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Mutations in *Fbx4* Inhibit Dimerization of the SCF^{Fbx4} Ligase and Contribute to Cyclin D1 Overexpression in Human Cancer

Olena Barbash,[1,2] Petia Zamfirova,[1] Douglas I. Lin,[1,2] Xiangmei Chen,[4] Ke Yang,[4] Hiroshi Nakagawa,[3] Fengmin Lu,[4] Anil K. Rustgi,[3] and J. Alan Diehl[1,2,*]
[1]Abramson Family Cancer Research Institute
[2]Department of Cancer Biology
[3]Division of Gastroenterology, Department of Medicine and Department of Genetics
University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
[4]Department of Microbiology, Peking University Health Science Center, Beijing 100083, China
*Correspondence: adiehl@mail.med.upenn.edu
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SUMMARY

SCF^{Fbx4} was recently identified as the E3 ligase for cyclin D1. We now describe cell-cycle-dependent phosphorylation and dimerization of Fbx4 that is regulated by GSK3β and is defective in human cancer. We present data demonstrating that a pathway involving Ras-Akt-GSK3β controls the temporal phosphorylation and dimerization of the SCF^{Fbx4} E3 ligase. Inhibition of Fbx4 activity results in accumulation of nuclear cyclin D1 and oncogenic transformation. The importance of this regulatory pathway for normal cell growth is emphasized by the prevalence of mutations in *Fbx4* in human cancer that impair dimerization. Collectively, these data reveal that inactivation of the cyclin D1 E3 ligase likely contributes to cyclin D1 overexpression in a significant fraction of human cancer.

INTRODUCTION

Cell-cycle progression requires the orderly accumulation of proteins that drive cell division with the coordinated inactivation or loss of proteins that inhibit this activity. Progression through G1 phase ensues upon activation of CDK4, CDK6, and CDK2 through association with their regulatory subunits, cyclin D (D1, D2, or D3, depending upon the tissue type) and cyclin E. Accumulation of cyclins is determined largely through coordinated cycles of synthesis and destruction (Sherr et al., 1992; Winston and Pledger, 1993; Geng et al., 1996). Efforts to define the proteolytic machinery led to the identification of SCF (Skp1-Cul1-F box protein) E3 ligases as the primary regulators of G1 cyclin proteolysis. The F box component of SCF ligases determines substrate specificity and generally binds to substrates in a phosphorylation-dependent manner (Skowyra et al., 1997; Willems et al., 1999; Craig and Tyers, 1999). More specifically, cyclin D1 proteolysis is directed by the SCF$^{Fbx4/\alpha B\text{-crystallin}}$ ligase, whereas cyclin E ubiquitination is directed by SCF^{Fbw7} ligase (Lin et al., 2006; Koepp et al., 2001; Strohmaier et al., 2001; Welcker et al., 2003). Accumulation of cyclin D1 is acutely sensitive to growth-factor stimulation. Cyclin D1 initially accumulates in G1 phase through growth-factor-dependent transcription and translation (Sherr et al., 1992). At the G1/S boundary, cyclin D1 is phosphorylated by GSK3β at the residue Thr286 followed by CRM1-dependent nuclear export to the cytoplasm, where cyclin D1 is recognized by SCF$^{Fbx4/\alpha B\text{-crystallin}}$ ligase, ubiquitinated, and targeted to the 26S proteasome (Diehl et al., 1997; Alt et al., 2000; Lin et al., 2006).

Cyclin D1 is overexpressed frequently in different human cancers such as those originating from the esophagus, head and neck, and breast, and overexpression is considered an early, causative event in some of these cancers (Diehl, 2002). Overexpression was initially thought to result from chromosomal

SIGNIFICANCE

Cyclin D1 overexpression occurs frequently in human cancer; however, the contributing mechanisms remain ambiguous, as the frequency of gene amplification is lower than the incidence of overexpression. Herein we demonstrate that attenuation of cyclin D1 SCF^{Fbx4} E3 ubiquitin ligase activity occurs frequently in human cancer and may represent a common mechanism of overexpression and deregulation of cyclin D1. Significantly, inactivation of the Fbx4 ligase is the result of mutations in N-terminal regulatory regions of Fbx4 that disrupt ligase dimerization, thereby revealing the biological significance of SCF ligase oligomerization. In summary, our study establishes Fbx4 as a tumor suppressor in human cancer, function of which is abrogated by a unique category of mutations that target E3 ligase activity.

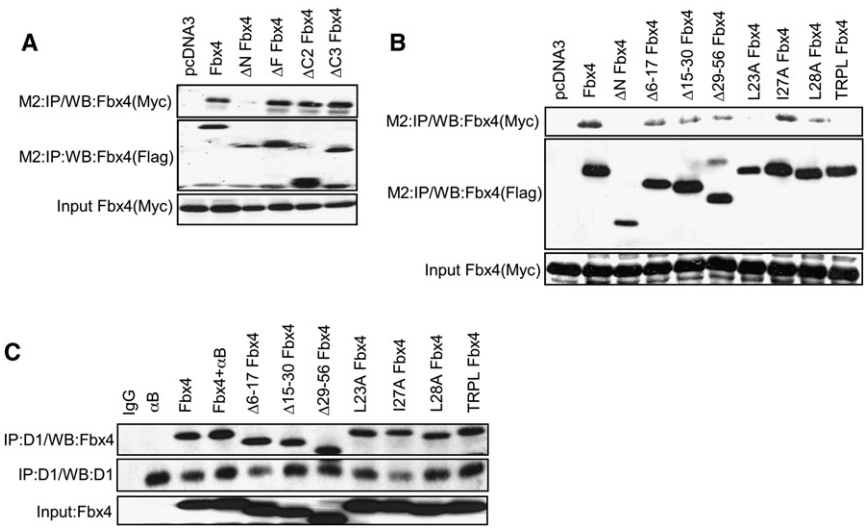


Figure 1. Dimerization of Fbx4 Occurs through Its N Terminus

(A and B) Lysates were prepared from 293T cells transfected with vectors encoding human wild-type Myc-tagged Fbx4 and the indicated FLAG-Fbx4 mutant constructs. Protein complexes were isolated by M2 affinity chromatography and detected by immunoblot with Fbx4 antibody.

(C) 293T cells were cotransfected with Fbx4 constructs along with cyclin D1, CDK4, and αB-crystallin. Twenty-four hours posttransfection, cells were treated with MG132 for 6 hr followed by cyclin D1 immunoprecipitation. Cyclin D1 was detected with 13G11 monoclonal antibody.

translocations or gene amplification (Dickson et al., 1995; Zukerberg et al., 1995; Nakagawa et al., 1995). Because the frequency of cyclin D1 overexpression exceeds the frequency of such large chromosomal alterations, it was proposed that alterations in turnover of cyclin D1 protein might also contribute to aberrant cyclin D1 levels (Russell et al., 1999; Diehl, 2002). Indeed, recent work has revealed mutations in cyclin D1 that directly inhibit phosphorylation-dependent proteolysis (Moreno-Bueno et al., 2004; Benzeno et al., 2006). However, such mutations are rare and imply the occurrence of other mechanisms. Investigations that might reveal such mechanisms have been hindered in part by our lack of knowledge regarding the E3 ligase that directs cyclin D1 ubiquitin-mediated proteolysis. With the recent identification of Fbx4/αB-crystallin as the specificity factor that directs cyclin D1 ubiquitination (Lin et al., 2006), it is now possible to ascertain whether components of the cyclin D1 E3 ligase, Fbx4, or αB-crystallin are subjected to genetic alterations in human cancers and may represent potential tumor suppressor proteins in this context.

Since SCF recognition generally depends upon substrate phosphorylation, it has been inferred that SCF ligases are constitutively active, with regulation occurring mainly at the level of substrate phosphorylation. However, recent work has revealed that many F box proteins can function as homodimers, thereby providing a potential means of ligase regulation. Fbw7, an E3 ligase that ubiquitinates cyclin E, c-Myc, and Notch, has been demonstrated to form dimers, but whether dimerization regulates ligase activity or substrate recognition is controversial (Zhang and Koepp, 2006; Welcker and Clurman, 2007; Tang et al., 2007). β-Trcp, an E3 ligase for IκB, forms both homo- and heterodimers between two isoforms, β-Trcp1 and β-Trcp2, with only the homodimers forming productive ligases whereas heterodimerization is inhibitory (Suzuki et al., 2000). The mechanism by which the formation of F box protein dimers promotes substrate ubiquitination is unclear, but possible scenarios include increased concentration of substrate in the area of high E2 activity as well as positioning of substrate in a manner that facilitates the ubiquitination of multiple lysine residues (Tang et al., 2007). Dimerization of F box proteins is directed through the so-called D domain. The D domain is localized N-terminal to the F box region, and critical hydrophobic residues (leucine and isoleucine) are conserved spatially in distinct F box proteins such as Fbw7, β-Trcp, and Fbw2 (Zhang and Koepp, 2006). Although the phenomenon of ubiquitin ligase substrate adaptor self-association is established, the functional significance of this and whether the dimerization is constitutive or regulated remain to be elucidated.

This work provides evidence that Fbx4 contains an N-terminal dimerization domain (D domain) and that dimerization is regulated by phosphorylation of a conserved serine residue (Ser11 in mouse; Ser12 in human) proximal to the D domain. We demonstrate that dimerization-deficient Fbx4 mutants fail to support efficient cyclin D1 degradation. Mutation of this serine residue to alanine attenuates dimerization of Fbx4 and significantly impairs cyclin D1 ubiquitination and proteolysis. Importantly, the data reveal that phosphorylation of Ser11 is temporally regulated during cell-cycle progression, with maximal phosphorylation and dimerization occurring during S phase. Strikingly, Fbx4 phosphorylation is GSK3β dependent, thereby revealing that GSK3β controls not only substrate availability, through phosphorylation of cyclin D1, but also activation of the E3 ligase that promotes cyclin D1 ubiquitination. Finally, we demonstrate that inhibition of $SCF^{Fbx4/\alpha B\text{-crystallin}}$ ligase activity leads to accumulation of cyclin D1 in the nuclei and promotes cellular transformation.

RESULTS

Fbx4 Contains an N-Terminal Dimerization Domain

To test Fbx4 dimerization, a series of *Fbx4* deletion alleles with an N-terminal FLAG tag (see Figure S1A available online) were coexpressed with Myc-tagged wild-type Fbx4 in 293T cells, and interactions were assessed by anti-FLAG precipitation. Myc-Fbx4 was detected in precipitates containing wild-type FLAG-Fbx4 (Figure 1A), revealing homotypic dimerization. While neither the F box nor the C-terminal regions were required, removal of the N-terminal 50 amino acids of Fbx4 abrogated dimerization (Figure 1A, ΔN Fbx4). Critically, ΔN Fbx4 still bound Skp1 (Figure 2A), suggesting that this deletion did not result in global folding defects. Mutation of leucine/isoleucine residues within Fbx4 (Figure S1B) that are spatially conserved with leucines that contribute to dimerization of Fbw7 (Zhang and Koepp, 2006) defined Leu23 as essential for Fbx4 dimerization (Figure 1B). As with dimerization-defective Fbw7 (Zhang and

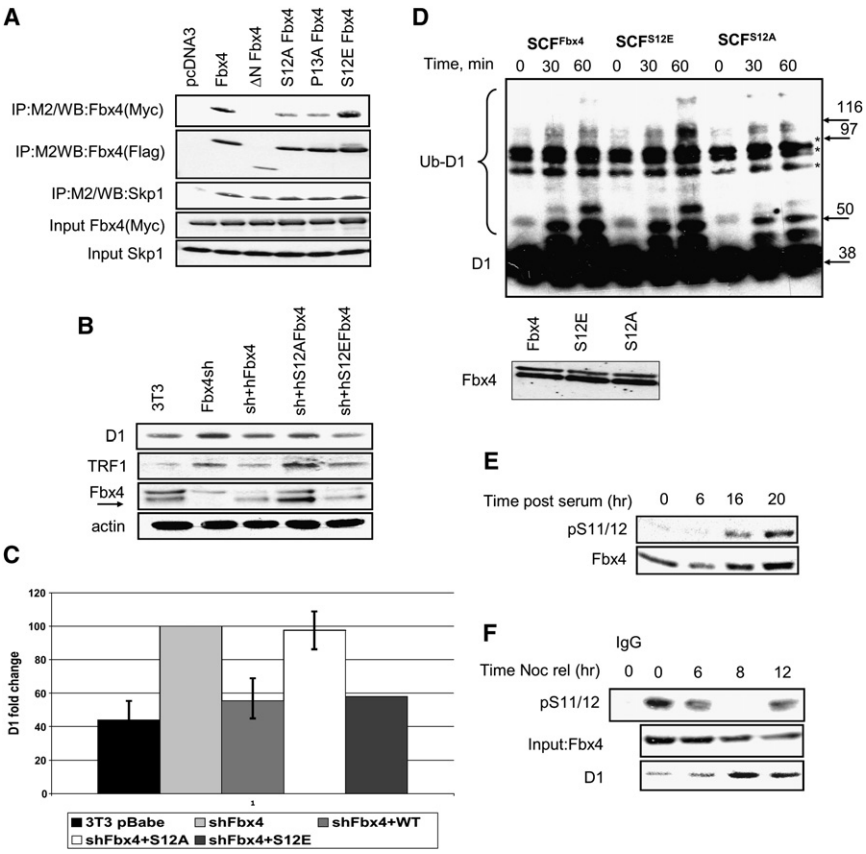


Figure 2. Fbx4 Ser12 Is Required for Cyclin D1 Ubiquitination and Proteolysis

(A) 293T cells were transfected with plasmids encoding FLAG-tagged Fbx4 mutants and wild-type Myc-tagged Fbx4. Complexes were isolated by affinity chromatography using M2-conjugated agarose, and individual components were detected by immunoblotting with Fbx4 and Skp1 antibodies.

(B) NIH 3T3 cells in which Fbx4 had previously been knocked down by shRNA were transfected with wild-type, S12A, or S12E Fbx4 pcDNA3 followed by G418 selection of stably expressing clones. Asynchronous cells were harvested and subjected to western blotting with cyclin D1, TRF1, and Fbx4 antibodies.

(C) Quantification of data in (B). Error bars represent ±SD.

(D) Ligase complexes (purified protein shown in bottom panel) were purified from stable cell lines expressing SCF^{Fbx4}, SCF^{S12E}, or SCF^{S12A} Fbx4 (described in [B]). In vitro ubiquitination reactions were performed using GST-tagged purified cyclin D1. Ubiquitin-conjugated cyclin D1 was detected by immunoblotting. Nonspecific complexes are denoted by asterisks.

(E) NIH 3T3 cells were serum starved for 48 hr and released into 10% FBS-containing medium for the indicated intervals. Immunoblot analysis was performed using pSer11/12 Fbx4 and a total Fbx4 antibody.

(F) Cells synchronized via nocodazole block were harvested at the indicated intervals following nocodazole release. Fbx4 phosphorylation was detected by precipitation with pSer11/12 antibody and immunoblotting with total Fbx4 antibody. Total cyclin D1 and Fbx4 levels were assessed by direct western blotting.

Koepp, 2006; Welcker and Clurman, 2007), Fbx4 mutants retained their capacity to associate with cyclin D1 (Figure 1C) and αB-crystallin (Figure S1C) (Lin et al., 2006).

Ser12 of Fbx4 Contributes to Fbx4 Dimerization

Ser12 of Fbx4 is a predicted proline-directed phosphorylation motif (Beausoleil et al., 2004). Due to the proximity of Ser12 with the D domain, we considered the possibility that phosphorylation of Ser12 could regulate dimerization of Fbx4. We therefore generated both nonphosphorylatable (S12A) and phosphomimetic (S12E) mutations in Fbx4 as well as a P13A mutant to address whether a proline-directed kinase targeted this site; all mutants were then assessed for dimerization potential. Both S12A Fbx4 and P13A Fbx4 exhibited reduced dimerization potential when compared to wild-type Fbx4 or S12E Fbx4 (Figure 2A). We next reconstituted murine NIH 3T3 cell lines wherein endogenous Fbx4 had been stably knocked down (Lin et al., 2006), using either wild-type (human) Fbx4 or the Ser12 mutants of Fbx4 followed by G418 selection to establish stable cell lines. We demonstrated previously that Fbx4 knockdown results in overexpression of endogenous cyclin D1 (Figures 2B and 2C). Reconstitution of cells with either wild-type Fbx4 or S12E Fbx4 resulted in a reduction of cyclin D1 to levels comparable to parental NIH 3T3, while S12A Fbx4 had reduced activity. Significantly, levels of TRF1, the only other reported Fbx4 substrate (Lee et al., 2006), mimicked those of cyclin D1. To assess the contribution of Ser12 to Fbx4-dependent ubiquitination activity, we purified wild-type and mutant SCF^{Fbx4} ligases from NIH 3T3 stable cell lines by anti-FLAG affinity purification (Figure 2D; Fbx4 levels are shown in the lower panel). Both wild-type Fbx4 and S12E Fbx4 catalyzed in vitro ubiquitination of cyclin D1, while activity of S12A Fbx4 was attenuated. Thus, disruption of Ser12 leads to decreased dimerization of Fbx4 and decreased ubiquitination of cyclin D1.

Phosphorylation of Ser12 Is Induced in the S Phase of the Cell Cycle

Since Ser12 is important for ligase function, we considered a scenario wherein phosphorylation of Ser12 regulates Fbx4 dimerization and ultimately ligase activity. To test this notion, we first generated phospho-Ser11/12 (Ser11 in mouse; Ser12 in human)-reactive antibodies (Figures S2A and S2B). If phosphorylation of Ser12 regulates Fbx4 dimerization and ubiquitination activity, we anticipated temporal regulation of Ser12 phosphorylation. Because cyclin D1 ubiquitination is triggered upon G1/S transition following phosphorylation-dependent nuclear export, we predicted peak phosphorylation of Fbx4 at the G1/S boundary. To test this premise, NIH 3T3 cells were synchronized by serum

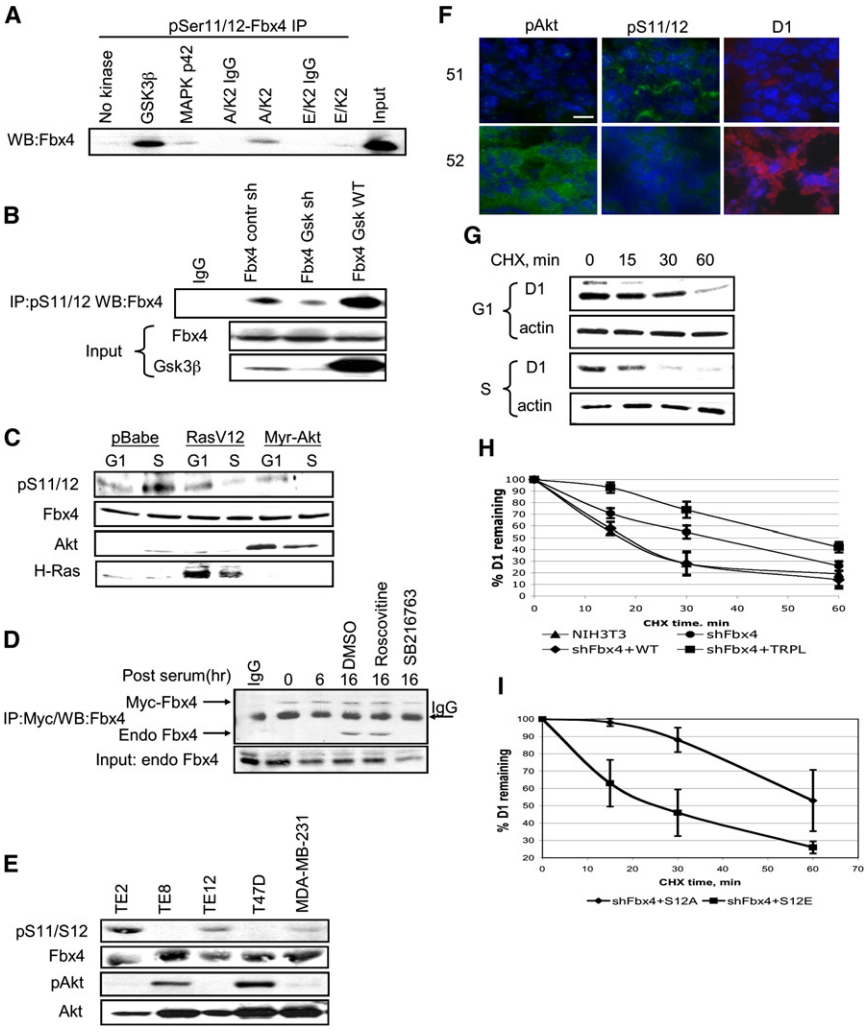


Figure 3. GSK3β Phosphorylates Ser11/12 of Fbx4 in the S Phase of the Cell Cycle

(A) In vitro kinase assay was performed using purified GST-tagged Fbx4 and indicated kinases. Following in vitro phosphorylation, samples were precipitated with pSer11/12 Fbx4 antibody and subjected to immunoblotting with Fbx4 antibody.

(B) 293T cells were transfected with wild-type Fbx4 along with control shGFP vector, GSK3β shRNA vector, or a plasmid encoding wild-type (WT) GSK3β. Fbx4 was immunoprecipitated from cellular extracts using pSer11/12 Fbx4 antibody 48 hrs posttransfection and detected by immunoblotting with total Fbx4 antibody.

(C) NIH 3T3 cells were transfected with empty vector or RasV12- or Myr-Akt-expressing constructs. Cells were synchronized by serum starvation and released into complete medium for 6 hr (G1) or 16 hr (S). Protein extracts were processed for immunoblotting using pSer11/12 Fbx4, total Fbx4, total Akt, and H-Ras antibodies.

(D) NIH 3T3 cells were transfected with vector encoding Myc-tagged Fbx4 and were synchronized 24 hr later in G0. Cells were released into the cell cycle for the indicated intervals (cells were treated with kinase inhibitors for 6 hr before harvesting samples at the 16 hr time point). Myc-Fbx4 pulldown was performed using Myc-agarose. Myc-Fbx4-associated endogenous Fbx4 was detected by immunoblotting with total Fbx4 antibody.

(E) Total cell extracts from esophageal carcinoma cell lines (TE2, TE8, and TE12) and breast carcinoma cell lines (T47D and MDA-MB-231) were subjected to precipitation with pSer11/12 antibody followed by immunoblotting with Fbx4 antibody. Direct western blotting was performed using pAkt (Ser473) and total Akt antibodies.

(F) Frozen sections from MMTV-Neu breast tumors were analyzed by immunohistochemistry using pAkt (Ser473), pSer11/12 Fbx4, and cyclin D1. Scale bar = 8 μM.

(G) NIH 3T3 cells were arrested by serum starvation followed by replating in complete media. Cycloheximide (CHX) was added for the indicated intervals during G1 (6 hr postrelease) and S (16 hr postrelease) phases of the cell cycle. Cyclin D1 turnover was analyzed by western analysis using cyclin D1 antibody.

(H) NIH 3T3 shFbx4 stable cell line was transfected with either wild-type (WT) Fbx4 or Fbx4 TRPL. Cycloheximide chase was performed in asynchronous cells 48 hr after transfection. Quantification of three independent experiments is provided; error bars represent ±SD.

(I) S12A Fbx4 and S12E Fbx4 were individually reintroduced into Fbx4 knockdown cells, and cyclin D1 turnover was assessed in S phase. Quantification of three independent experiments is provided; error bars represent ±SD.

starvation; following mitogen-triggered cell-cycle reentry, pSer12 of Fbx4 was assessed at 0, 6, 16, and 20 hr using our phosphospecific antibody. Phosphorylation of Ser12 was low during cell-cycle entry but increased dramatically upon S phase entry (16 hr) and increased further during S phase (20 hr, Figure 2E). Cells were also synchronized with nocodazole to assess Ser12 phosphorylation in cells traversing the G2/M–G1 transition. Ser12 phosphorylation was high during G2/M and early G1 phase (Figure 2F, 0 and 6 hr), points at which cyclin D1 levels remain low. By contrast, Ser12 phosphorylation was reduced dramatically during mid/late G1 phase when cyclin D1 levels increase (8 hr) and was again high in early S phase (12 hr, Figure 2F).

Given the proximity of Ser12 and Pro13, we reasoned that a proline-directed kinase likely regulates phosphorylation of this serine residue. To identify the kinase responsible for Ser12 phosphorylation, we focused on proline-directed protein kinases known to be active or activated at the G1/S phase boundary. Strikingly, only GSK3β efficiently phosphorylated Ser12 in vitro as determined by IP/western (Figure 3A). Phosphorylation of Fbx4 by GSK3β was also confirmed with recombinant proteins in an in vitro kinase assay (Figure S3A). Additionally, while GSK3β efficiently phosphorylated Ser12, GSK3α did not exhibit detectable activity toward this residue in Fbx4 (Figure S3B). This result is consistent with the capacity of GSK3β, but not GSK3α, to function as a proline-directed kinase. We evaluated the role of CDK2 (roscovitine) and GSK3β (LiCl and SB216763) in vivo. Both LiCl and SB216763 reduced pSer12, while roscovitine did not (Figure S4A).

To establish the relevance of GSK3β in vivo, we used several independent approaches. Initially, we determined whether

overexpression of kinase-dead GSK3β in insect cells inhibited Ser12 phosphorylation. Expression of kinase-dead GSK3β efficiently inhibited phosphorylation in a dose-dependent manner, and, when total Fbx4 was immunoprecipitated from cells, kinase-dead GSK3β associated stably with Fbx4 (Figure S4B), demonstrating a direct interaction. We then utilized a previously validated shRNA vector (Jin et al., 2003) to knock down GSK3β in human cells. Knockdown of GSK3β reduced pSer12 relative to a nonspecific shGFP RNA, while overexpression of wild-type GSK3β increased pSer12 (Figure 3B). Finally, we determined whether the inhibition of GSK3β activity through expression of activated Ras (RasV12) or Akt (Myr-Akt) (Rodriguez-Viciana et al., 1994; Cross et al., 1995) would inhibit phosphorylation of S12. Both RasV12 and Myr-Akt inhibited pSer12 (Figure 3C), consistent with a model wherein the Ras-Akt-GSK3β cascade regulates Ser12 phosphorylation of Fbx4.

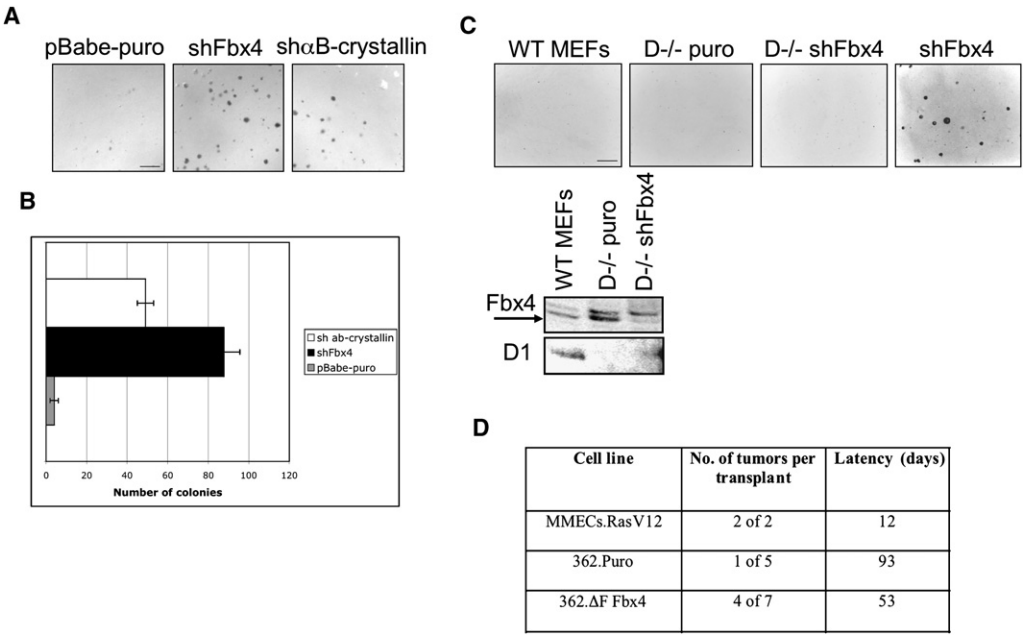
If phosphorylation of Ser12 regulates Fbx4 dimerization, then dimerization of Fbx4 should parallel phosphorylation and increase during S phase. NIH 3T3 cells were transfected with a plasmid encoding 6×Myc-Fbx4, synchronized by serum deprivation, and released into the cell cycle. Dimerization of endogenous Fbx4 with Myc-Fbx4 was assessed by precipitation with anti-Myc antibodies from lysates prepared from cells harvested in G0, G1, and S phase of the cell cycle followed by immunoblot with antiserum directed against total Fbx4. This approach allows the simultaneous detection of both endogenous and ectopic Fbx4 on the same gel and thereby allows assessment of stoichiometry. Stoichiometric binding of endogenous Fbx4 to Myc-Fbx4 was detected during S phase (16 hr) and was inhibited by incubation of cells with a small-molecule GSK3β inhibitor, demonstrating that dimerization is induced during S phase and correlates with Ser12 phosphorylation by GSK3β (Figure 3D). To corroborate GSK-dependent regulation of Fbx4 dimerization, a plasmid encoding RasV12 or the shRNA targeting GSK3β was introduced into 293T cells along with constructs encoding Myc-Fbx4 and FLAG-Fbx4. Dimerization was analyzed by FLAG pull-down and western blotting with antisera directed against Fbx4 allowing detection of both tagged forms on a single western blot. Inhibition of GSK3β activity through overexpression of RasV12 or GSK3β knockdown resulted in an inhibition of Ser12 phosphorylation and decreased Fbx4 dimerization (Figure S4C). To determine whether Fbx4 Ser12 phosphorylation is regulated in an Akt-dependent manner in vivo, we utilized a panel of esophageal and breast cancer cells with differential Akt activity levels. In cells with high Akt activity (Figure 3E, TE8 and T47D), there was no detectable Ser12 phosphorylation. This finding was also supported by the absence of immunostaining with pSer11/12 antibody in MMTV-Neu mouse tumors, which exhibit high Akt activity (Figure 3F, tumor 51 versus 52). Consistent with increased Akt activity downregulating ligase phosphorylation (and thus activity), cyclin D1 levels were also increased in tumors with high Akt activity.

The data presented suggest that dimerization, while not essential for Fbx4-dependent ubiquitination, does potentiate this activity. If so, cyclin D1 should exhibit increased turnover during S phase. Indeed, the half-life of cyclin D1 was shorter during S phase ($t_{1/2} < 15$ min) than in G1 ($t_{1/2} > 40$ min) (Figure 3G). Finally, we assessed the ability of phosphorylation-deficient or phosphomimetic Fbx4 mutants to rescue cyclin D1 turnover in NIH 3T3 cells wherein endogenous Fbx4 is knocked down. We transfected the stable shFbx4 NIH 3T3 line with human cDNAs encoding either wild-type Fbx4 or S12A/S12E Fbx4 (stable cell lines, presented in Figure 2B), synchronized cells by serum starvation, and measured the half-life of cyclin D1 during S phase. The cyclin D1 half-life was extended to almost 40 min in Fbx4 knockdown cells (Figure 3H). In cells wherein wild-type Fbx4 was reintroduced, cyclin D1 turnover was restored ($t_{1/2} \sim 15$ min) such that it was comparable with parental cells (Figure 3H; Figure S5). Strikingly, the S12A Fbx4 mutant was not able to restore normal kinetics of cyclin D1 turnover, whereas S12E Fbx4 was proficient in restoring the rate of cyclin D1 proteolysis (Figure 3I). Consistent with dimerization being critical for Fbx4-dependent cyclin D1 turnover, the dimerization-deficient Fbx4 TRPL also failed to restore cyclin D1 turnover (Figure 3H; Figure S5). Therefore, both phosphorylation of Ser12 and dimerization of Fbx4 are required for sufficient cyclin D1 turnover in S phase cells.

Inhibition of Cyclin D1 Proteolysis Leads to Its Nuclear Accumulation and a Transformed Phenotype

Because Fbx4 is a critical regulator of cyclin D1 accumulation and thus CDK4 activity, we considered it likely that Fbx4 would exhibit tumor suppressor activities. Indeed, knockdown of either Fbx4 or αB-crystallin in mouse fibroblasts facilitated growth of cells in soft agar, a characteristic of neoplastic transformation (Figures 4A and 4B). In contrast, knockdown of Fbx4 in cyclin D1 null fibroblasts failed to induce growth in soft agar (Figure 4C), indicating that cyclin D1 is a critical target for SCF^{Fbx4}. We also assessed the capacity of inhibition of Fbx4 to cooperate with cyclin D1 in tumor growth. For this experiment, mammary tumors were harvested from MMTV-D1 mice (Lin et al., 2007) and cultured in vitro. We have found that over the course of serial cultivation, transgene expression is reduced and the tumorigenic phenotype of the cells is lost; we therefore reasoned that inhibition of cyclin D1 turnover might restore tumorigenicity. We therefore infected mammary epithelial cells with control empty vector or a vector encoding a dominant-negative *Fbx4* allele (ΔF Fbx4), which extends the half-life of cyclin D1 (Figure S6A). In this system, we were unable to achieve any significant knockdown of Fbx4 using shRNA, and we therefore focused on ΔF Fbx4 as a basis for abrogating Fbx4 function. Cells were injected in fat pads of NOD/SCID mice, and tumor growth was assessed. While only 1 of 5 MMTV-D1 mice formed tumors, 4 of 7 MMTV-D1/ΔF Fbx4 mice formed established tumors (Figure 4D).

Past work has provided evidence that accumulation of cyclin D1-dependent kinase in the nucleus during S phase disrupts temporal control of DNA replication through stabilization of Cdt1 that in turn induces DNA rereplication and genomic instability (Aggarwal et al., 2007). We therefore addressed the fate of cyclin D1 in cells that have decreased Fbx4 or αB-crystallin activity. Indeed, cyclin D1 accumulated in the nucleus in cells wherein endogenous Fbx4 or αB-crystallin was reduced by shRNA (Figures 5A and 5B). The differences in nuclear accumulation in Fbx4 versus αB-crystallin knockdown likely reflect knockdown efficiency, as our degree of Fbx4 knockdown was routinely 60% while αB-crystallin was reduced to undetectable levels (Figure 5C). As expected with the nuclear accumulation of cyclin D1, Cdt1 was



Cell line	No. of tumors per transplant	Latency (days)
MMECs.RasV12	2 of 2	12
362.Puro	1 of 5	93
362.ΔF Fbx4	4 of 7	53

Figure 4. Inhibition of SCF$^{Fbx4/\alpha B\text{-crystallin}}$ Activity Leads to Neoplastic Growth

(A) Soft agar colony formation assay was performed using pBabe-puro, shFbx4, and shαB-crystallin knockdown cell lines. Cells were plated in soft agar and grown for 21 days. Scale bar = 250 μM.

(B) Quantification of data in (A). Error bars represent ±SD.

(C) Same experiment as in (A) using wild-type (WT) and *cyclin D1*$^{-/-}$ MEFs infected with control (puro) or shFbx4 retrovirus. Scale bar = 250 μM. Levels of Fbx4 and cyclin D1 proteins are provided in the bottom panel.

(D) 10^6 cells were transplanted into cleared mammary fat pads of 3-week-old NOD/SCID mice. Mice were monitored for palpable tumor formation biweekly.

overexpressed following knockdown of either Fbx4 or αB-crystallin (Figure 5C). Thus, inhibition of cyclin D1 SCF^{Fbx4} activity leads to nuclear accumulation of cyclin D1 and cell transformation.

Fbx4 Is Mutated in Human Esophageal Carcinoma

In a majority of cancers harboring cyclin D1 overexpression defects, proteolytic machinery have been implicated as a molecular basis. We therefore assessed primary esophageal cancers, which are known to frequently overexpress cyclin D1 (Nakagawa et al., 1995; Ikeguchi et al., 2001), for mutations in *Fbx4* and *αB-crystallin* (*CryAB*). The *Fbx4* DNA sequence was assessed in 116 primary esophageal carcinomas and in matched adjacent normal tissue. We identified and verified hemizygous missense mutations in 14% of the primary tumors (Table 1). No mutations were identified in *αB-crystallin*, and *cyclin D1* was wild-type in tumors harboring inactivating *Fbx4* mutations. A majority of the mutations in *Fbx4* occur in the N-terminal regulatory domain and interfere with Fbx4 dimerization. Mutations that impair phosphorylation (S12L, P13S) and indirectly influence dimerization along with mutations that target a critical residue within the dimerization motif (L23Q) were detected. In addition, a proline→threonine mutation was identified at residue 76 within the F box (P76T); P76T impairs binding to Skp1 (Figure S6B), resulting in a dominant-negative allele. In addition to the above described mutations, numerous S8R mutations were identified. Mutations in this domain, like S12L, impair Fbx4-dependent regulation of cyclin D1 degradation (Figure S6C).

Because these mutations should reduce total SCF^{Fbx4} activity, we expected that there should be a corresponding increase in the accumulation of downstream substrates. Immunohistochemical analysis revealed overexpression of cyclin D1 and TRF1 in Fbx4 mutant tumors, consistent with a substrate-ligase relationship (Figures 6A and 6B; Table 1). Additionally, we assessed S12L and P13S Fbx4 dimerization properties. Both mutants exhibited reduced dimerization potential compared with wild-type Fbx4 (Figure S6D) and consequentially were unable to catalyze rapid cyclin D1 proteolysis (Figure S6E). We also entertained the notion that a second ligase implicated in targeting cyclin D1 for ubiquitination in cultured cells, Fbw8, might also be inactivated in esophageal caners. However, our analysis of Fbw8 expression in normal esophageal epithelium and tumor tissue revealed that Fbw8 is not expressed in either normal esophageal epithelium or associated tumors (data not shown). Given the lack of detectable expression of Fbw8 in this tissue, we conclude that an Fbw8-based E3 ligase is unlikely to contribute to cyclin D1 proteolysis.

Since all identified mutations in *Fbx4* are hemizygous, we set out to determine whether mutant *Fbx4* alleles exhibit dominant-negative activity. NIH 3T3 fibroblasts were transfected with wild-type Fbx4 with or without Fbx4 mutants. Overexpression of wild-type Fbx4 led to the reduction of total cyclin D1 protein levels (Figure S6F, compare lanes 1 and 2), while coexpression of S8R, S12L, or P13S Fbx4 (transfected in 1:1 ratio relative to wild-type Fbx4) attenuated Fbx4-dependent cyclin D1 loss (Figure S6F, lanes 3–5).

We next assessed the ability of wild-type Fbx4 or the Fbx4 S8R, S12A, and L23A mutants to restore normal growth characteristics of Fbx4 knockdown fibroblasts. shFbx4 NIH 3T3 cells were transfected with the indicated constructs (Figure 6C, lower

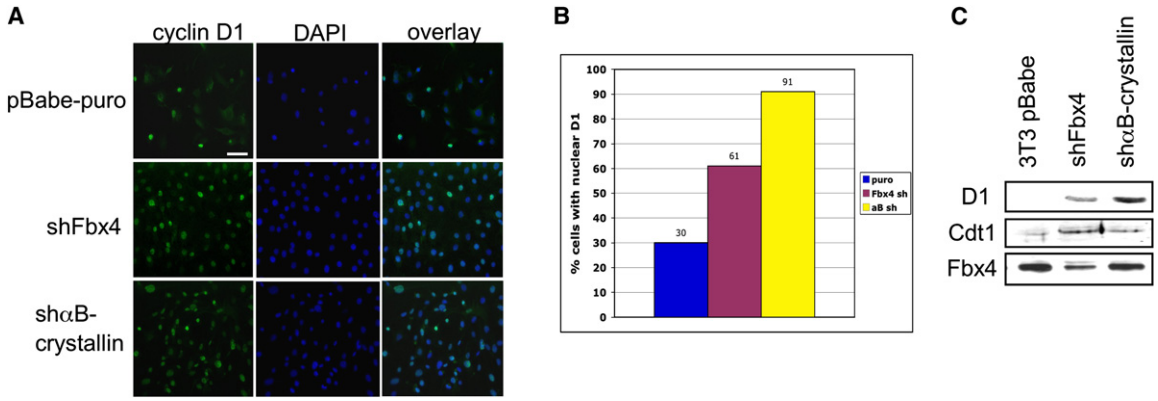


Figure 5. Disruption of SCF$^{Fbx4/\alpha B\text{-crystallin}}$ Activity Leads to Nuclear Accumulation of Cyclin D1

(A) Immunofluorescence was performed on the indicated cell lines using 13G11 cyclin D1 monoclonal antibody and counterstained with DAPI. Scale bar = 25 μM.

(B) Quantification of data in (A).

(C) Protein extracts from stable NIH 3T3 cell lines expressing shFbx4 or shαB-crystallin were analyzed by western blot using cyclin D1 and Cdt1 antibodies.

panel), and their capacity to proliferate in soft agar was assessed. Re-expression of wild-type Fbx4 significantly reduced the number of colonies, while ligase-defective Fbx4 mutants did not exhibit significant activity (Figures 6C and 6D). Altogether, we provide evidence that cancer-derived *Fbx4* mutations that target the GSK3β phosphorylation site in Fbx4 and affect its dimerization have reduced activity toward cyclin D1. The mechanism by which these mutations contribute to tumor formation in vivo is a subject of great interest for future studies.

DISCUSSION

To date, there is little evidence that SCF ligase activity is regulated. Rather, recognition of substrates by the F box component of SCF ligases generally relies on substrate phosphorylation. Recent work, however, has revealed the presence of a dimerization domain in F box proteins (Zhang and Koepp, 2006; Welcker and Clurman, 2007), thereby providing a platform for potential regulation through F box protein (and thus SCF) dimerization. The work described herein reveals the presence of a D domain within Fbx4, an F box-only subtype; the conservation of this domain suggests that dimerization is a common feature of the SCF ligase. Strikingly, our data demonstrate that dimerization of Fbx4 is regulated through growth-factor-dependent phosphorylation of Ser12 (Ser11 in the mouse). Furthermore, Ser12 phosphorylation and Fbx4 dimerization are required and contribute to an increased rate of cyclin D1 proteolysis during S phase. Surprisingly, phosphorylation of Fbx4, like cyclin D1 (Diehl et al., 1998), is catalyzed by GSK3β.

As might be expected of a negative regulator of an oncogene, *cyclin D1*, our data provide evidence that Fbx4 has biological properties consistent with a tumor suppressor. First, inactivation or a reduction in Fbx4 levels results in the cellular acquisition of a transformed phenotype in vitro. Of equal importance, analysis of human esophageal cancer, in which cyclin D1 overexpression is a common event, revealed mutations in *Fbx4* that directly abrogate Fbx4 dimerization or inhibit binding to core SCF components (Skp1). All mutations identified interfere with ubiquitin ligase activity. In aggregate, the data presented reveal a unique mechanism of growth-factor-dependent regulation of cyclin D1 and G1/S transition through phosphorylation-dependent dimerization of SCF^{Fbx4}. Our data also demonstrate direct abrogation of this pathway in human esophageal cancer and are suggestive of such potential in other cancers that are highlighted by cyclin D1 overexpression.

While SCF ligases are considered constitutively active, accumulating evidence suggests that regulation is much more sophisticated than initially appreciated. Skp2 levels are determined in part through E2F-dependent transcription (Zhang and Wang, 2006). Likewise, β-Trcp levels can be modulated by stress-activated protein kinases (Spiegelman et al., 2001). Dimerization represents another potential regulatory apex. In fact Skp2, Fbw7, and β-Trcp1/2 all dimerize (Zhang and Koepp, 2006; Suzuki et al., 2000; Welcker and Clurman, 2007; Chew et al., 2007). Our data support a role for temporally regulated Fbx4 dimerization in the regulation of substrate accumulation. Like Fbw7, Fbx4 contains a D domain adjacent to the F box. We have defined leucine/isoleucine residues (Leu23/28, Ile27) in Fbx4 that are essential for homodimerization. The spatial organization of these residues is conserved spatially with Leu246 and Val251, which contribute to the dimerization of Fbw7 (Tang et al., 2007). What distinguishes Fbx4 is the role of Ser12/Pro13, which acts as a phosphoacceptor residue for GSK3β. The generation of a Ser12 phosphospecific antibody reveals GSK3β-dependent phosphorylation of this residue. Strikingly, mutation of this residue to a nonphosphorylatable residue attenuated homodimerization and significantly attenuated cyclin D1 ubiquitination and destruction. Taken together, these data suggest that phosphorylation contributes to regulated self-association. The significance of this is highlighted by mutations of Ser12 and Pro13 in human cancer.

We noted that the peak of Fbx4 phosphorylation/dimerization occurs at the G1/S boundary, providing evidence for temporal regulation of Fbx4 dimerization. Significantly, at the G1/S boundary, cyclin D1 undergoes nuclear export, and its levels are reduced through phosphorylation-dependent proteolysis (Diehl et al., 1997); half-life measurements reveal that Fbx4 dimerization coincides with increased cyclin D1 turnover during S phase.

Table 1. Analysis of *Fbx4* Mutations in Esophageal Tumors

Tumor #	*Fbx4*	*cyclin D1*	*CryAB*	Cyclin D1 Protein Levels
1 ESCC	L23Q	WT	WT	NA
4 EAC	P13S	WT	WT	NA
7 ESCC	S12L	WT	WT	+++
8 ESCC	L23Q	WT	WT	+++
9 ESCC	WT	WT	WT	+
10 ESCC	WT	WT	WT	+++
11 ESCC	WT	WT	WT	++
12 ESCC	WT	WT	WT	NA
14 ESCC	WT	WT	WT	NA
15 ESCC	S8R	WT	WT	+++
16 ESCC	WT	WT	WT	NA
17 ESCC	WT	WT	WT	NA
18 ESCC	WT	WT	WT	+
19 ESCC	P76T	WT	WT	+++
21 EAC	WT	WT	WT	NA
22 EAC	L23Q	WT	WT	NA
23 EAC	P76T	WT	WT	NA
24 EAC	L23Q	WT	WT	NA
38 ESCC	S8R	NA	NA	NA
39 ESCC	G30N	NA	NA	NA
40 ESCC	exon 1 24 bp insert	NA	NA	NA
41 ESCC	S8R	NA	NA	NA
42 ESCC	S8R	NA	NA	NA
43 ESCC	S8R	NA	NA	NA
44 ESCC	codon 18 CA insertion	NA	NA	NA

Full-length cDNAs encoding *Fbx4*, *cyclin D1*, and *αB-crystallin* (*CryAB*) were generated by first-strand RT-PCR using oligo(dT) primers followed by PCR using specific primers. 116 total samples were sequenced in both directions. Cyclin D1 protein expression was analyzed by immunohistochemistry. ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma; WT, wild-type. NA indicates that paraffin-embedded tumor tissue was not available for immunohistochemical analysis of cyclin D1 accumulation.

Thus, through this mechanism, growth-factor availability and cell-cycle progression are coordinated via GSK3β, which in turn targets both substrate (cyclin D1) and E3 ligase (SCF^{Fbx4}).

Accumulating data suggest that oncogenic functions of cyclin D1 depend upon its nuclear accumulation during S phase (Lin et al., 2007; Gladden et al., 2006). The nuclear deregulation of cyclin D1/CDK4 activity disrupts temporal regulation of DNA replication by directly impairing proteolysis of the replication licensing factor Cdt1 (Aggarwal et al., 2007). If nuclear overexpression of cyclin D1 is in fact the oncogenic event, why does loss of components of the D1 E3 ligase promote neoplastic growth? Critically, impaired Fbx4 or αB-crystallin also promotes nuclear accumulation of cyclin D1 and consequent stabilization of Cdt1, a direct consequence of nuclear cyclin D1/CDK4.

The significance of Fbx4 activity for cell homeostasis is emphasized by the relative frequency of mutations that impair its biochemical activity in human cancer. We identified 16 mutations in 116 esophageal tumors screened. Importantly, the fact that these mutations are hemizygous implies that a reduction in Fbx4 dosage is sufficient to stabilize cyclin D1 and trigger neoplastic growth. Consistent with this interpretation, our knockdowns of Fbx4 typically achieved an ~60% reduction in Fbx4 levels, and this was sufficient to trigger neoplastic growth. Alternatively, our data reveal that coexpression of mutants with wild-type Fbx4 can attenuate Fbx4-mediated cyclin D1 turnover. This result is expected in the case of the P76T mutation, which disrupts Skp1 binding, resulting in a dominant-negative allele similar to those generated through deletion of the F box. Given that the other mutations disrupt oligomerization rather than Skp1-Cul1-Rbx1 recruitment, the dominant-negative activity may reflect titration of these ligase components into inactive complexes.

In all tumor specimens that harbored *Fbx4* mutations, cyclin D1 protein levels were elevated and accumulated in the nuclei. This observation, together with the aforementioned transformation phenotype, strongly supports the supposition that abrogation of cyclin D1 E3 ligase activity is oncogenic. It also underscores the importance of Fbx4 dimerization for ligase activity since the disruption of dimerization is observed in human esophageal cancer, in contrast to Fbw7, where all identified mutations directly impair substrate binding (O'Neil et al., 2007; Thompson et al., 2007). One can infer from the strong selection for such mutations that complete loss of Fbx4 may in fact be catastrophic for the cell.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfections

All mammalian cells were maintained in DMEM containing 10% fetal bovine serum, glutamine, and antibiotics. G0 synchronization was achieved by culture in medium containing 0.1% fetal bovine serum for 24 hr. Where indicated, cells were plated at optimal density 24 hr before transfection and were transfected the following day using Lipofectamine Plus reagent (Invitrogen). Stable cell lines expressing Fbx4 constructs were generated by G418 (Calbiochem) selection (1 mg/ml) for 21 days and subsequently cultured in medium containing 500 μg/ml G418. Insect Sf9 cells were maintained as described previously (Summers and Smith, 1987). Human Fbx4 Myc-tagged vector was generated by PCR of human Fbx4 with primers designed for directional cloning into pCS2-MT plasmid (in frame with six Myc tags at the N terminus of Fbx4).

Site-Directed Mutagenesis

FLAG-tagged Fbx4 mutants were constructed by QuikChange Site-Directed Mutagenesis Kit (Stratagene) using pcDNA3-Fbx4 plasmid as template. PCR reactions were performed following the manufacturer's instructions. Clones were sequenced in their entirety to confirm the presence of mutations.

Immunoprecipitation and Western Analysis

Cells were harvested in buffer containing 50 mM HEPES (pH 8.0), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20, protease, and phosphatase inhibitors (1 mM PMSF, 20 U/ml aprotinin, 5 mg/ml leupeptin, 1 mM DTT, 0.4 mM NaF, 10 mM β-glycerophosphate, and 100 nM okadaic acid). Protein concentration of samples was determined by BCA assay, and Fbx4 was precipitated using either M2 agarose (Sigma-Aldrich) or cyclin D1 mouse monoclonal antibody D1-72-13G. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting. Antibodies used in these studies were as follows: Fbx4 rabbit polyclonal antibody (Rockland Immunochemicals), αB-crystallin (SPA 223) rabbit polyclonal antibody (Stressgen), H-Ras (Santa Cruz), pSer473 Akt and Akt (Cell Signaling), cyclin D1 mouse monoclonal antibody D1-72-13G, cyclin D1 mouse anti-human

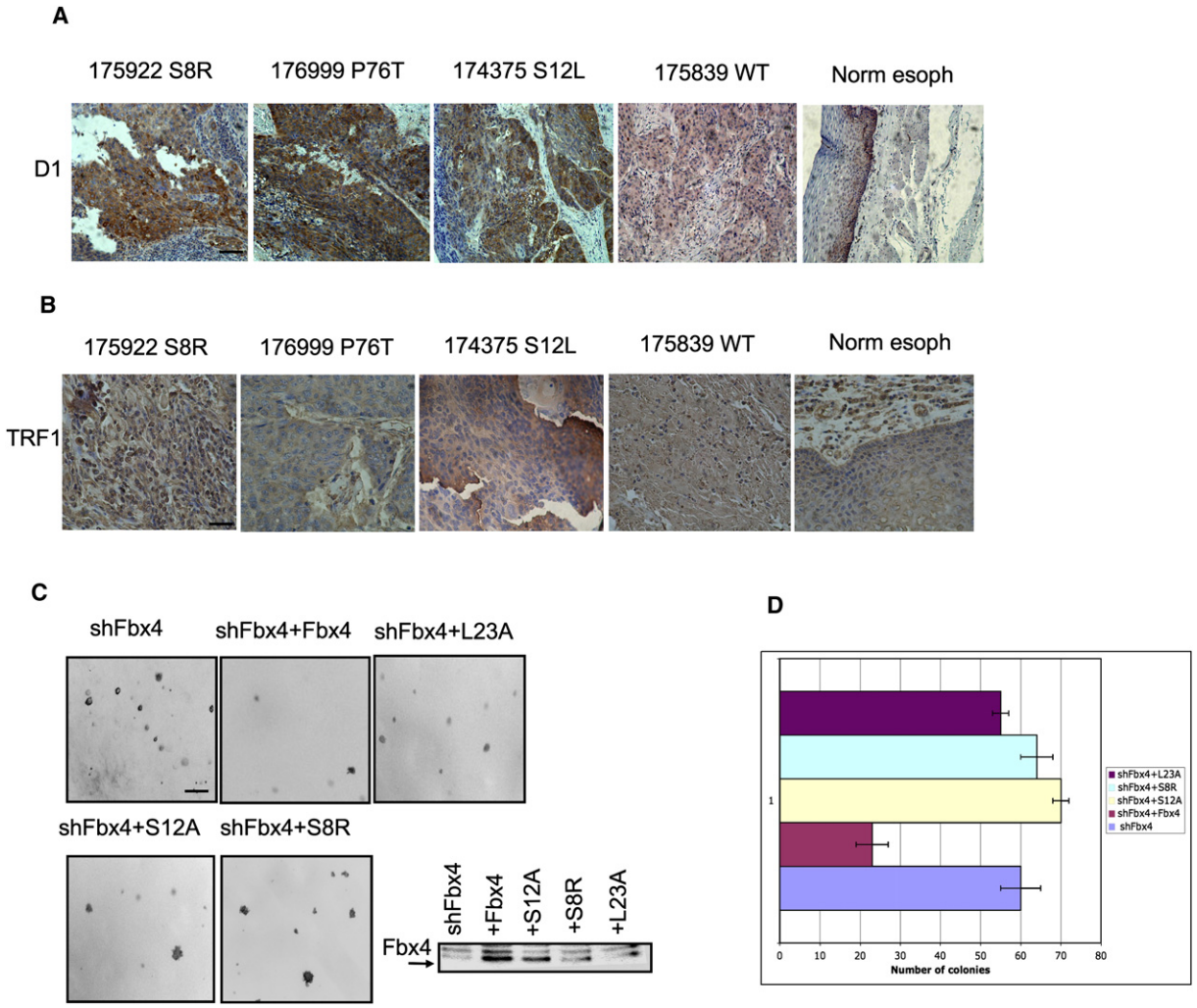


Figure 6. Analysis of *Fbx4* Mutations in Human Esophageal Tumors

(A and B) Immunohistochemical analysis of cyclin D1 or TRF1 expression in esophageal tumors: S8R Fbx4, P76T Fbx4, S12L Fbx4, wild-type (WT) Fbx4, and normal esophagus. Scale bars = 50 μM in (A) and 25 μM in (B).

(C) shFbx4 NIH 3T3 lines were transfected with the indicated Fbx4 expression vectors (immunoblot of Fbx4 levels is presented at bottom right) and assessed for growth in soft agar for 21 days. Scale bar = 250 μM.

(D) Quantification of data in (C). Error bars represent ±SD.

(AB3, Calbiochem), β-actin (Sigma-Aldrich), TRF1 rabbit polyclonal antibody (Abcam Inc.), mouse anti-Skp1 (BD Transduction Labs). Anti-phospho-Ser11 Fbx4 (mouse, corresponding to Ser12 in human) antibody was generated by immunizing rabbits with peptides containing pSer11 (murine-based peptide; YenZym Antibodies, LLC) followed by affinity purification.

Immunofluorescence

NIH 3T3-derived cell lines were plated at optimal density on glass coverslips. Twenty-four hours after splitting, cells were permeabilized with methanol:acetone (1:1), washed with PBS, and incubated in primary antibody (D1-72-13G) for 2 hr. After washing and secondary anti-mouse FITC-conjugated antibody application, slides were mounted using antifade DAPI reagent and analyzed by fluorescence microscopy using a Nikon Eclipse E800 microscope.

Soft Agar Transformation Assay

Anchorage-independent growth was determined by analyzing cellular growth in semisolid medium. Cells were seeded in Iscove's medium containing 0.65% noble agar/10% fetal calf serum. Cells were grown for 21 days in 8% CO_2.

In Vitro Kinase Assay

Purified GST-tagged Fbx4 was used as a substrate for in vitro kinase reactions (30 min at 30°C). GSK3β and MAPK p38 were purchased from Cell Signaling; GSK3α was purchased from Abcam. Cdk2 complexes were purified from Sf9 cells infected with the indicated baculoviruses.

In Vitro Ubiquitination

NIH 3T3-derived stable cell lines expressing wild-type Fbx4 and Ser12 mutants were used to purify SCF^{Fbx4} complexes. The SCF complexes were immunoprecipitated from the lysates using M2 agarose and incubated with purified GST-tagged cyclin D1 phosphorylated in vitro by recombinant GSK3β (5U), E1, E2 (UbcH5A), ATP, and ubiquitin for the indicated times at 37°C. Proteins were resolved by 10% SDS-PAGE and visualized by western blotting with anti-cyclin D1 antibody D1-72-13G11.

Analysis of *Fbx4* Mutations in Human Tumors

Fbx4, *cyclin D1*, and *αB-crystallin* PCR products from normal and tumor-derived mRNA samples were generated using human *Fbx4*-, *cyclin D1*-, and

αB-crystallin-specific primers. All mutations were confirmed via bidirectional sequencing. All esophageal cancer tissues were obtained with informed consent and with Institutional Review Board approval from the University of Pennsylvania, Kitano Hospital (Osaka, Japan), and Peking University. Patient deidentifiers were used.

SUPPLEMENTAL DATA

The Supplemental Data include six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/1/68/DC1/.

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