designated in the FBXO subclass may be more appropriately placed in the FBXL or FBXW subclasses. The need for clear communication in this field necessitates a unified nomenclature for F-box proteins.

To develop a comprehensive nomenclature for mammalian F-box proteins, we have systematically analyzed F-box proteins in the human and mouse genomes and have organized these genes in a manner that largely conforms to previous nomenclature standards, as explained below. This nomenclature has now been adopted and implemented by the HUGO Gene Nomenclature Committee. Several factors were considered in devising the most appropriate nomenclature for the future. First, genes whose symbols were approved by the nomenclature committee prior to the discovery of these genes as F-box proteins will remain as the approved symbol. Second, the previous nomenclature used letters (a, b, ...) to indicate what appeared to be paralogous genes (e.g., FBXL3a and FBXL3b). However, because it is now appreciated that many F-box proteins exist as multiple splicing variants, the use of such a designation scheme has been avoided, necessitating the complete renaming of a small number of F-box proteins. Finally, mouse and human orthologs have been given the same symbols to facilitate comparative studies in the future. A detailed description of how the nomenclature changes have affected individual F-box genes is provided in the Supplemental Material.

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**Figure 1.** Domain structures of mammalian F-box proteins. Domains identified by the Hidden Markov Model algorithms of SMART or Pfam include F-box motif (F), WD40 repeat (WD), leucine-rich repeat (L), transmembrane domain (T), F-box-associated domain (FBA), between-ring domain (IBR), domain in carbohydrate binding proteins and sugar hydrolases (CASH), kelch repeat (K), calponin homology domain (CH), domain present in cupin metalloenzyme family (Imc), domain present in PSD-95, Dlg, and ZO-1 (PDZ), zinc-binding domain found in Lin-11, Isl-1, and Mec-3 (Lim), HNH nuclease family (HNHc), novel eukaryotic zinc-binding domain (CHORD), and tetratrico peptide repeat (TPR). The following domains were found via the Structural Classification of Proteins (SCOP) database, which can be used to predict protein sequences that can adopt known protein folds: ApaG-like, which is structurally similar to bacterial ApaG; Apolipophorin, the apolipophorin-III-like fold; Ubl, the ubiquitin-like fold; TDL, which is Traf-domain like; RNI-like, which may form structure similar to that of leucine-rich repeats in placental RNase inhibitor; and RCC1, which is a possible regulator of chromatin condensation-I fold.
Our analysis led to the identification of 68 human and 74 mouse genes encoding recognizable F-box motifs, as detected by Hidden Markov Models (Table 1; Fig. 1) (Bateman et al. 2004; Letunic et al. 2004). A phylogenetic representation of human F-box motifs is shown in Figure 2. The phylogeny of F-box domain sequences only, which gives the cleanest available view of the evolutionary signature of the family, shows two major groups of F-box proteins (an evolutionary divergence). Different protein interaction domains are scattered throughout the two groups indicating that similar domain swapping mechanisms act on both, but ruling out that all FBXW subfamily members diverged from a single FBXW ancestor, for example.

Clear mouse orthologs were identified for all human F-box proteins except FBXW12, with the majority of mouse genes displaying >80% identity with their human counterparts (Table 1). In the mouse, FBXW12-related sequences have been dramatically expanded to seven genes (one at chromosome 13A5 [Fbxw17] and a cluster of six genes at chromosome 9F2 [Fbxw13, Fbxw14, Fbxw15, Fbxw16, Fbxw18, Fbxw19]). Each of these seven mouse genes is equally related to FBXW12, and, therefore, we are unable to unambiguously designate a mouse ortholog of human FBXW12. The mechanism and significance of expansion of this subclass of F-box proteins in the mouse are unknown. Three human proteins with F-box like motifs—Tome-1 (CDC43), TBL1, and TBLR1 (TBL1XR1)—were not included because the presumptive F-box sequence did not reach the threshold sufficient for this classification.

A combination of BLAST analyses and phylogenetic tree construction using putative substrate interaction domains together with the F-box motif revealed possible orthologs of mammalian F-box proteins in Drosophila melanogaster and Caenorhabditis elegans (Table 1; Fig. 3). The inclusion of substrate interaction domains allows confirmation of some relationships with the mammalian proteins [e.g., FBXL12 with SKP2], but also demonstrates, in comparison to the F-box domain only tree, that the phylogenetic spread of each subgroup is as wide as that of the whole family. Interestingly, the D. melanogaster genome contains several possible orthologs of the human FBXL series that are not found in C. elegans (Table 1; Fig. 3). The fact that C. elegans has more than 300 F-box proteins but that only a few display relationships with mammalian genes indicates significant diversification of the F-box proteins in this organism. This expansion is species-specific because the Caenorhabditis briggsae genome is predicted to encode a similar number of F-box proteins as found in human and mouse genomes (Stein et al. 2003). Six genes encoding F-box proteins appear to be conserved in C. elegans, D. melanogaster, and mammals: BTRC (FBXW1), FBXW7, FBXL2, FBXO10, FBXO25, and FBXO45 (Table 1; Fig. 3). Interestingly, in mammals four of these six genes have a paralog: FBXW1 (BTRC, β-TRCP1) for FBXW11 [β-TRCP2], FBXL20 for FBX12, FBXL11 for FBX11, and FBXO32 for FBXO25, respectively. The FBA-containing subclass of FBXO proteins are contained in the C. elegans genome but are absent in D. melanogaster (Table 1; Fig. 3). Thus, it is possible that much of the core SCF signaling common to metazoans is performed by a relatively small number of highly conserved F-box proteins. To date, conserved degradation pathways have been found for targets of mammalian FBXW7 and β-TRCP1/2 in both C. elegans and Drosophila. c-MYC and cyclin E are targeted by ago/FBXW7 in both Drosophila and mammals (Koepf et al. 2001; Moberg et al. 2001, 2004; Strohmaier et al. 2001; Tetzlaff et al. 2004; Welcker et al. 2004), and Notch is targeted by sel-10/FBXW7 in both mammals and C. elegans (Hubbard et al. 1997; Wu et al. 2001; Tetzlaff et al. 2004; Tsunematsu et al. 2004). Similarly, β-TRCP1/2/slimb has been linked to the β-catenin, IκB, and cell cycle pathways in both Drosophila and mammals (for review, see Maniatis 1999, Guardavaccaro and Pagano 2003).

Despite the large number of mammalian F-box proteins, in addition to β-TRCP1/2 and FBW7, only one other mammalian F-box protein has been matched to its downstream substrates, namely, SKP2 [Ang and Harper 2004; Cardozo and Pagano 2004]. Interestingly, SKP2 is the product of a proto-oncogene, FBW7 is a tumor suppressor (Pagano and Benmaamar 2003; Yamasaki and Pagano 2004), and overexpression of β-TRCP1 can contribute to transformation at least in some epithelial tissues.

Figure 2. Phylogenetic tree depiction of interrelationships between human F-box proteins. The tree is generated from the set of amino acid sequences comprising the F-box domain by the neighbor-joining method (Saitou and Nei 1987) as adapted in ICM software (Molsoft LLC; http://www.molsoft.com).
Finally, EMI1/FBXO5, an inhibitor of the mitotic ubiquitin ligase APC/C, is overexpressed in tumor cell lines and certain breast tumors [Hsu et al. 2002; van 't Veer et al. 2002]. Other F-box proteins appear to play a role in different diseases. For example, Dactylin/FBW4 is encoded by SHFM3, the split hand–foot malformation syndrome gene 3 [Basel et al. 2003]. FBXO3 expression is increased in proliferating synovium of patients with rheumatoid arthritis [Masuda et al. 2002]. FBXO32 is up-regulated during muscle atrophy [Bodine et al. 2001; Gomes et al. 2001]. Thus, F-box proteins are attractive candidates for drug discovery because they play crucial roles in many important signaling pathways.

Validated protein structure prediction tools revealed inappropriately classified F-box proteins as well the association of new functional or structural domains with the F-box motif (Fig. 1). For example, certain F-box proteins previously placed in the FBXO class (e.g., FBXO13) were found to have LRRs and were reclassified accordingly (Table 1; also see Supplemental Material). FBXO14 was found to have WD40 repeats and was reclassified as FBXW12 (Table 1). Three FBXO members (FBXO33, FBXO38, and FBXO39) may display structural similarity to RNase inhibitor, the prototypical LRR, but these sequences do not reach the threshold required to be fingered as authentic LRRs based on sequence information alone (Fig. 1). Additional protein folds new to the mammalian F-box class include ubiquitin-like folds (FBXO7), TPR-like domain (FBXO9), RCC1 (FBXO24), and Kelch repeats (FBXO42). In addition to the five FBA-containing F-box proteins that bind glycosylated proteins [Cardozo and Pagano 2004], two additional proteins (FBXO10 and FBXO11) contain the CASH domain frequently found in carbohydrate-binding proteins and hydrolases (Fig. 1).

[Kudo et al. 2004]. Finally, EMI1/FBXO5, an inhibitor of the mitotic ubiquitin ligase APC/C, is overexpressed in tumor cell lines and certain breast tumors [Hsu et al. 2002; van 't Veer et al. 2002]. Other F-box proteins appear to play a role in different diseases. For example, Dactylin/FBW4 is encoded by SHFM3, the split hand–foot malformation syndrome gene 3 [Basel et al. 2003]. FBXO3 expression is increased in proliferating synovium of patients with rheumatoid arthritis [Masuda et al. 2002]. FBXO32 is up-regulated during muscle atrophy [Bodine et al. 2001; Gomes et al. 2001]. Thus, F-box proteins are attractive candidates for drug discovery because they play crucial roles in many important signaling pathways.

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