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Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF^{FBX4- α Bcrystallin} complex

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Summary

Growth factor-dependent accumulation of the cyclin D1 proto-oncogene is balanced by its rapid phosphorylation-dependent proteolysis. Degradation is triggered by threonine 286 phosphorylation, which promotes its ubiquitination by an unknown E3 ligase. We demonstrate that Thr286 phosphorylated cyclin D1 is recognized by a SCF ubiquitin ligase where FBX4 and α B crystallin govern substrate specificity. Overexpression of FBX4 and α B crystallin triggered cyclin D1 ubiquitination and increased cyclin D1 turnover. Impairment of SCF^{FBX4- α Bcrystallin} function attenuated cyclin D1 ubiquitination, promoting cyclin D1 overexpression and accelerated cell cycle progression. Purified SCF^{FBX4- α Bcrystallin} catalyzed polyubiquitination of cyclin D1 *in vitro*. Consistent with a putative role for a cyclin D1 E3 ligase in tumorigenesis, FBX4 and α B crystallin expression was reduced in tumor-derived cell lines and a subset of primary human cancers that overexpress cyclin D1. We conclude that SCF^{FBX4- α Bcrystallin} is an important E3 ubiquitin ligase that promotes ubiquitin-dependent degradation of Thr286 phosphorylated cyclin D1.

Keywords

cyclin D1; CDK4; FBX4; α B crystallin; E3 ubiquitin ligase

INTRODUCTION

Cyclin D1, the allosteric regulator of CDK4/6, is an integral mediator of growth factor-dependent G1 phase progression. Cyclin D1 expression, activation and nuclear accumulation occur during mid-G1 phase (Marshall, 1999); nuclear cyclin D1/CDK complexes trigger G1 progression via inactivation of the retinoblastoma protein and related family members (Sherr, 1996). During S-phase, cyclin D1 activation is opposed by Pro287-directed phosphorylation of Thr286 by glycogen synthase kinase 3 β (GSK3 β); phosphorylation triggers cyclin D1 nuclear export and ubiquitination-mediated proteolysis (Alt et al., 2000).

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While phosphorylation of Thr286 directs CRM1 binding to cyclin D1, it is also an essential signal for ubiquitin-dependent destruction (Diehl et al., 1997). Ubiquitination is catalyzed through a pathway involving the E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and an E3 (ubiquitin ligase). The latter enzyme is the most diverse and it determines substrate-specificity and the rate of ubiquitin conjugation. The E3 itself can be either a single protein or a multi-protein complex. An E3 that recognizes Thr286 phosphorylated cyclin D1 has not been identified.

Regulated proteolysis is commonly utilized to maintain threshold levels of critical cell cycle regulators. Two distinct classes of E3 ligases participate in the regulation of cell cycle progression. The Skp1-Cull1-F-box (SCF) family of E3 ubiquitin ligases promote ubiquitination of phosphorylated substrates and typically target mediators of the G1-S phase transition (Skowyra et al., 1997). While the F-box component determines substrate specificity, additional co-factors may also facilitate substrate recognition (Ganoth et al., 2001; Hao et al., 2005; Spruck et al., 2001). The G2-M transition requires the E3 ligase activity of the Anaphase Promoting Complex (APC/C) (Harper et al., 2002); CDC20 and CDH1 subunits of APC/C direct substrate recognition (Burton et al., 2005; Kraft et al., 2005; Pflieger et al., 2001).

Cyclin D1 is frequently overexpressed in human cancer; overexpression is often associated with increased gene expression due to gene amplification or oncogene induced signaling (Sherr, 1996). Accumulation of cyclin D1 in cancer can also result from disruption of cyclin proteolysis. Cyclin D1 proteolysis can be inhibited through several distinct mechanisms. First, recent work revealed that human cancer cells harbor mutations in cyclin D1 that disrupt Thr286 phosphorylation (Benzeno, 2006) and prevent ubiquitin-mediated proteolysis. Second, upstream oncogenic events, such as those targeting Wnt or Ras inhibit GSK3 β activity thereby decreasing cyclin D1 turnover (Diehl et al., 1998; Rimerman et al., 2000). Finally, it remains plausible that loss of a D1-specific E3 ligase might contribute to D1 overexpression in human cancer. The latter mechanism emphasizes the importance of identifying the cyclin D1-specific E3 ligase. Given the necessity of Thr286 phosphorylation for cyclin D1 proteolysis (Diehl et al., 1997), an unidentified SCF complex is a likely candidate cyclin D1 ubiquitin ligase.

We have identified a SCF E3 ligase, wherein the F-box protein, FBX4, targets Thr286 phosphorylated cyclin D1. FBX4 belongs to the Fbx class of F-box proteins, which lack known structural motifs in their C-terminus. Cyclin D1 recognition by FBX4 requires α B crystallin which functions in a manner analogous to CKS1 in Skp2-dependent recognition of phosphorylated p27^{Kip1}. The data presented reveal that both FBX4 and α B crystallin are required for rapid ubiquitination and degradation of cyclin D1 in vivo and that purified SCF^{FBX4- α B crystallin} complexes can direct cyclin D1 ubiquitination in vitro. Strikingly, analysis of breast cancer-derived cells revealed loss α B crystallin in a subset of cell lines. Loss of α B crystallin resulted in cyclin D1 stabilization revealing the requirement of the SCF^{FBX4- α B crystallin} ligase for cyclin D1 proteolysis.

RESULTS

Identification of α B crystallin as a major component of cyclin D1 complexes

To identify a putative cyclin D1 E3 ubiquitin ligase, we purified cyclin D1-complexes by affinity chromatography. Due to the short half-life of phosphorylated cyclin D1, cells were treated with proteasome inhibitors to enrich for phospho-T286 cyclin D1 and stabilize interactions with a putative E3 ligase. A major protein of ~22–23 kDa (identified by mass spectrometric analysis as α B crystallin) co-purified with Flag-cyclin D1, but not from parental NIH3T3 cells (Fig. 1A) or with a non-degradable cyclin D1 mutant (data not shown). We also noted a protein of ~46Kd that co-purified with cyclin D1; the identity of this protein could not be confirmed due to contaminating actin peptides. Association between endogenous cyclin D1

and α B crystallin under conditions of proteasome inhibition was confirmed by precipitation with a cyclin D1 specific antibody followed by immunoblot with α B crystallin antibodies (Fig. 1B). Binding of cyclin D1- α B crystallin depended upon the integrity Thr286 since the cyclin D1T286A mutant did not co-precipitate with α B crystallin (Fig. 1C).

Phospho-T286 dependent interaction between cyclin D1 and the SCF^{FBX4- α B crystallin} ligase

α B crystallin associates with a 46 KD protein, FBX4, *in vivo* and increases ubiquitin-conjugation of unknown proteins (den Engelsman et al., 2003). We therefore considered whether α B crystallin might facilitate targeting of FBX4 to phosphorylated cyclin D1. Indeed, endogenous cyclin D1 and α B crystallin do co-precipitate with FBX4 (Fig. 2A and Fig. S1A). The binding of cyclin D1 to FBX4 and α B crystallin was observed only under conditions of proteasome inhibition likely reflecting the stabilization labile complexes.

To determine whether Thr286 phosphorylation is required for recognition by the SCF^{FBX4- α B crystallin} complex, wild type cyclin D1 or phosphorylation deficient cyclin D1 mutants produced in Sf9 insect cells (cyclin D1 is readily phosphorylated at Thr286 in Sf9 cells (Diehl et al., 1997)) was mixed with immobilized SCF^{FBX4- α B crystallin} complexes produced in 293T cells. Wild-type cyclin D1 bound to the SCF^{FBX4- α B crystallin} complex *in vitro*; omission of α B crystallin inhibited cyclin D1 binding to FBX4 (Fig. 2B). Binding of SCF^{FBX4- α B crystallin} to two phosphorylation-deficient D1T286A and D1P287A (Benzeno, 2006) mutants was not observed (Fig. 2B). These results demonstrate that FBX4 binds to cyclin D1 in a phospho-Thr286 dependent manner and that α B crystallin increases the affinity of cyclin D1 for the SCF^{FBX4} complex.

The role of Thr286 phosphorylation in mediating recognition by FBX4- α B crystallin, SKP1 and CUL1 complexes was determined using a phospho-T286 peptide corresponding to the C-terminus of cyclin D1. FBX4- α B crystallin-SKP1-CUL1 complexes specifically associated with phospho-T286 peptides, but not to unphosphorylated peptides. Omission of either FBX4 or α B crystallin eliminated binding of SCF^{FBX4- α B crystallin} to the phospho-D1 peptides (Fig. 2C). FBX4 did not bind to other phosphodegrons (i.e. phospho-I κ B α or phospho-MYC peptides) excluding the possibility that the SCF^{FBX4- α B crystallin} complex simply has an affinity for phosphorylated Ser/Thr residues (Fig. 2D). Additionally, phosphorylated cyclin D1 peptides did not recruit other F-box proteins such as FBW2 (Fig. 2E) or SKP2 (data not shown). These results suggest that both α B crystallin and FBX4 are required for the recognition of phospho-T286 cyclin D1 by the SCF^{FBX4- α B crystallin} complex.

The SCF^{FBX4- α B crystallin} complex regulates cyclin D1 protein stability *in vivo*

To determine whether the SCF^{FBX4- α B crystallin} complex regulates cyclin D1 proteolysis, a dominant-negative FBX4 allele was created (Δ F)FBX4. F-box deletion abrogates SKP1 binding (Zheng et al., 2002), but not substrate or α B crystallin recognition (Fig. S1B; data not shown). Dominant-negative F-box mutants have been used to assess substrate relationships (Carrano et al., 1999; Latres et al., 1999; Montagnoli et al., 1999). Expression of (Δ F)FBX4 increased the half-life of endogenous cyclin D1 from <30 minutes to greater than 60 minutes (Fig. 3A). Expression of (Δ F)FBX4 also induced accumulation of cyclin D1 in an α B crystallin-dependent manner, but did not increase cyclin D1-T286A levels (Fig. 3B). The effect of α B crystallin likely reflects the capacity to promote (Δ F)FBX4 binding to phosphorylated cyclin D1 in cells.

As independent assessment of FBX4 and α B crystallin function, we utilized short-hairpin vectors to target either component. Knockdown of FBX4 or α B crystallin extended the half-life of cyclin D1 to greater than 60 min whereas the half-life remained <30 minutes in cells expressing control shRNA (Fig. 3C, 3D). Knockdown of FBX4 also stabilized cyclin D2 (data

not shown) suggesting that SCF^{FBX4- α Bcrystallin} may regulate the proteolysis of all D-type cyclins. Knockdown of FBX4 in human U2OS cells also triggered accumulation of cyclin D1 (Fig. S1C) compared to cells expressing shRNAs directed against another F-box protein, β -TrCP (Jin et al., 2003) demonstrating that FBX4 regulates cyclin D1 proteolysis in mouse and human cells. Conversely, knockdown of FBX4 did not effect cyclin E or A accumulation (Fig. 5C; S1C), which are ubiquitinated by the SCF^{FBW7} and APC/C complexes respectively (Harper et al., 2002; Koepp et al., 2001).

The ability of FBX4 and α B crystallin to drive cyclin D1 proteolysis was also investigated. 293T cells were transfected with cyclin D1, FBX4 and α B crystallin, and cyclin D1 abundance was assessed by immunoblot. Ectopic expression of FBX4 and α B crystallin decreased levels of cyclin D1 in a dose-dependent manner (Fig. 3E). Reduction of cyclin D1 was dependent on Thr286 phosphorylation (Fig. 3E) and proteasome activity (Fig. 3F). These data suggest the SCF^{FBX4- α Bcrystallin} ligase regulates cyclin D1 stability and levels *in vivo*.

The SCF^{FBX4- α B crystallin} ubiquitin ligase catalyzes cyclin D1 ubiquitination

Reduced cyclin D1 turnover in cells expressing either (Δ F) FBX4 or knockdown of FBX4 suggested that FBX4 directs cyclin D1 polyubiquitination. Consistent with this notion, (Δ F) FBX4 expression decreased cyclin D1 polyubiquitination *in vivo* and this effect was potentiated by co-expression of α B crystallin (Fig. 4A, 4B). Similar results were obtained when these reactions were analyzed by immunoblot with either a HA antibody (to detect HA-ubiquitin) or a cyclin D1 antibody (Fig. 4A, 4B) excluding the possibility that these results reflected ubiquitination of co-precipitating proteins. Reduced cyclin D1 ubiquitination was also observed in cells expressing shRNAs directed against FBX4 (Fig. 4C). Thus, impairment of SCF^{FBX4- α B crystallin} function via either (Δ F)FBX4 or FBX4 knockdown stabilizes cyclin D1 as a result of decreased cyclin D1 polyubiquitination.

In vitro transcribed and translated ³⁵S labeled cyclin D1 was mixed with purified SCF^{FBX4- α B crystallin} complexes to determine whether cyclin D1 was a direct substrate. After addition of ATP, ubiquitin, E1, E2, and purified SCF^{FBX4- α B crystallin}, we detected higher molecular cyclin D1 species consistent with cyclin D1 polyubiquitination (Fig. 4D, lane 2). In the absence of FBX4 (lane 3), Cull1/Skp1/Roc1 (lane 4), or E1/E2 (lane 5), cyclin D1 ubiquitination was eliminated. In addition, a mono-ubiquitinated cyclin D1 form was also seen in our *in vitro* ubiquitination reactions (Fig. 4D, lane 2, lower panel) but not in control reactions lacking FBX4 (Fig. 4D, lanes 1 and 3, lower panel), supporting the role of SCF^{FBX4- α B crystallin} as an E3 ligase for cyclin D1. Replacement of wild-type FBX4 with (Δ F) FBX4 impaired polyubiquitination of cyclin D1 (Fig. 4E, lane 2) demonstrating that ubiquitination depended on Cull1-Skp1-Roc1 and excluding the possibility that ubiquitination was mediated by another contaminating F-box protein. Similarly omission of α B crystallin inhibited cyclin D1 ubiquitination (Fig. 4E, lane 3) demonstrating that α B crystallin is an essential co-factor. Ubiquitinating activity was also dependent on Thr286 phosphorylation of cyclin D1 (Fig. 4F). Similar results were obtained using SCF^{FBX4- α B crystallin} produced in Sf9 cells (data not shown). These results provide direct biochemical evidence that cyclin D1 is a *bona fide* substrate for SCF^{FBX4- α B crystallin}.

Cytoplasmic localization of FBX4 and α B crystallin

Cyclin D1 ubiquitination and proteolysis are postulated cytoplasmic events (Diehl et al., 1998), suggesting the involvement of a cytoplasmic E3 ligase. Immunofluorescence staining, utilizing two distinct FBX4 antibodies, revealed cytoplasmic FBX4 in both NIH3T3 and U2OS cells (Fig. 5A). Knockdown of FBX4 dramatically reduced staining with either antibody confirming antibody specificity (Fig. 5B, 5C). The cytoplasmic localization was confirmed by biochemical fractionation (Fig. 5D). While our results reveal that FBX4 and α B crystallin are

largely cytoplasmic, they do not exclude the possibility that a small fraction of FBX4 and α B crystallin transiently shuttles between nuclear and cytoplasmic compartments (den Engelsman et al., 2004).

Cell cycle dependent binding of FBX4 to cyclin D1

Following re-entry from a G₀, cyclin D1 induction is limited by growth factor-dependent gene expression (Matsushime et al., 1991). At the G₁/S-phase transition, cyclin D1 is phosphorylated at Thr286 by GSK3 β , exported from the nucleus and degraded in the cytoplasm (Diehl et al., 1998). We hypothesized that following G₀ release FBX4 would not limit cyclin D1 accumulation until the G₁/S phase transition when GSK3 β phosphorylates cyclin D1 inducing its cytoplasmic re-distribution. To test this hypothesis, we assessed the cell cycle dependence of cyclin D1-FBX4 binding following G₀ release. NIH3T3 fibroblasts were cultured for 24 hrs in medium containing 0.1% FBS and released in medium containing 10% FBS. Lysates were prepared from NIH3T3 cells synchronized at G₀ (0hrs), G₁ (8hrs), and S (18hrs) phases of the cell cycle. Four hours prior to harvesting, cells were treated with MG132 to enrich for cyclin D1-FBX4 complexes. Immunoblot of FBX4 precipitates revealed enrichment of cyclin D1-FBX4 complexes during S-phase following cell cycle reentry (Fig. 6A). To assess the dependence of cyclin D1-FBX4 interaction on GSK3 β , we transfected NIH3T3 cells with dominant negative GSK3 β or empty vector and assessed binding. Expression of this GSK3 β mutant dramatically reduced cyclin D1-FBX4 binding (Fig. 6B) as well as cyclin D1 phosphorylation (data not shown).

Knockdown of the SCF^{FBX4- α B crystallin} ligase accelerates G1 phase progression

Because overexpression of cyclin D1 correlates with increased rates of cell cycle progression (Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994), we expected that compromised cyclin D1 proteolysis would accelerate G₁ transition. However, because G₀-G₁-S-phase movement is largely dependent upon cyclin D1 gene expression, we reasoned that reentry rates would be refractory to FBX4/ α B crystallin levels. To test this, cell lines wherein either α B crystallin or FBX4 are knocked down were synchronized by serum starvation. Cells were released in complete media containing BrdU, and S-phase entry was assessed by FACS. Knockdown of α B crystallin or FBX4 did not accelerate the G₀ to S-phase transition (Fig. 6C). Similarly, expression of (Δ F)FBX4 did not accelerate S phase entry following a serum starvation (G₀) block (data not shown).

We next assessed whether loss of FBX4 or α B crystallin would accelerate progression from G₂ to S-phase. Cells were arrested at the G₂/M transition by nocodazole treatment and released once again in medium containing BrdU. Whereas control NIH3T3 cell lines entered S-phase between 9–10 hours post-nocodazole (G₂/M) release, knockdown of α B crystallin or FBX4 reproducibly accelerated G₁-phase traverse resulting in S-phase entry 4–6 hours post-nocodazole release (Fig. 6D and Supplemental Table 1). Cyclin D1 levels remained elevated in knockdown cells relative to controls following nocodazole release (Fig. 6E). Consistent with D1 as the primary target, knockdown of FBX4 or α B crystallin did not accelerate cell cycle progression in D1^{-/-} fibroblasts (Fig. 6F, G) or D1-T286A expressing 3T3 cells (data not shown).

FBX4 and α B crystallin levels are altered in human cancers

Overexpression of cyclin D1 in various human malignancies suggests that impairment of SCF^{FBX4- α B crystallin} function might contribute to cyclin D1 overexpression in cancer. Indeed, the α B crystallin locus maps to chromosome 11q22.3-q23.1, a region deleted in human cancer (Bax et al., 2001; Dohner et al., 1999). To quantify FBX4 and α B crystallin levels in human malignancies, we assessed expression in a matched tumor vs. normal mRNA array (materials and methods). α B crystallin mRNA levels were low in 40% of tumors examined as compared

to corresponding normal controls (Fig. 7A); in contrast, FBX4 was down regulated in 24% of tumors (Fig. 7A). mRNA levels of FBX4 and α B crystallin mRNA were down regulated in a broad spectrum of tumors including prostate, thyroid and breast adenocarcinomas and lymphoma. The reduced frequency of FBX4 loss may reflect a necessity for FBX4-mediated degradation of additional substrates (Lee et al., 2006).

We also assessed protein levels of FBX4, α B crystallin and cyclin D1 by immunohistochemical analysis of primary esophageal tumors (n=36), a cancer in which cyclin D1 is frequently deregulated (Sherr, 1996). In normal esophageal tissue, accumulation of cyclin D1 was mutually exclusive with FBX4 and α B crystallin (Fig. S2A, S2B); cyclin D1 was highest in proliferative basal cells. In tumors, α B crystallin and FBX4 levels were negative or very weak in 64% and 52% of adenocarcinomas and in 54% and 64% squamous cell carcinomas respectively (Fig. S2A, S2B). A subset of these tumors (24%, 6/25 adeno, 54%, 6/11 squamous cell carcinomas) exhibited negative or low α B crystallin or FBX4 protein levels with concomitant cyclin D1 overexpression (Fig. S2A, arrows and S2B).

In addition, screening of 10 tumor-derived cell lines revealed loss of α B crystallin in breast cancer derived, MCF7, MDA-MB-231, and BT474 cells (Fig. 7B). α B crystallin loss in MCF7 cells results from loss of chromosome 11 (www.path.cam.ac.uk/~pawefish/BreastCellLineDescriptions/mcf7.htm). The absence of α B crystallin correlated with decreased cyclin D1 proteolysis and increased D1 levels relative to control breast epithelial cells (Fig. 7B, 7C and S3). Reintroduction of α B crystallin into MCF7 cells (Fig. S1D) restored the rapid kinetics of cyclin D1 proteolysis (Fig. 7C) suggesting that stabilization of cyclin D1 in these cells resulted from loss of α B crystallin. Thus, impairment of SCF^{FBX4- α B crystallin} function is a potential mechanism underlying cyclin D1 overexpression in human cancer.

DISCUSSION

Cyclin D1 is a substrate for the SCF^{FBX4- α B crystallin} complex

Herein, we describe experiments revealing the following: 1) α B crystallin and FBX4 are components of phospho-T286 cyclin D1 containing protein complexes; 2) α B crystallin is necessary for recognition of phosphorylated cyclin D1 by the SCF^{FBX4} ubiquitin ligase; 3) the SCF^{FBX4- α B crystallin} complex ubiquitinates phosphorylated cyclin D1 and targets it for proteasomal degradation; 4) the α B crystallin accessory component is lost in multiple breast cancer cell lines resulting in cyclin D1 stabilization and overexpression. This data supports the notion that reduced cyclin D1 proteolysis likely contributes to cyclin D1 overexpression in certain malignancies.

Several lines of evidence suggest that cyclin D1 is a true substrate for the SCF^{FBX4- α B crystallin} ubiquitin ligase. First, cyclin D1, FBX4 and α B crystallin are present in endogenous complexes in vivo. Second, the interaction between cyclin D1 and the SCF^{FBX4- α B crystallin} complex is dependent on cyclin D1 phosphorylation (a characteristic of SCF-substrate interactions). Third, overexpression of FBX4 and α B crystallin triggers the destruction of wild-type cyclin D1, but not of the phosphorylation-deficient cyclin D1 mutant, D1-T286A. Fourth, cyclin D1 is stabilized in vivo by the expression of either a dominant-negative FBX4 or by shRNAs against FBX4 and α B crystallin mRNAs. Finally, purified SCF^{FBX4- α B crystallin} complexes catalyze ubiquitination of cyclin D1 in vitro providing direct biochemical evidence that cyclin D1 is a *bona fide* substrate for the SCF^{FBX4- α B crystallin} complex.

The SCF^{SKP2} ligase has also been implicated in the ubiquitination of cyclin D1 (Ganiatsas et al., 2001; Yu et al., 1998). Knockdown of SKP2 promoted accumulation of cyclin D1 (Yu et

al., 1998). However, evidence for direct ubiquitination by the SCF^{SKP2} is absent. Indeed a physical association between SKP2 and either cyclin D1 or phospho-T286 cyclin D1, has not been reported. The stabilization of cyclin D1 observed in cells expressing antisense oligos against SKP2 likely reflects stabilization of other substrates such as p21^{Cip1} or p27^{Kip1} (Bornstein et al., 2003; Carrano et al., 1999), both of which can decrease cyclin D1 proteolysis (Alt et al., 2002; Cheng et al., 1999; Coleman et al., 2003).

Possible roles of α B crystallin within the SCF^{FBX4}- α B crystallin complex

Our data suggests that α B crystallin acts as a specificity determinant that facilitates FBX4 recognition of Thr286 phosphorylated cyclin D1. It is possible that it is the chaperone-like activity of α B crystallin SCF^{FBX4}- α Bcrystallin provides this function. However, there is no data to suggest that the chaperoning function of α B crystallin depends upon substrate phosphorylation. Thus, we favor an explanation wherein targeting of phosphorylated cyclin D1 to SCF^{FBX4}- α B crystallin represents a distinct function for α B crystallin. Three distinct mechanisms for α B crystallin function in this process can be envisioned. In the first, α B crystallin might directly bind to phospho-Thr286 of cyclin D1 and bridge D1 to SCF^{FBX4}. Such a mechanism is analogous to the function provided by CKS1 in the SCF^{SKP2}-CKS1 complex where CKS1 binds to Thr-187 phosphorylated p27^{Kip1} (Bashir et al., 2004; Bornstein et al., 2003; Ganoth et al., 2001; Hao et al., 2005). In an alternative model, the phospho-acceptor site within SCF^{FBX4}- α B crystallin is cooperatively recognized by both FBX4 and α B crystallin. Finally, it remains formally possible that α B crystallin may not participate in phospho-acceptor recognition site. Rather, α B crystallin might function as an allosteric regulator of FBX4 by inducing a conformational change of FBX4 thereby increasing the affinity of FBX4 for phosphorylated cyclin D1.

Spatial control of cyclin D1 ubiquitination

Previous work suggested that phosphorylation-dependent ubiquitination of cyclin D1 occurs in the cytoplasm (Diehl et al., 1998). Indeed our analysis revealed that both FBX4 and α B crystallin are exclusively cytoplasmic. Based on the steady state cytoplasmic localization of FBX4 and the fact that, following a G0 release, cyclin D1 binds to FBX4 during S-phase (a cell cycle phase wherein cyclin D1 is cytoplasmic (Diehl et al., 1998)), we suggest FBX4-dependent ubiquitination of cyclin D1 is a cytoplasmic event. Although our data suggest FBX4 is the major E3 ligase, it remains possible that alternative cytoplasmic adaptor mechanisms exist that target cyclin D1 for destruction. Consistent with this, cyclin D1T286A exhibits reduced turnover relative to wild-type cyclin D1 in α B crystallin-deficient MCF7 cells (data not shown).

In contrast to cytoplasmic ubiquitination machinery, there may also exist a nuclear, phosphorylation-independent cyclin D1 destruction pathway that is not mediated by the SCF^{FBX4}- α B crystallin complex. Evidence for this pathway comes from the discovery of cyclin D1b, an alternative splice variant of canonical cyclin D1 (Betticher et al., 1995; Howe and Lynas, 2001; Lu et al., 2003; Solomon et al., 2003). Cyclin D1b lacks the last exon of canonical cyclin D1, and therefore Thr286. Since cyclin D1b cannot be phosphorylated at Thr286, it cannot be exported to the cytoplasm. Although the half-life of cyclin D1b is longer than canonical cyclin D1, cyclin D1b degradation remains proteasome-dependent (data not shown) suggesting the existence of phosphorylation-independent machinery that earmarks nuclear cyclin D1b for destruction. Phosphorylation-independent destruction of cyclin D1 has been proposed to occur through the activity of a protein called antizyme (Newman et al., 2004).

Other SCF^{FBX4} substrates

FBX4 also targets the telomeric DNA repeat binding protein, Pin2/Trf1, for degradation (Lee et al., 2006) potentially contributing to telomere maintenance. In contrast to D1, recognition

does not depend upon α B crystallin. At first, it seems somewhat counterintuitive that impairment of FBX4 function could promote both a proliferative signal, via cyclin D1 stabilization, and a growth inhibitory one by inducing telomere shortening via Pin2/Trf1 stabilization. However, the dominant FBX4 downstream effector substrate *in vivo* may depend on cellular context. For instance, a cancer cell may have acquired the expression of telomerase or additional factors to evade checkpoint responses induced by the effects of Pin2/Trf1 stabilization. In addition, stabilization of one FBX4 substrate may compensate for the effect of stabilizing another. Cyclin D1, for example, can induce the maintenance of telomere length, via a telomerase independent mechanism, (Opitz et al., 2001). Additional work should focus on establishing substrate hierarchy.

Role of FBX4 and α B crystallin in cancer and cell cycle progression

Cyclin D1 is overexpressed in a variety of human tumors that do not exhibit cyclin D1 gene amplification or structural abnormalities of the D1 locus, which suggests that increased cyclin D1 stability is a potential mechanism whereby cyclin D1 overexpression occurs in human cancer. Indeed, mutations of cyclin D1 at Thr286 and Pro-287 have been found in human tumors (Benzeno, 2006), thereby rendering cyclin D1 more stable. Impairment of SCF^{FBX4- α B crystallin} function may also account for cyclin D1 overexpression in a subset of human tumors. In fact, the human α B crystallin locus is located on chromosome 11q22.3-q23.1, a region frequently deleted in human cancer (Dohner et al., 1999).

In a preliminary screen of several human cancers, we have observed that both FBX4 and α B crystallin are down regulated at the protein and mRNA levels, suggesting that SCF^{FBX4- α B crystallin} function might be impaired in human cancer. Our results do not exclude the possibility that variations in FBX4 and α B crystallin levels in the tumors examined is secondary to differences in tumor proliferative fractions. However, re-introduction of α B crystallin in MCF7 cells, a breast cancer cell line that lacks α B crystallin, restored the rate of cyclin D1 proteolysis to that of wild type. Future studies will by necessity address whether mutations in FBX4 or α B crystallin that specifically abolish SCF^{FBX4- α B crystallin} binding to cyclin D1 occur in human cancer in the absence of significant alteration in cyclin D1 mRNA levels. Future studies with higher power will also address whether loss of FBX4 and α B crystallin are mutually exclusive or coordinate events and its relation to cyclin D1 overexpression. Our results suggest that impairment of SCF^{FBX4- α B crystallin} function may contribute to cyclin D1 overexpression in human cancer via decreased ubiquitin-mediated cyclin D1 degradation.

While the function of the SCF^{FBX4- α B crystallin} complex in cancer remains speculative, our data reveal a role of this E3 ubiquitin ligase as regulator of cell cycle progression. Knockdown of either FBX4 or α B crystallin reproducibly accelerated G1 phase progression from a G2/M (nocodazole) block, but not from a G0 (serum starvation) block, suggesting that decreased cyclin D1 proteolysis increases the rate of cell cycle progression. The failure of FBX4 and α B crystallin knockdown to accelerate the G0 to S phase transition likely reflects the fact that the G0 to S transition heavily relies on the induction of cyclin D1 transcription (Matsushime et al., 1991). Indeed our results are consistent with the observation that α B crystallin^{-/-} lens epithelial cells exhibit hyperproliferation and genomic instability (Bai et al., 2003). Future efforts are needed to establish the potential tumor suppressive action of both FBX4 and α B crystallin in relation to cyclin D1 as a substrate.

METHODS

Cell Culture Conditions and Transfections

NIH3T3, 293T and U2OS cells were maintained in DMEM medium containing glutamine, antibiotics and 10% FBS (Gemini). NIH3T3 cell derivatives engineered to overexpress Flag-FBX4 and Flag-(Δ F) FBX4 were generated by transfection using LipofectAMINE Plus (Invitrogen) with pcDNA3 vectors encoding the appropriate cDNA. Transfected cell lines were selected and maintained in 400 μ g/mL G418. Stable NIH3T3 cell lines expressing cyclin D1 have been described previously (Alt et al., 2000). Insect Sf9 cells were grown have been described previously (Summers, 1987). Transient expression of vectors encoding respective cDNAs was achieved by using LipofectAMINE Plus according to the instructions from the manufacturer. Metabolic labeling and protein half-life measurements were performed as previously described (Diehl and Sherr, 1997; Rimerman et al., 2000).

Gene silencing

pSM2c shRNA vectors targeting mouse FBX4 and mouse α B crystallin were purchased from Open Biosystems CSHL library; (Mouse α B crystallin target sequence: TATTAGCTTAATAATCTGGGCC); (Mouse FBX4 target sequence: ATTCATCTTGCCATTCATGACGT). NIH3T3 cells were infected with retroviruses encoding α B crystallin or FBX4 shRNAs; pools and clones of infected cells were selected with puromycin. Knockdown of human FBX4 was achieved by transfection with shRNA vectors (Paddison et al., 2002; Paddison and Hannon, 2002) containing either of the following target sequences: GCCGGTACAGTGTGATTCCACAGATTCAA; GGCATTGAGTGGATTCTTGAAGAAGTGGGA.

Construction of FBX4 mutants

pcDNA3 Flag-tagged FBX4 was a gift from Michelle Pagano. Flag-(Δ F)FBX4 mutant was generated using the following primers by mutagenesis using the Quick Change Mutagenesis kit (Stratagene); Forward: 5-**GAGGAGGTGGATGAGAGGGATCTTCCTTCTTGG**-3; Reverse: 5-**CCAAGAAGGAAGATCCCTCTCATCCACCTCCTC**-3.

Immunoprecipitation and immunoblotting

NIH3T3 cells were treated with 10 μ M MG132 or 50 μ M LLnL for 4 hrs. Cells were lysed 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 (1mM PMSF, 20 U/ml aprotinin, 5 mg/ml leupeptin, 1 mM DTT, 0.4 mM NaF, 10 mM β -Glycerophosphate, 100 nM Okadaic Acid). Cyclin D1 was precipitated with the mouse monoclonal antibody D1-72-13G. Alternatively, FBX4 was precipitated with a rabbit polyclonal antibody generated by immunizing rabbits with FBX4 derived peptides. Immune complexes were captured with protein A Sepharose, and resolved by SDS-PAGE. The following antibodies were used for westerns: cyclin D1 (72-13G), α B crystallin (SPA 223) (Stressgen), HA (12CA5), SKP1 (Transduction Labs), β -actin (AC15) (Sigma). FBX4 antiserum was generated by immunizing rabbits with peptides generated based on the N or C-terminus of FBX4 (Rockland Immunochemicals Inc, PA).

In vitro peptide pull down

293T cells transfected as indicated were lysed in Tween 20 buffer plus 1mM PMSF, 20 U/ml aprotinin, 5 mg/ml leupeptin, 1 mM DTT, 0.4 mM NaF, 10 mM β -Glycerophosphate, 100 nM Okadaic Acid. Whole cell extracts were incubated with 100 μ g of peptides biotinylated peptides (synthesized by the Baylor College of Medicine Protein Chemistry Core) and bound proteins were captured on avidin agarose beads. Beads were boiled in SDS sample buffer prior to separation by SDS-PAGE.

Mass Spectrophotometry

Cyclin D1 complexes were separated by SDS-PAGE and visualized with Coomassie Brilliant Blue staining. Excised bands were destained with 50% acetonitrile (v/v)-25mM NH_4HCO_3 , dehydrated with 100% acetonitrile, dried under vacuum and incubated with bovine trypsin (1 $\mu\text{g}/\text{ml}$, Sigma) overnight at 37°C. Peptides were extracted with 50% acetonitrile and 5% trifluoroacetic acid, dried under vacuum and reconstituted in 50% acetonitrile and 0.1% trifluoroacetic acid. Peptide solutions were mixed with an equal volume of α -Cyano-4-hydroxycinnamic acid matrix and spotted onto MALDI plates. Peptides were calibrated with Sequazyme peptide mass standards kit (PE Biosystem) and analyzed by MALDI-TOF mass spectrometry (Voyager DE Pro, Applied Biosystems). Identification of proteins was performed using MS-Fit software (<http://prospector.ucsf.edu/ucshtml4.0/msfit.htm>).

In vivo ubiquitination

Cell expressing HA-ubiquitin were lysed in buffer containing 50 mM Tri-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% deoxycholate, 1 mM PMSF, 20 U/ml of aprotinin, 5 mg/ml of leupeptin, 1 mM DTT, 0.4 mM NaF, 5 mM NEM and 20 μM MG132. Cyclin D1 or Flag-D1 derivatives were precipitated with either the cyclin D1 antibody or with the M2 monoclonal antibody. Proteins were separated by SDS-PAGE, and ubiquitinated intermediates were detected by immunoblot.

In vitro ubiquitination

293T cells were transfected with vectors encoding Flag-FBX4, αB -crystallin, Cul1, Skp1 and ROC1. The SCF complexes were purified from the lysates of these cells using M2 agarose (Sigma) and incubated with in vitro translated ^{35}S -labeled cyclin D1 and unlabeled CDK4 in the presence of recombinant GSK3 β (5U), E1, E2 (UbcH5A), ATP and ubiquitin for 10 min at 30°C followed by incubation for another 50 min at 37°C. Proteins were resolved on 10% SDS-PAGE and visualized by autoradiography.

Subcellular fractionation

Cells were collected by centrifugation at 300g for 4 min at 4°C and suspended in 400 μL buffer A {10 mM HEPES (pH 7.5), 10 mM KCl, 1 mM DTT, 1 mM PMSF, 20 U/ml aprotinin, 5 mg/ml leupeptin, 0.4 mM NaF, 10 mM β -Glycerophosphate}. After incubation on ice for 15 min, 12.5 μL of 10% NP-40 was added and incubated on ice for 10 min. Nuclei were pelleted at 1500g for 5 min, and the supernatant (cytoplasmic extracts) was centrifuged at 13,000g. Nuclei were washed with 1 mL of buffer A twice, and suspended in 50 μL of buffer B {20 mM HEPES (pH 7.5), 0.4 M NaCl, 1 mM DTT, 1 mM PMSF, 20 U/ml aprotinin, 5 mg/ml leupeptin, 0.4 mM NaF, 10 mM β -Glycerophosphate}. Nuclear extract was collected by centrifugation at 18,000g for 5 min.

Immunofluorescence

Cells were fixed in 70% methanol, 30% acetone for 15 min at -20°C, blocked for 1hr in 4% BSA/PBS followed by incubation in primary antibody (6702, 1:100; affinity purified 7099, 1:2) for 1 hr at room temperature. Cells were washed in PBS and incubated in secondary FITC conjugated antibody (1:200) for 30 min at room temperature. Cells were washed with PBS and counterstained with Hoechst (1:500).

Tumor vs. normal mRNA array

mRNA array representing the matched malignant/normal tissues was purchased from Biochain Institute, Inc (Hayward, CA). Hybridizations with cDNA probes specific for FBX4 and αB crystallin performed according to instructions. mRNA levels were normalized to β -actin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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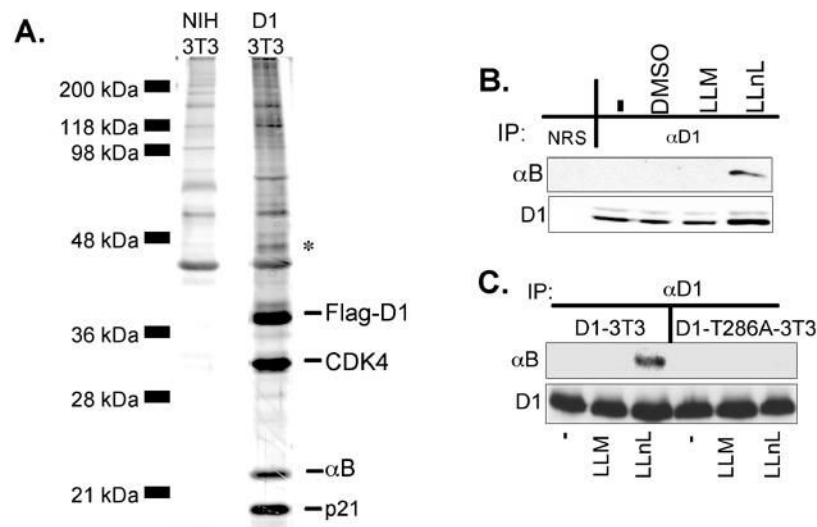


Fig. 1. Identification of α B crystallin as a cyclin D1-associated protein

(A) Silver stain of affinity-purified cyclin D1 complexes prepared from parental NIH3T3 cells or Flag-D1 3T3 cell lines treated with the proteasome inhibitor, LLnL (leucyl-leucyl-norleucinal). Cyclin D1-interacting proteins were identified by mass spectrometry. (B) Western blot of endogenous cyclin D1 precipitates prepared from proliferating NIH3T3 cells treated with DMSO, LLM (N-acetyl-leuciny-leuciny-methioninal) or LLnL. Normal rabbit serum (NRS) precipitates served as a negative control. (C) The cyclin D1- α B crystallin interaction is dependent on cyclin D1 phosphorylation at Thr286. NIH3T3 cells expressing either wild-type cyclin D1 or the D1T286A mutant were treated with vehicle, LLM or LLnL for 6hrs and cell lysates prepared from these cells were precipitated with a cyclin D1 specific antibody (13-17G) and probed for cyclin D1 and α B crystallin.

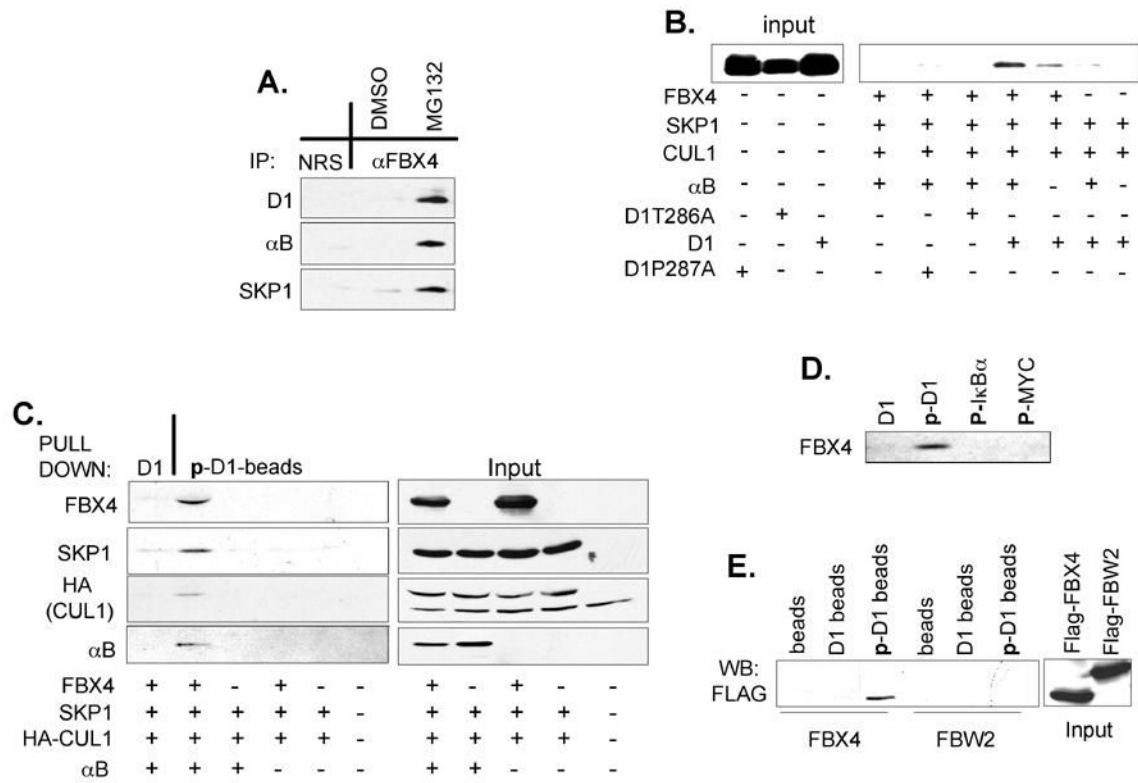


Fig. 2. Phosphorylation-dependent interaction between cyclin D1 and the SCF^{FBX4- α B} crystallin complex

(A) Endogenous FBX4, cyclin D1 and α B crystallin associate in vivo. Cell lysates prepared from proliferating NIH3T3 cells treated with DMSO or MG132 were precipitated with antibodies directed against FBX4 or normal rabbit serum (NRS) and subjected to immunoblot with the indicated antibodies. (B) Phospho-T286-dependent binding of cyclin D1 to SCF^{FBX4- α B} crystallin in vitro. Cyclin D1 or phosphorylation-deficient derivatives, D1T286A and D1P287A, were mixed with SCF^{FBX4- α B} crystallin complexes and purified by immunoaffinity chromatography from 293T cells; where indicated, FBX4 and α B crystallin were omitted. (C) Cyclin D1-FBX4 binding is dependent on α B crystallin and Thr286 phosphorylation. 293T lysates containing the indicated proteins were incubated with beads coupled to the phosphorylated cyclin D1 peptide or to unphosphorylated cyclin D1 peptide. Bound proteins were detected by immunoblot. (D) Phospho-MYC and -Ik β do not interact with SCF^{FBX4- α B} crystallin. Beads coupled to the indicated peptides were incubated with 293T lysates expressing SCF^{FBX4- α B} crystallin complexes as in (C) and bound FBX4 was detected by immunoblot. (E) Beads coupled to the indicated peptides were incubated with 293T lysates harboring either SCF^{FBX4- α B} crystallin or SCF^{FBW2} complexes and bound FBX4 or FBW2 were detected by anti-FLAG western blot. Uncoupled beads served as negative (background) control.

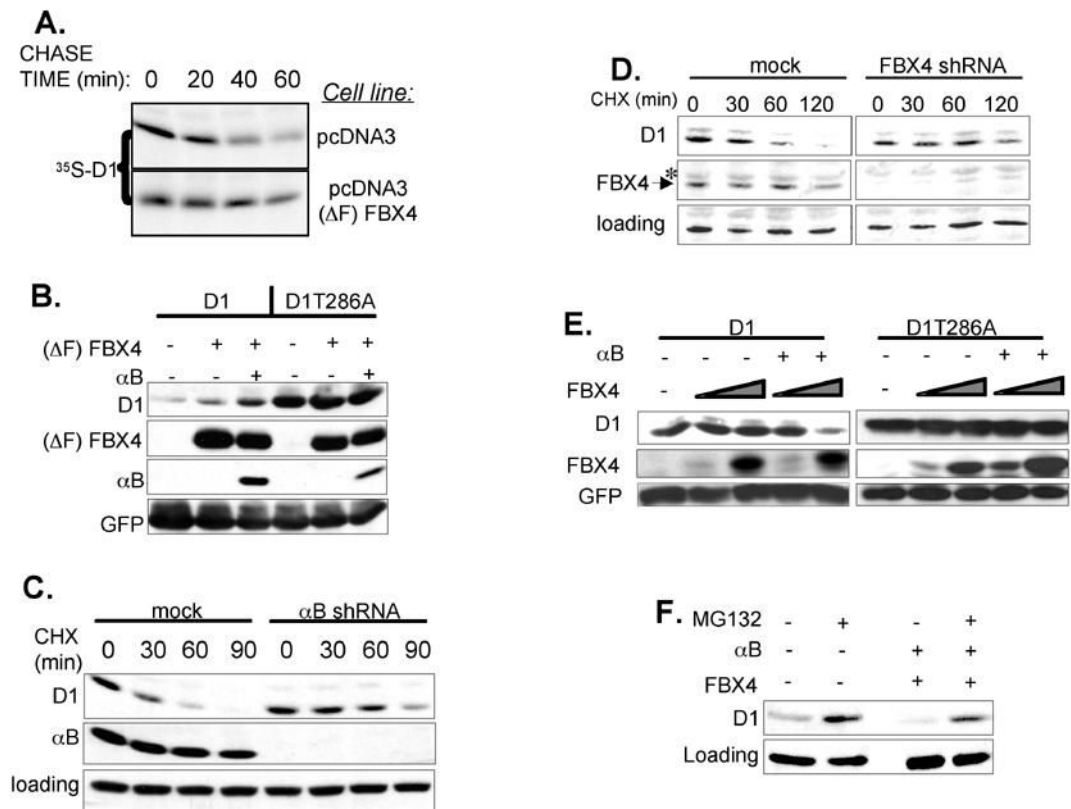


Fig. 3. The SCF^{FBX4-αB} crystallin ubiquitin ligase regulates cyclin D1 stability and protein levels *in vivo*.

(A) NIH3T3 cells expressing empty vector or (ΔF)FBX4 were pulse-labeled with ³⁵S methionine/cysteine and “chased” for the indicated time periods. Cyclin D1 was precipitated and visualized by autoradiography. (B) Western analysis of whole cell extracts prepared from 293T cells transfected with cyclin D1, CDK4, (ΔF) FBX4 and αB crystallin. (C) (D) NIH3T3 cells expressing either control, αB crystallin (C) or FBX4 shRNAs (D) were treated with 100 μg/mL of cycloheximide for the indicated time periods, and cyclin D1 half-life was determined by western blotting. (E) Expression of FBX4 and αB crystallin promotes cyclin D1 proteolysis. 293T cells were transfected with cyclin D1, CDK4, αB crystallin, FBX4 and GFP. Cell lysates were prepared and immunoblotted for cyclin D1, FBX4 and GFP. (F) Proteasome inhibition inhibits FBX4-dependent cyclin D1 proteolysis.

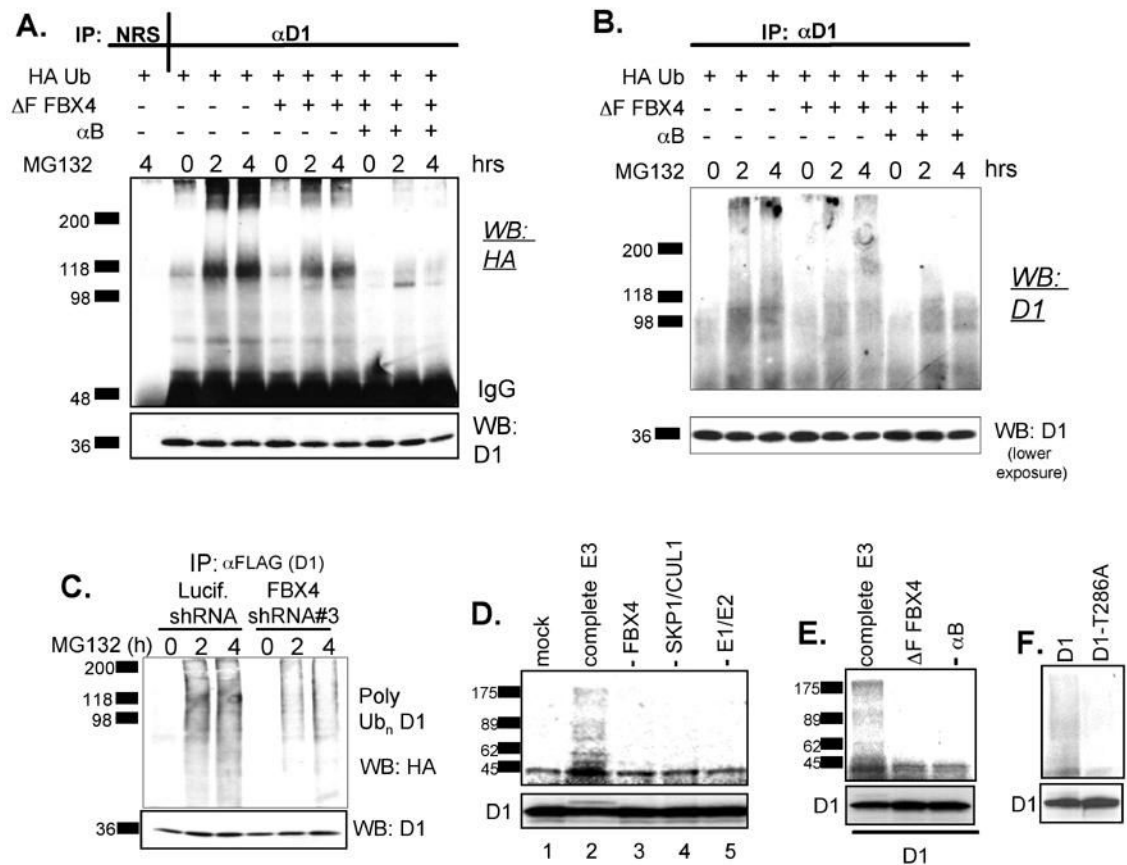


Fig. 4. The SCF^{FBX4- α B crystallin} complex catalyzes cyclin D1 ubiquitination *in vivo* and *in vitro*. (A) NIH3T3 cells expressing Flag-cyclin D1 were transfected with HA-ubiquitin, (Δ F) FBX4 and α B crystallin and treated with 20 μ M MG132 for 0, 2 and 4 hours. Cell lysates were subjected to precipitation with either normal rabbit serum (NRS) or with a cyclin D1 specific antibody and ubiquitinated proteins were visualized by anti-HA immunoblot. (B) Same as in (A) except that cyclin D1 immunoprecipitates were probed with a cyclin D1 antibody. (C) Human U2OS cells were co-transfected with HA-tagged ubiquitin, cyclin D1 and shRNAs specific for firefly luciferase or FBX4. Cyclin D1 ubiquitination was detected by immunoprecipitation with an anti-flag antibody followed by anti-HA western blotting. (D) The SCF^{FBX4- α B crystallin} complex catalyzes ubiquitination of cyclin D1 *in vitro*. Ubiquitination of *in vitro* transcribed and translated cyclin D1 was assessed by mixing cyclin D1 with GSK3 β ATP, ubiquitin, E1, E2, and a complete SCF^{FBX4- α B crystallin} complex (lane 2). The reactions were also carried out omitting the following: the entire E3 ligase (lane 1), FBX4 (lane 3), Cul1, Skp1, Roc 1 (lane 4) or E1, E2 (lane 5). (E) The F-box of FBX4 and α B crystallin are required for cyclin D1 polyubiquitination *in vitro*. Ubiquitination reactions were initiated by addition of an ATP, ubiquitin, E1 and E2 enzymes. Where indicated wild-type FBX4 was replaced with (Δ F)FBX4 (lane 2), α B crystallin was omitted from the reaction (lane 3). (F) Phosphorylation deficient cyclin D1-T286A is refractory to SCF^{FBX4- α B crystallin}-dependent ubiquitination. Cyclin D1 ubiquitination D-F was assessed by autoradiography (upper panels) and input is shown (lower panels).

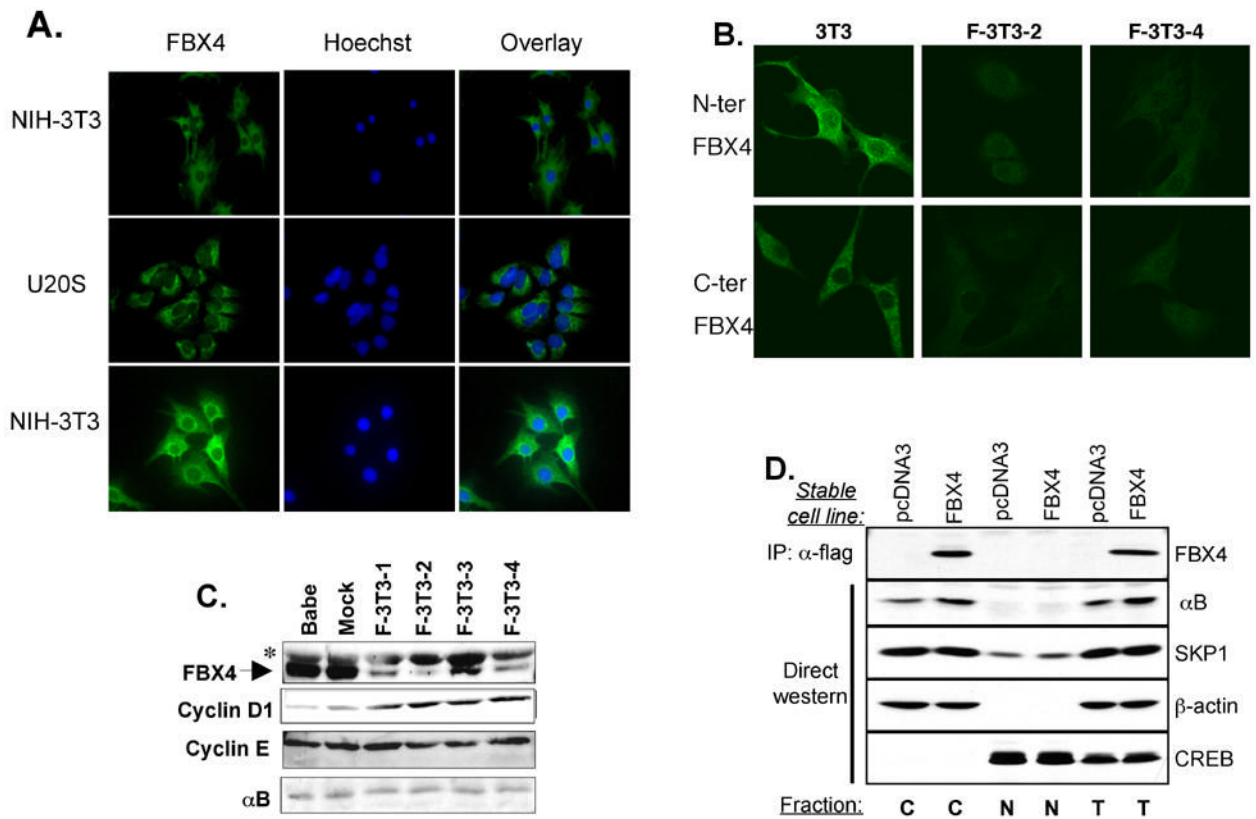


Fig. 5. Cytoplasmic localization of FBX4 and α B crystallin

(A) Immunofluorescence of asynchronously growing NIH3T3 and U2OS cells with N-terminus (top) or C-terminus (middle and bottom panels) FBX4-specific antibodies. (B) Immunofluorescence of asynchronously growing mouse fibroblasts stably expressing FBX4-specific hairpins (F-3T3-2, F-3T3-4). (C) Western analysis confirming knockdown of FBX4 in NIH3T3 cells. Asterisk indicates a nonspecific band detected by the secondary antibody. (D) Stable NIH3T3 cells expressing empty vector or Flag-tagged FBX4 were fractionated into cytoplasmic (C), nuclear (N) and total (T) extracts. Fractions were used for immunoprecipitation with an anti-flag antibody or for direct western blots as indicated.

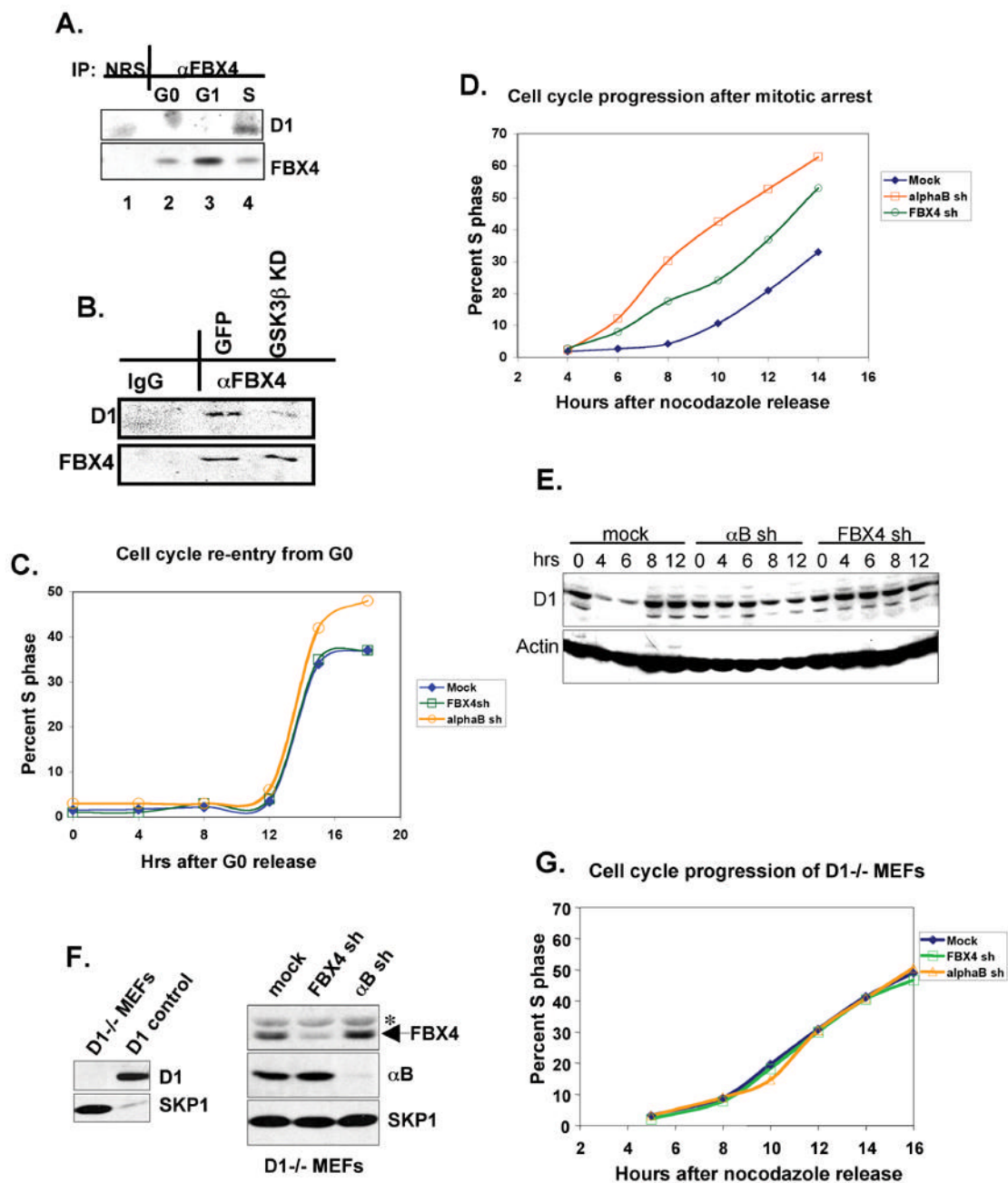


Fig. 6. The SCF^{FBX4-αB} crystallin ubiquitin ligase regulates cell cycle progression
 (A) Cell cycle dependent binding between FBX4 and cyclin D1. NIH3T3 cell were released from a G0 (serum starvation) block and binding between cyclin D1 and FBX4 was assessed by IP-western. (B) Lysates prepared from NIH-3T3 cells transfected with GFP or GSK3β kinase-dead (KD) constructs and treated with MG132 were precipitated with FBX4 antiserum or rabbit anti-mouse IgG and subjected to immunoblot with indicated antibodies. (C) Kinetics of cell cycle re-entry from G0 in NIH3T3 cells shRNAs against FBX4 or αB crystallin (D) NIH3T3 cells expressing shRNAs against FBX4/αB crystallin were released from a nocodazole (G2/M) block in media containing BrDU and percentage of cells in S phase assessed by FACS. (E) Immunoblot (cyclin D1) of NIH3T3 cells expressing shRNAs against FBX4/αB crystallin

released from a nocodazole (G2/M) block for the indicated time. (F) Knockdown of FBX4/ α B crystallin in D1^{-/-} fibroblasts (right panel); control blot of D1 and Skp1 (left panel). (G) D1^{-/-} cells expressing shRNAs against FBX4/ α B crystallin were released from a nocodazole and S-phase entry was assessed as in D.

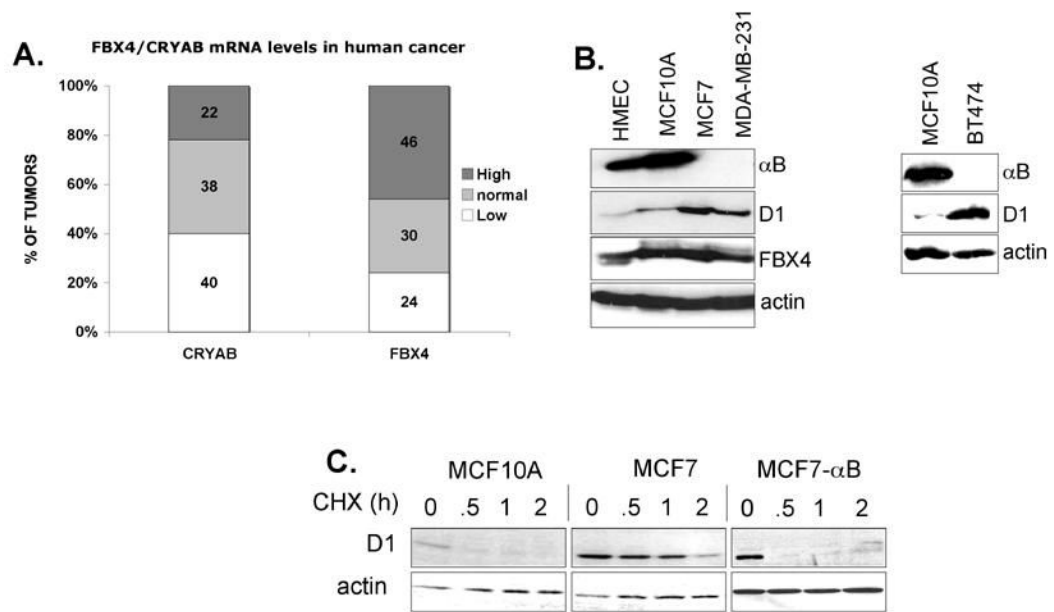


Fig. 7. FBX4 and α B crystallin levels are altered in human cancers

(A) Quantification of FBX4 and α B crystallin mRNA levels in a tumor vs. matching normal tissue in an mRNA array. (B) Expression of α B crystallin, cyclin D1 and FBX4 in normal human mammary epithelial (HMEC), non-tumorigenic breast MCF10A cells and tumorigenic breast cancer cell lines (MCF7, MDA-MB-231, BT474). (C) MCF10A, MCF7 or MCF7 cells infected with a retrovirus encoding α B crystallin were treated with cycloheximide for the indicated intervals and levels of cyclin D1, α B crystallin and actin were assessed by immunoblot.