Phosphorylation-dependent regulation of SCF^Fbx4 dimerization and activity involves a novel component, 14-3-3ε

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Abstract

Fbx4 is an F-box constituent of SCF ubiquitin ligases that directs ubiquitylation of cyclin D1. Ubiquitylation of cyclin D1 requires phosphorylation of both cyclin D1 and Fbx4 by GSK3β. GSK3β-mediated phosphorylation of Fbx4 Ser12 during the G1/S transition regulates Fbx4 dimerization, which in turn governs Fbx4-driven E3 ligase activity. In esophageal carcinomas that overexpress cyclin D1, Fbx4 is subject to inactivating mutations that specifically disrupt dimerization, highlighting the biological significance of this regulatory mechanism. In an effort to elucidate mechanisms that regulate dimerization, we sought to identify proteins that differentially bind to wild type Fbx4 versus a cancer-derived dimerization deficient mutant. We provide evidence that phosphorylation of Ser-12 generates a docking site for 14-3-3ε. 14-3-3ε binds to endogenous Fbx4 and this association is impaired by mutations that target either Ser-8 or Ser-12 in Fbx4, suggesting that this N-terminal motif in Fbx4 directs its interaction with 14-3-3ε. Knockdown of 14-3-3ε inhibited Fbx4 dimerization, reduced SCF^Fbx4 E3 ligase activity, and stabilized cyclin D1. Collectively, the current results suggest a model wherein 14-3-3 binds to Ser-12 phosphorylated Fbx4 to mediate dimerization and function.

Introduction

D-type cyclins contribute to the G1/S phase transition through their capacity to allosterically activate either cyclin dependent kinase 4 (CDK4) or CDK6. Overexpression of cyclin D1 is observed in a wide spectrum of cancers including lymphoma, melanomas, esophageal, head and neck, colon, and breast cancers. Extensive research has established that dysregulation of cyclin D1 in human cancer is often post-translationally mediated and is a driving oncogenic process (Diehl, 2002). While mutations in cyclin D1 are rare, recent work has demonstrated that ubiquitylation of cyclin D1 is frequently defective in transformed cells, resulting in aberrant nuclear accumulation of cyclin D1 protein (Barbash *et al.*, 2009; Barbash *et al.*, 2008; Benzeno *et al.*, 2006). Nuclear accumulation of cyclin D1, particularly in S phase, is

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now appreciated to directly contribute to genomic instability and drive transformation (Aggarwal *et al.*, 2007; Aggarwal *et al.*). Therefore, further investigations into the mechanisms of cyclin D1 protein turnover is essential to a deeper understanding of cyclin D1-driven tumorigenesis.

Skp-cullin-F-box (SCF) ubiquitin ligases are multi-subunit complexes that direct specific ubiquitylation of distinct substrates (Skaar and Pagano, 2009). Substrate specificity is dictated by the F-box protein subunit of the complex. Fbx4 is the F-box protein that directs SCF-mediated ubiquitylation of phosphorylated cyclin D1 (Diehl *et al.*, 1997; Lin *et al.*, 2006). While cyclin D1 phosphorylation regulates cyclin D1 turnover, increasing evidence indicates that SCF complexes are subject to post-translational regulation as well. For example, many F-box proteins dimerize and thereby contribute to SCF function (Chew *et al.*, 2007; Suzuki *et al.*, 2000; Welcker and Clurman, 2007; Zhang and Koepp, 2006). Structural and biochemical analyses have revealed that Fbx4 dimerizes and site-directed mutagenesis has identified key residues within conserved dimerization domains (DDs) that regulate self-association (Barbash *et al.*, 2008; Li and Hao, 2010). Significantly, Fbx4 dimerization stimulates its E3 activity; the N-terminal DD is required both *in vitro* and *in vivo* for Fbx4-mediated cyclin D1 degradation. Dimerization, and thus activity, of Fbx4 relies on phosphorylation of Ser12 (p-Ser12). Intriguingly, GSK3β, the kinase responsible for D1 phosphorylation, also mediates Fbx4 Ser-12 phosphorylation, thus placing GSK3β at a critical juncture for cyclin D1 degradation. The importance of this regulatory mechanism is underscored by the observation that mutations in critical dimerization and phosphorylation motifs of Fbx4 are found in human cancer and are accompanied by cyclin D1 accumulation (Barbash *et al.*, 2008). Of note, while many cancer-derived mutations target residues that directly mediate dimerization or phosphorylation, some mutations fall outside these regions, yet still inhibit dimerization highlighting the need for further mechanistic insights.

14-3-3 phosphoserine/threonine binding proteins play important roles in the regulation of signal transduction in a wide variety of cellular processes, which include cell cycle checkpoints and the DNA damage response. 14-3-3 consensus-binding sequences have been identified through comprehensive analysis of their client proteins, which revealed two modes of consensus phospho-serine/threonine sites, RSXpSXP or RXF/YXpSXP (Yaffe *et al.*, 1997). Approximately half of 14-3-3 interacting proteins carry these consensus sequences, while others utilize less conventional motifs. Binding to 14-3-3 usually results in altered activity of the binding protein, regulation of other protein associations, or subcellular localization.

Here we report 14-3-3ε as a novel Fbx4 binding partner. Our data demonstrate that 14-3-3ε facilitates Fbx4 dimerization and that this interaction requires Ser8, which is frequently mutated in human cancer, and p-Ser12. Consistent with a cell cycle regulated function, Fbx4 binding to 14-3-3ε is dependent on p-Ser12 and restricted to the G1/S and S phase, where phosphorylation of Fbx4 and Fbx4 activity is maximal. We found that expression of 14-3-3ε positively regulates Fbx4 dimerization *in vivo* and that 14-3-3ε contributes to cyclin D1 ubiquitylation and clearance. Our results provide important mechanistic insights with regard to of the regulation of cyclin D1 degradation and introduce a potential paradigm wherein 14-3-3 regulatory proteins facilitate the activation of specific SCF E3 ligases.

Results

Restoration of Fbx4 in human cancers suppresses anchorage-independent growth characteristics

Because previous work revealed that Fbx4-depletion in NIH3T3 cells promotes anchorage independent growth and this phenotype can be reversed by reconstitution with shRNA-resistant Fbx4, we determined whether anchorage-independent growth of select human esophageal cancer cell lines with altered Fbx4 activity could be attenuated by reconstitution with wild type Fbx4. TE8 cells (Fbx4 activity is diminished due to GSK3β inhibition by high Akt expression) and TE15 cells (wild type Fbx4) show a marked decrease in anchorage-independent growth upon Fbx4 expression, suggesting that increased Fbx4 function can overcome low endogenous activity and even, enhance normal activity (Figure 1a,b). However, the growth of TE10 cells (which express the heterozygous dimerization-defective S8R mutation) in soft agar (Figure 1b) and on plastic (Figure 1c) was largely refractory to expression of exogenous wild type Fbx4. This result is consistent with our previous finding that the S8R mutation exerts dominant negative characteristics when co-expressed with wild type Fbx4 (Barbash *et al.*, 2008).

Since the S8R mutation impairs Fbx4-mediated ubiquitin ligase activity and Fbx4 directs the ubiquitylation of cyclin D1, we hypothesized that cells harboring this mutation should exhibit an increased cyclin D1 half-life. Indeed, the half-life of cyclin D1 from S8R-expressing TE10 cells was prolonged when compared to wild type Fbx4 expressing TE15 cells (Figure 1d). Recent work revealed the importance of temporal degradation of cyclin D1 in preventing DNA re-replication and highlighting the notion that even subtle alterations in cyclin D1 stability can impact genomic fidelity (Aggarwal *et al.*, 2007; Aggarwal *et al.*). Furthermore, expression of exogenous wild type as well as phospho-mimetic S12E Fbx4 decreased steady-state cyclin D1 levels in TE8 cells with low endogenous Fbx4 activity (Figure 1e). As a negative control, expression of Fbx4 in TE7 cells (in which cyclin D1 is not efficiently exported to the cytoplasm (Benzeno *et al.*, 2006) did not affect cyclin D1 levels. Taken together, these data suggest that expression of Fbx4 exerts a tumor suppressive effect in human cancer cells through regulation of cyclin D1.

Serine 8 and 12 of Fbx4 contribute to 14-3-3ε binding

To explore the defect in Fbx4 function associated with the S8R mutation, we utilized mass spectrometry to identify proteins that bound differentially to wild type versus Fbx4S8R. 14-3-3ε was enriched in wild type Fbx4 purified complexes versus S8R Fbx4 (Figure 2a). Analysis of the sequence surrounding S8 of human Fbx4 revealed a potential 14-3-3 consensus binding motif, suggesting a role for S8 and p-S12 in the association of Fbx4 with 14-3-3ε (Figure 2b). The decreased binding of 14-3-3ε to Fbx4S8R and S12A mutants compared to wild type supported the requirement of both Ser-8 and phosphorylated Ser-12 for Fbx4/14-3-3ε association in vivo (Figure 2c,d). Furthermore, substitution of S12 with a phospho-mimetic glutamate residue significantly increased 14-3-3ε association (Figure 2e). Consistent with a direct interaction, recombinant GST-tagged 14-3-3ε pulled down Fbx4 produced in Sf9 cells; in contrast, binding of Fbx4S8R and Fbx4S12A to recombinant 14-3-3 was attenuated (Figure 2f). Mutant Fbx4S8R exhibited reduced GST-14-3-3ε binding

and the 14-3-3ε K49E mutant decreased Fbx4 association, indicating that Fbx4 is a typical 14-3-3 client protein (Figure 2g).

Fbx4/14-3-3ε binding is restricted to G1/S and S-phase

Previous work revealed that phosphorylation of Fbx4 at S12 by GSK3β regulates Fbx4 dimerization and activity (Barbash *et al.*, 2008). Since the same serine residue that governs Fbx4 activity also contributes to 14-3-3ε binding, we entertained the possibility that the 14-3-3ε interaction correlates with Fbx4 phosphorylation-dependent dimerization. To determine whether 14-3-3ε association is cell-cycle regulated, we harvested cells synchronized in G0/G1, G1/S, and S phase and assessed 14-3-3ε recruitment. Strikingly, 14-3-3ε binding was restricted to the G1/S and S phases (Figure 3a,b). Notably, 14-3-3ε was detected in Fbx4 complexes isolated with antibodies directed against phospho-S12. Since consensus 14-3-3 binding is mediated by phospho-serine (or phospho-threonine), we determined whether phosphorylation of S12 by GSK3β was required for 14-3-3 recruitment. Indeed, treatment of 3T3 cells with a pharmacologic GSK3β inhibitor reduced phosphorylation of S12 and significantly, decreased 14-3-3ε co-immunoprecipitation with Fbx4 (Figure 3c). We also verified the role of GSK3β-dependent phosphorylation of Ser-12 by introducing a dominant-negative kinase dead (kd) GSK3β mutant in 3T3 cells (Diehl *et al.*, 1998). The 14-3-3ε interaction with Fbx4 was disrupted in a kd-GSK3β dose-dependent manner (Figure 3d). Taken together, these results suggest a model wherein Fbx4 is first phosphorylated at S12 by GSK3β predominantly at the G1/S transition and subsequently binds 14-3-3ε in a phospho-S12 dependent manner.

Fbx4 mutants deficient for 14-3-3ε binding retain cytoplasmic localization

One major activity of 14-3-3 is the regulation of nuclear-cytoplasmic distribution of client proteins. To address whether 14-3-3ε directs the cytoplasmic distribution of Fbx4, we investigated subcellular distribution of 14-3-3ε binding-deficient Fbx4 mutants. NIH3T3 cells transfected with Fbx4, Fbx4S8R or Fbx4S12A were visualized processed for immunofluorescence microscopy (Figure 4a) or subjected to nuclear fractionation (Figure 4b). Our results demonstrate that ectopically expressed Fbx4 mutants that are 14-3-3ε binding-deficient remain cytoplasmic. These results were confirmed with the endogenous Fbx4 mutant S8R in TE10 cells, which exhibited no detectable nuclear accumulation by immunofluorescence (Figure 4c).

14-3-3ε regulates Fbx4 dimerization and E3 ligase activity

Based on the phospho-S12 dependent 14-3-3ε binding combined with the previously established role of S12 phosphorylation on Fbx4 dimerization, we surmised that 14-3-3ε acts as a regulatory factor, facilitating Fbx4 dimerization. We addressed this hypothesis by depleting 14-3-3ε in cells transfected with wild type myc-Fbx4 and wild type Flag-Fbx4. Knockdown of 14-3-3ε reduced co-precipitation of myc-Fbx4 with flag-Fbx4 by 40% (Figure 5a,b). Conversly, overexpression of 14-3-3ε increased the dimerization of Fbx4 (Figure 5c). Taken together, we conclude that the dimerization of Fbx4 is dependent not only on phospho-S12 but also the recruitment of 14-3-3ε.

Because Fbx4 dimerization is required for SCF ubiquitin ligase activity, we investigated the effect of 14-3-3ε on cyclin D1 ubiquitylation. Indeed, in vitro ubiquitylation of cyclin D1 by Fbx4-directed SCF complexes was enhanced upon addition of 14-3-3ε; the presence of increasing amounts of 14-3-3ε likely resulted in protein aggregation, thereby compromising functional ligase (Figure 5d). Conversely, Fbx4S8R exhibited decreased ubiquitylating capacity and the presence of 14-3-3ε actually decreased activity raising the possibility that 14-3-3 proteins may provide more than a single function.

To translate our earlier observation that Fbx4 is a typical 14-3-3ε client protein into a functional assay, SCF^{Fbx4} complexes purified from SF9 cells were incubated with either recombinant wild-type, K49E mutant, or no 14-3-3ε and *in vitro* ubiquitylation of cyclin D1 was assessed. SCF^{Fbx4} incubated with wild type 14-3-3ε enhanced poly-ubiquitin linked cyclin D1 compared to SCF^{Fbx4} alone or SCF^{Fbx4} incubated with K49E 14-3-3ε mutants (Figure 5e). The requirement of K49 demonstrates that 14-3-3ε enhancement of Fbx4 ubiquitylating function occurs through the conserved amphipathic groove utilized in binding a wide variety of ligands (Liu *et al.*, 1995). We also assessed *in vivo* ubiquitylation in NIH3T3 cells transfected with either control vector or a 14-3-3ε short hairpin vector. We observed a similar trend in which down-regulation of 14-3-3ε resulted in attenuation of poly-ubiquitylated cyclin D1 (Figure 5f). Consistent with this result, cells depleted of 14-3-3ε exhibited significantly prolonged cyclin D1 half-life as well as steady-state levels (Figure 5g). In summary, we have shown that human cancer-derived Fbx4 mutations of S8 and S12 exhibit impaired 14-3-3ε recognition and that the presence of 14-3-3ε is essential for maximal Fbx4 activity. These findings provide mechanistic insights into activation the SCF^{Fbx4} ligase and contribute to our understanding of the proteolytic regulation of cyclin D1.

Discussion

Recent work examining the regulation of several F-box proteins, including Fbx4, βTRCP, and Fbw7, have suggested that dimerization is a key regulatory mechanism and a requisite for full ubiquitylating function (Barbash *et al.*, 2008; Chew *et al.*, 2007; Suzuki *et al.*, 2000; Welcker and Clurman, 2007; Zhang and Koepp, 2006). However, there remains scant molecular insight into the regulation of dimerization. By analysis of binding partners for wild type Fbx4 versus a cancer derived, dimerization-deficient Fbx4 mutant, we identified 14-3-3ε. Our work demonstrates that this 14-3-3 enhances Fbx4 dimerization and the activity of the Fbx4 towards a biologically relevant downstream substrate, cyclin D1. Association of Fbx4 with 14-3-3ε, in turn, is dependent upon phosphorylation of Fbx4 on S12 by GSK3β, providing evidence for the existence of multiple regulatory mechanisms that determine E3 ligase activity of the SCF^{Fbx4} E3 ligase. These multiple levels of regulation, combined with the observation that Fbx4 is frequently mutated in cyclin D1 overexpressing human cancers, suggest that precise temporal control of Fbx4 is a crucial tumor suppressive mechanism. Indeed, previous work has shown that 60% depletion of Fbx4 is sufficient to promote cyclin D1-dependent transformation in colony assays (Barbash *et al.*, 2008). Taken together with the observation that dimerization is essential for Fbx4 activity, our current finding that ~40% knockdown of 14-3-3ε results in a ~40% decrease in Fbx4 dimerization highlights the importance of 14-3-3ε in Fbx4 tumor suppressor function.

14-3-3ε is one of seven 14-3-3 family members all of which are phosphoserine/threonine binding proteins. Two phosphoserine/threonine consensus binding motifs within target proteins, RSXpSXP and RXF/YXpSXP (where pS represents phosphoserine/threonine), predict 14-3-3 binding (Yaffe *et al.*, 1997). The Fbx4 N-terminus contains the sequence, RSGTNSPP, which resembles the former. While many 14-3-3 binding proteins contain the exact motifs, roughly half do not; thus, non-traditional binding sequences, which Fbx4 contains, account for a significant fraction of 14-3-3 interactions. The specificity of our interaction is underscored by the marked reduction in 14-3-3ε binding upon either Fbx4S8R or S12A mutation, indicating the association requires precise interactions that utilize several residues within the 14-3-3ε binding motif. Moreover, charge reversing mutation of 14-3-3ε lysine 49, which sits in the substrate binding pocket and directly complements phosphoserine/threonines on targets, to arginine abolished association with Fbx4, providing evidence that Fbx4 interacts with the established amphipathic groove on 14-3-3ε. Thus, Fbx4 appears to be a traditional 14-3-3ε binding partner.

14-3-3 proteins can regulate client protein interactions with other proteins, alter the intrinsic catalytic activity of its clients, or govern subcellular localization of its client proteins (Thorson *et al.*, 1998; Tzivion *et al.*, 1998; Tzivion *et al.*, 2001; van Heusden, 2009; van Heusden and Steensma, 2006; Waterman *et al.*, 1998). We found that 14-3-3ε enhances SCF^{Fbx4} ubiquitylation of cyclin D1 both *in vitro* and *in vivo* and that this enhancing effect is likely mediated through Fbx4 dimer formation. This finding raises the question of whether 14-3-3ε binding only increases Fbx4 auto-affinity or also possesses an intrinsic pro-ubiquitylation effect on Fbx4. That is, 14-3-3ε binding may induce a conformational change in Fbx4 that facilitates its activity; the activating potential of 14-3-3 has been described for other proteins, including Raf-1 and the tryptophan and tyrosine hydroxylases (Dhillon *et al.*, 2009; Freed *et al.*, 1994; Ichimura *et al.*, 1988; Wang *et al.*, 2009). We also cannot rule out the possibility that 14-3-3ε promotes Fbx4-substrate recruitment independent of its effect on dimerization and serves as an intermolecular bridge as reported in other cases (Tzivion *et al.*, 2001; Zhang *et al.*, 1997). We do not expect this to be the case however, as Fbx4-substrate interactions are confined to the C-terminal GTPase-like domain and 14-3-3ε binding occurs at the extreme N-terminus (Zeng *et al.*, 2010).

In addition, although we demonstrate that Fbx4 directly interacts with 14-3-3ε, it is conceivable that this interaction enhances the ability of Fbx4 to associate with other factors. We considered the possibility that 14-3-3ε facilitates skp1 binding and thereby serves as a SCF recruitment mechanism. However, we do not expect this to be the case, since mutation of S12A, which implies a relative lack of associated 14-3-3ε, does not impair skp1 binding (Barbash *et al.*, 2008).

Finally, a third major function of 14-3-3 proteins is governing subcellular localization. One such example of this occurs within the SCF ligase family; recent investigations indicate that phosphorylated skp2 is relocalized from the nucleus to the cytoplasm in a 14-3-3β dependent manner (Lin *et al.*, 2009). Our past studies indicate that Fbx4 is exclusively cytoplasmic and our results show that impairment of 14-3-3ε binding does not significantly affect this distribution (Figure 4). However, this does not preclude the possibility that 14-3-3s may recruit Fbx4 to subcellular cytoplasmic compartments. In aggregate, these observations

support the idea that 14-3-3ε regulates Fbx4 activity through promoting its self-association, which is critical for ligase activity.

We have previously shown that mutations in the dimerization domain affecting the 14-3-3ε binding motif of Fbx4 occur in esophageal cancer and these included the phosphorylation site S12 and P13. The S8R mutation, which we show in this study to be 14-3-3ε binding deficient, was detected at a higher frequency. Recently, S8R mutations were also detected at a similar rate in a subset of breast cancers and these mutations correlate with cyclin D1 overexpression, suggesting that this defect spans multiple cyclin D1-driven malignancies (Korcheva *et al.*, 2010). Whether there is aberrant 14-3-3ε function in human cancer which impacts the Fbx4/cyclin D1 axis remains to be explored. Loss of 14-3-3ε itself has been reported to track with regions of chromosomal deletion in small cell lung cancer (Konishi *et al.*, 2002). Decreased expression of 14-3-3ε has been proposed to contribute to the initiation and progression of laryngeal carcinoma, which often exhibits cyclin D1 overexpression (Che *et al.*, 2010; Pignataro *et al.*, 2005). Future unbiased, genome-wide association studies will be useful to determine whether perturbations along 14-3-3ε/Fbx4/cyclin D1 pathway contribute to cyclin D1 driven cancers.

Materials and Methods

Cell culture, plasmids and transfections

NIH3T3, HeLa, 293T and esophageal carcinoma cell lines were maintained in DMEM medium containing 10% fetal bovine serum, glutamine and antibiotics. Where indicated, 24 hours before transfection, cells were plated and the following day transfected using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Insect Sf9 cells were maintained as described previously (Alt *et al.*, 2000). Cells were synchronized by contact inhibition and 24 hrs culture in media supplemented with 0.1% FBS. Following replating in complete medium, cells were harvested at the indicated intervals. WT, S8R and S12A Fbx4 expression vectors were previously described (Barbash *et al.*, 2008). Rat GST-14-3-3ε WT and K49E bacterial expression vectors were obtained through Addgene. For mammalian expression, 14-3-3ε cDNA was subcloned into pcDNA3 vector.

Immunoprecipitation and immunoblot analysis

Cells were lysed in buffer containing following components: 50 mM HEPES (pH 8.0), 150mM NaCl, 2.5mM EGTA, 1mM EDTA, 0.1% Tween 20, protease, and phosphatase inhibitors (1mM PMSF, 20U/ml aprotinin, 5mg/ml leupeptin, 1mM DTT, 0.4mM NaF, 10mM β-glycerophosphate, and 100nM okadaic acid), protein concentration of samples was determined by BCA assay (Pierce, Rockford, IL), and Fbx4 was precipitated using either M2 agarose (Sigma-Aldrich, St. Louis, MO) or a Fbx4 rabbit polyclonal antibody. 14-3-3ε was precipitated using rabbit polyclonal antibody (Abcam, Cambridge, MA). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and analyzed by immunoblot. Antibodies used were as follows: Fbx4 rabbit polyclonal antibody (Rockland, Gilbertsville, PA), cyclin D1 mouse monoclonal D1-72-13G and mouse monoclonal AB3 (EMD Biosciences, Darmstadt, Germany), mouse monoclonal β-actin and Flag rabbit polyclonal (Sigma-Aldrich, St. Louis, MO), 14-3-3ε rabbit polyclonal (Abcam, Cambridge,

MA), ubiquitin mouse monoclonal (Covance, Emerville, CA), cyclin A rabbit polyclonal (Santa Cruz, Santa Cruz, CA); GSK3β (BD Transduction Labs, Lexington KY). For co-precipitation studies, GST-14-3-3ε was expressed in *E. Coli* and affinity purified; Fbx4 protein was purified from SF9 cells using Flag-agarose.

Cycloheximide chase analysis

For cycloheximide chase experiments cells were plated at equal densities and the next day asynchronous cells were treated with cycloheximide (100μg/ml, Sigma-Aldrich, St. Lois, MO) for indicated periods of times. Harvested cells were lysed in lysis buffer (as described above) and processed for immunoblot analysis.

In vitro ubiquitylation

SCF$^{Fbx4/αB-crystallin}$ complexes were purified from SF9 cells through M2 pulldown, mixed with purified Flag-tagged cyclin D1, E1, E2 (UbcH5A), ATP, and ubiquitin for indicated times at 37°C. Proteins were resolved on 10% SDS-PAGE and visualized by Western blotting with anti cyclin D1 antibody D1-72-13G11.

Transformation assays

Anchorage-independent growth was determined by analyzing cellular growth in semisolid medium. Cells (5×10^3) were seeded in complete Iscove's media containing 0.65% noble agar/10% FCS. Cells were grown for 14 days in 8% CO_2.

Acknowledgments

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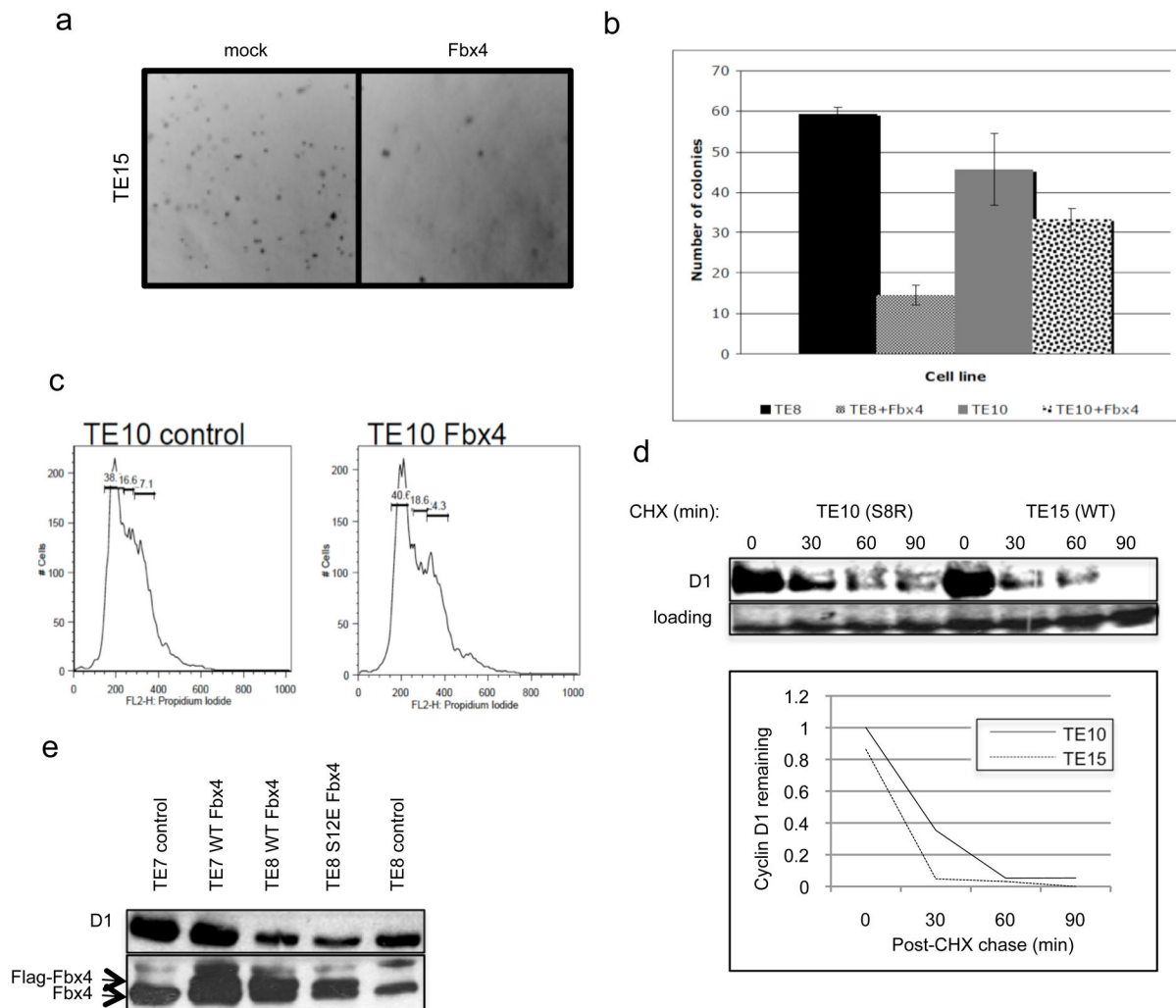
a

mock   Fbx4

TE15

b

c

TE10 control   TE10 Fbx4

d

CHX (min):   TE10 (S8R)   TE15 (WT)
                0   30   60   90   0   30   60   90

D1

loading

e

TE7 control
TE7 WT Fbx4
TE8 WT Fbx4
TE8 S12E Fbx4
TE8 control

D1

Flag-Fbx4
Fbx4

Figure 1. Fbx4 expression in esophageal carcinoma lines inhibits cyclin D1 expression and growth in soft agar

A. TE15 cells were transfected with the indicated Fbx4 expression vector and plated in soft agar 24 hours post-transfection. Photographs of colonies were taken at day 14. **B.** TE10 and TE8 cells were transfected with an Fbx4 expression vector and plated in soft agar 24 hours post-transfection. Colonies were quantified at day 14. **C.** Cell cycle distribution of TE10 cells expressing empty (control) or Fbx4 expression vector. **D.** Top panel: Cycloheximide chase in TE10 (Fbx4S8R) and TE15 (WT Fbx4) cell lines; bottom panel: quantification of western blot. **E.** TE7 and TE8 cells were transfected with Fbx4 expression vectors and asynchronous cells were analyzed by immunoblot 48 hours post-transfection.
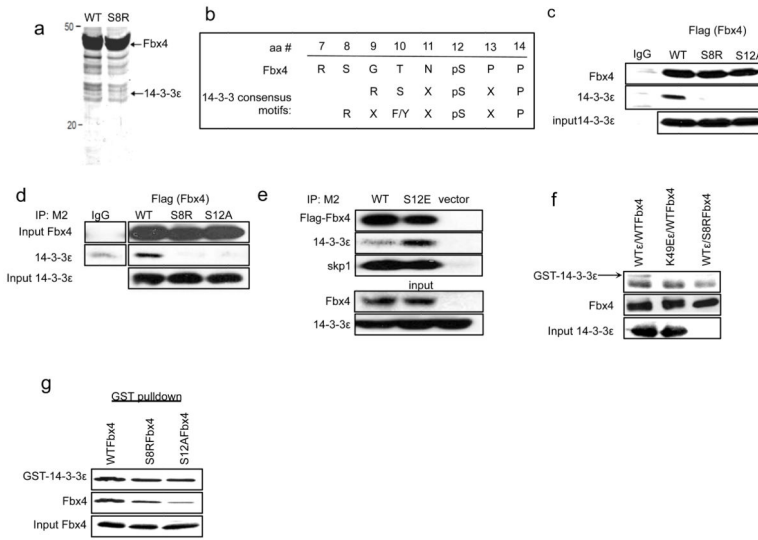
Figure 2. Fbx4 associates with 14-3-3ε through an N-terminal motif containing Ser-8 and Ser-12
A. Wild-type and S8R Fbx4 complexes were purified by Flag affinity precipitation from 293T cells and separated by SDS-PAGE followed by silver staining. Indicated proteins were identified by mass spectrometry. **B.** Alignment of putative 14-3-3ε binding motif on Fbx4 with consensus 14-3-3 binding motifs. **C.** Association of exogenously expressed wild type Fbx4, Fbx4S8R, and Fbx4S12A with endogenous 14-3-3ε in NIH3T3 cells. **D.** Association of exogenously expressed wild type Fbx4, Fbx4S8R, and Fbx4S12A with endogenous 14-3-3ε in 293T cells. **E.** Association of exogenously expressed wild type and Fbx4S12E with endogenous 14-3-3ε in 293T cells. **F.** Pulldown of GST-14-3-3ε with indicated Fbx4 mutant proteins purified from Sf9 cells. **G.** Association of Fbx4 wild type and S8R purified from Sf9 cells with recombinant GST-14-3-3ε. Fbx4 was purified from Sf9 cells using pS12-Fbx4 antibody and mixed with recombinant GST-14-3-3ε. Complexes were separated by SDS-PAGE followed by immunoblotting with indicated antibodies.
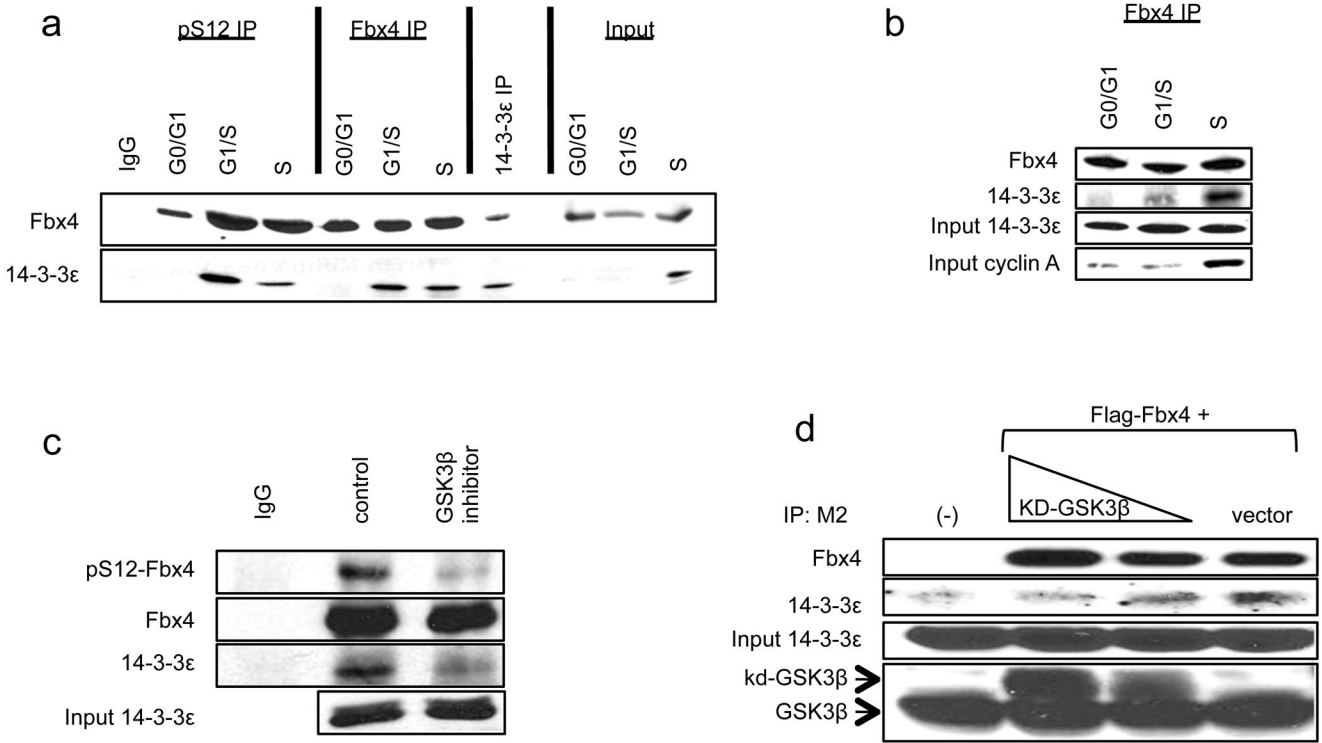
Figure 3. Association of Fbx4 with 14-3-3ε is cell cycle regulated
A. NIH3T3 cells were synchronized by contact inhibition/serum starvation and stimulated to re-enter by replating at subconfluence in media containing 10% fetal bovine serum. Cells were harvested at 0hrs (G0/G1), 8hrs (G1/S) and 16hrs (S). Fbx4 was precipitated using either pS12-Fbx4 or Fbx4 antibodies and protein complexes were separated by SDS-PAGE followed by immunoblot with indicated antibodies. **B.** HeLa cells were synchronized, harvested, and blotted as in A. **C.** NIH3T3 cells entering S phase (14 hrs after serum release) were treated with GSK3β inhibitor SB for 4hrs. Protein extracts were precipitated using Fbx4 antibody followed by immunoblot as indicated. **D.** Association of 14-3-3ε with wild type Fbx4 upon co-transfection of 3T3 cells with increasing amounts of kinase-dead (KD) GSK3β or vector control.
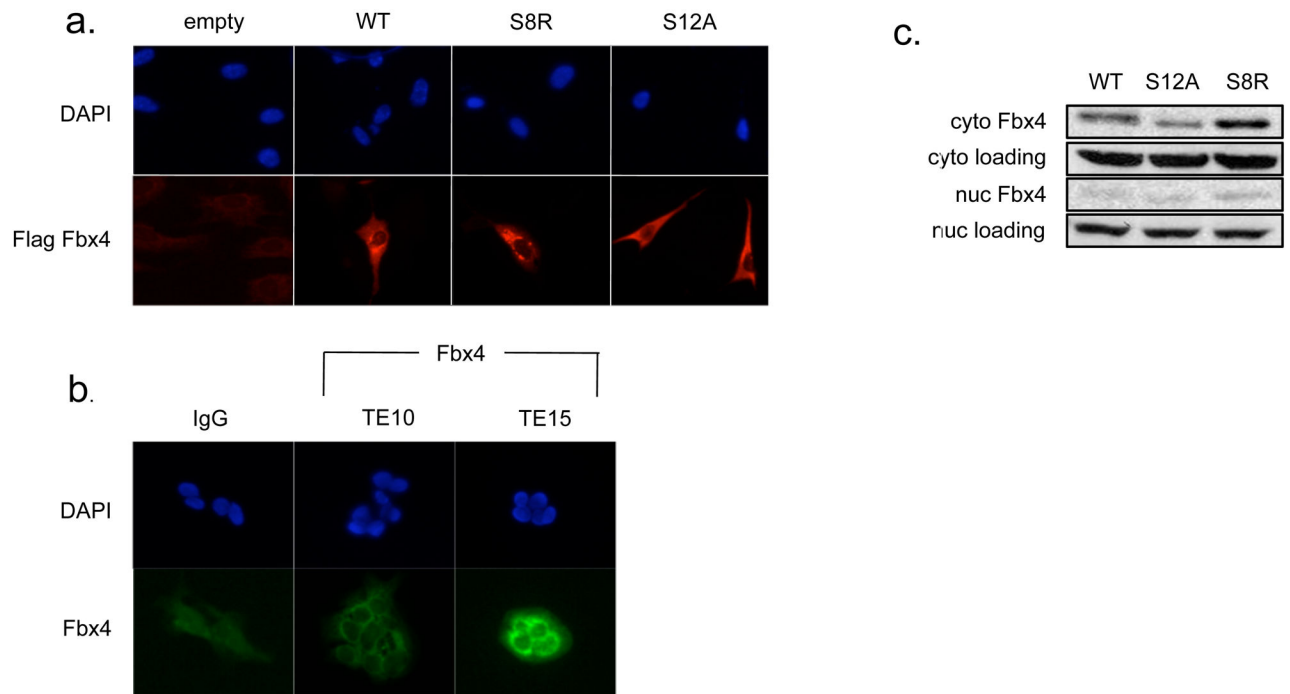
Figure 4. Fbx4 S8R and S12A localize to the cytoplasm
A. Immunofluorescence was performed on NIH3T3 cells transfected with the indicated Fbx4 variants. Cells were stained with M2-Flag antibody and counterstained with DAPI. **B.** Immunofluorescence was performed on TE10 (Fbx4S8R) and TE15 (WT Fbx4) esophageal cancer cell lines. Cells were stained with anti-Fbx4 antiserum and counterstained with DAPI. **C.** Nuclear fractionation and Fbx4 immunoblot of NIH3T3 cells transfected with the indicated Fbx4 variants.
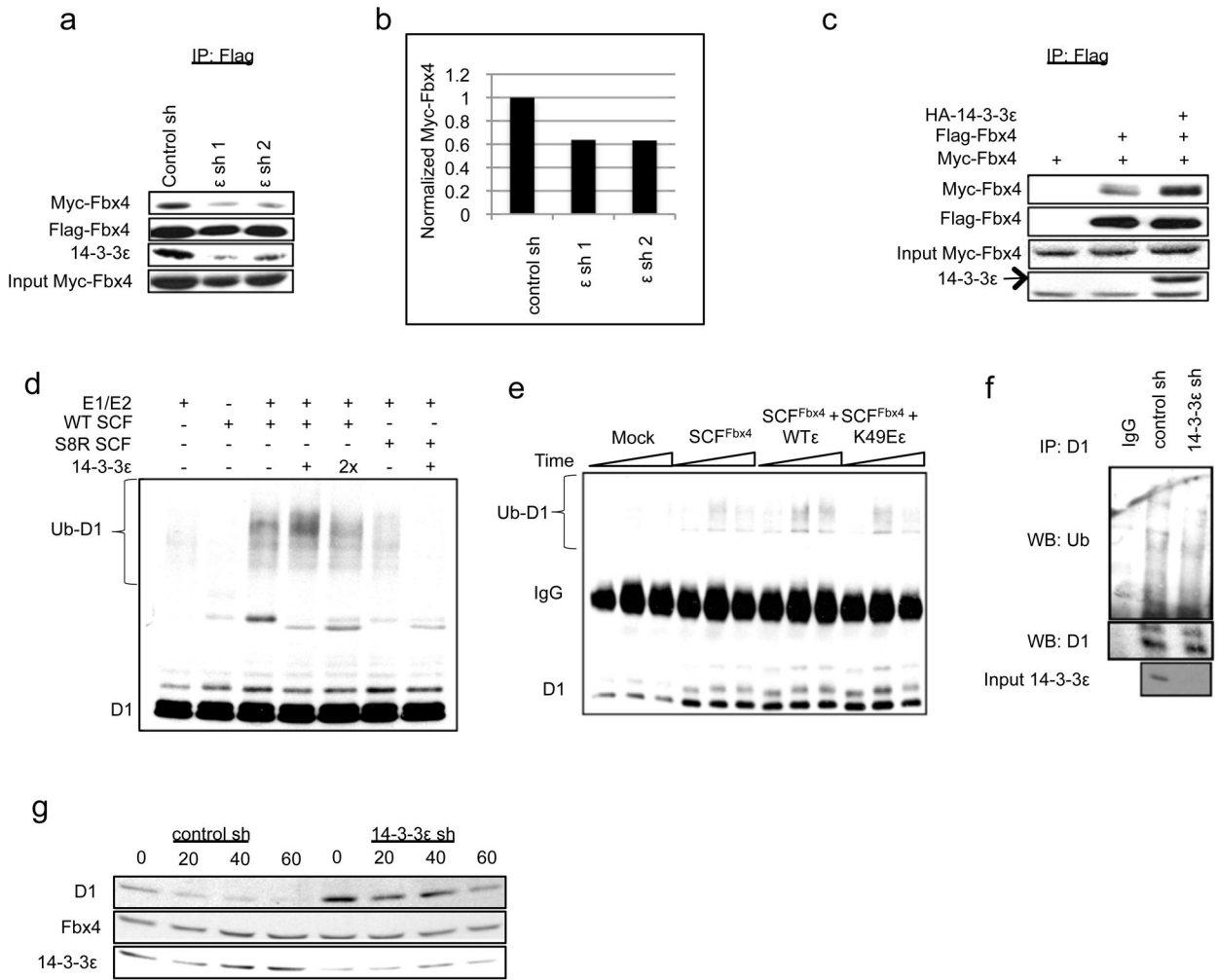
Figure 5. 14-3-3ε promotes Fbx4 dimerization and positively contributes to cyclin D1 ubiquitylation by SCF$^{\text{Fbx4/αBcrystallin}}$ and cyclin D1 stability

A. 293T cells were transfected with Flag-Fbx4, Myc-Fbx4 and sh14-3-3ε. Cell lysates were precipitated using Flag-agarose and separated by SDS-PAGE followed by immunoblot with indicated antibodies. **B.** Quantification of co-IP myc immunoblot signal intensity relative to input myc signal and flag-IP. **C.** 293T cells were transfected with Flag-Fbx4, Myc-Fbx4 and HA-14-3-3ε. Cell lysates were precipitated with Flag-agarose and separated by SDS-PAGE followed by immunoblot with indicated antibodies. **D.** Ubiquitylation of cyclin D1 with wild type or S8R SCF$^{\text{Fbx4}}$ in the presence or absence of 14-3-3ε. **E.** *In vitro* ubiquitylation of cyclin D1 by SCF$^{\text{Fbx4}}$ in the absence or presence of wild type or K49E GST-14-3-3ε. **F.** *In vivo* ubiquitylation of Cyclin D1 in NIH3T3 cells expressing shRNA toward 14-3-3ε. **G.** Cycloheximide chase in asynchronous NIH3T3 cells expressing either sh control or sh14-3-3ε.