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Alternative splicing variants of human Fbx4 disturb cyclin D1 proteolysis in human cancer

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ABSTRACT

Fbx4 is a specific substrate recognition component of SCF ubiquitin ligases that catalyzes the ubiquitination and subsequent degradation of cyclin D1 and Trx1. Two isoforms of human Fbx4 protein, the full length Fbx4α and the C-terminal truncated Fbx4β have been identified, but their functions remain elusive. In this study, we demonstrated that the mRNA level of Fbx4 was significantly lower in hepatocellular carcinoma tissues than that in the corresponding non-tumor tissues. More importantly, we identified three novel splicing variants of Fbx4: Fbx4γ (missing 168–245nt of exon1), Fbx4δ (missing exon6) and a N-terminal reading frame shift variant (missing exon2). Using cloning sequencing and RT-PCR, we demonstrated these novel splice variants are much more abundant in human cancer tissues and cell lines than that in normal tissues. When expressed in Sk-Hep1 and NIH3T3 cell lines, Fbx4β, Fbx4γ and Fbx4δ could promote cell proliferation and migration *in vitro*. Concordantly, these isoforms could disrupt cyclin D1 degradation and therefore increase cyclin D1 expression. Moreover, unlike the full-length isoform Fbx4α that mainly exists in cytoplasm, Fbx4β, Fbx4γ, and Fbx4δ locate in both cytoplasm and nucleus. Since cyclin D1 degradation takes place in cytoplasm, the nuclear distribution of these Fbx4 isoforms may not be involved in the down-regulation of cytoplasmic cyclin D1. These results define the impact of alternative splicing on Fbx4 function, and suggest that the attenuated cyclin D1 degradation by these novel Fbx4 isoforms provides a new insight for aberrant cyclin D1 expression in human cancers.

1. Introduction

Ubiquitin (Ub)-mediated proteolysis is an important means of regulating gene expression and has a pivotal role in the control of various cellular processes [1]. Ubiquitination is catalyzed via a triple-enzyme cascade including an E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and E3 (Ub ligase). Once ubiquitinated, proteins are rapidly hydrolyzed by the 26S proteasome. The substrate specificity is largely conferred by E3 ligases, and the interaction between substrates and E3 is crucial for the regulation of Ub-mediated protein turnover [2].

Skp-cullin-F-box (SCF) Ub ligases are multi-subunit complexes that mediate specific ubiquitination of distinct substrates [3]. Substrate specificity is decided by the F-box protein subunit of the complex. Recently, the Ub ligase SCF^{Fbx4} has been identified as a new E3 responsible for the ubiquitination and subsequent degradation of the cell cycle regulator cyclin D1 and telomeric DNA-binding protein Pin2 (also known as Trf1) [4,5]. Fbx4 belongs to the FBXO subfamily of F-box proteins in which the substrate-binding motif has not been identified yet [6]. Structurally, Fbx4 has several identified domains essential for its function in SCF complex: N-terminal dimerization domain (D domain), F-box domain, linker domain and C-terminal substrate-binding domain (G domain) [6–9]. Structural and biochemical analyses have revealed that the interaction between linker domain and G domain is crucial for the head-to-tail dimerization configuration of Fbx4, which is required for substrate binding and ubiquitin transfer [9].

Fbx4 recognizes its two known substrates cyclin D1 and Pin2 in two very different manners. Fbx4-mediated ubiquitination of cyclin D1 depends on its phosphorylation at Thr-286 residue, as well as an interaction with αB-crystallin, a small heat-shock protein, whereas that of Pin2 does not require phosphorylation [4,5,10]. Inhibition of Fbx4 activity results in accumulation of nuclear cyclin

Abbreviations: Fbx4, F-box only protein 4; Ub, ubiquitin; SCF, Skp-cullin-F-box; HCC, hepatocellular carcinoma.

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D1 and oncogenic transformation which appears at least in part to account for overexpression of cyclin D1 in human cancers [5,11], and mutations in the Fbx4 subunit of SCF^{Fbx4} were found to be associated with human primary esophageal carcinoma [12,13]. Therefore, the regulation of SCF^{Fbx4} activity is considered to be very important to sustain cyclin D1 level in normal cells, and impairment of SCF^{Fbx4} function is a critical mechanism underlying cyclin D1 overexpression in human cancers.

To date, two Fbx4 isoforms (named Fbx4α and Fbx4β in this paper) created by alternative splicing have been identified, but the roles these two isoforms play in SCF^{Fbx4} activity remain incompletely understood. Although the alternative splicing has been assigned as a regulatory mechanism for F-box protein [14], the specific differences of Fbx4 splicing profiles in cancer tissues remain elusive. In this study, we identified the presence of additional oncogenic splicing variants of Fbx4 in human tissues and these novel Fbx4 splicing variants are much more abundant in tumor tissues and cell lines than that in normal tissues. The attenuated cyclin D1 degradation by these novel Fbx4 isoforms *in vitro* provides a new insight for aberrant cyclin D1 expression and tumor development in human cancer.

2. Materials and methods

2.1. Cell lines and tissues

Human liver cancer cell lines SNU182, SNU449, HepG2, SK-Hep1 and mouse embryo fibroblast cell line NIH3T3 were purchased from ATCC (American Type Culture Collection; Manassas, VA, USA). The esophagus carcinoma cell lines KYSE450, KYSE510, SEG-1, BIC-1 and gastric cancer cell line NUGC3 were gifts from Professor Mingzhou Guo (Chinese PLA General Hospital).

A total of 40 pairs of HCC tissues and non-tumor tissues were provided by the Affiliated Oncology Hospital of Zhengzhou University. Both tumor samples and non-tumor samples were confirmed histologically. The usage of human samples in this study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethics Committee of Peking University Health Science Center.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR (qRT-PCR) was carried out using SYBR Green on a LightCycler 480IIreal-time PCR detection system (Roche, Germany) according to the manufacturer's instruction. To quantify novel Fbx4 isoforms, semi-quantitative PCR was performed with primers specific to each isoform, and the products were analyzed on 2% agarose gel. The primers used for RT-PCR are summarized in Table S1.

2.3. Clone-sequencing of Fbx4 mRNA composition

To clone Fbx4 mRNA, PCR reactions were performed with primers CDS1-F/R and CDS2-F/R (Table S1) by using cDNA from normal tissues and tumor cell lines, respectively. PCR products were purified and cloned into TA vector and transformed into *Escherichia coli* DH5α competent cells. Cell clone was randomly picked up and sequenced.

2.4. Plasmid construction

To construct vectors expressing Fbx4 isoforms, cDNA fragments from normal liver tissues were prepared by RT-PCR with specific primers (Table S1). The cDNA of Fbx4 isoforms was then cloned into pcDNA™3.1/Myc-His (−) vector (Invitrogen, Carlsbad, California). All expressing vectors were verified by DNA sequencing and Western blot assay.

2.5. Colony formation assay

Colony formation assay were performed using a soft agar kit (GenMed Scientifics, Inc., Arlington, USA) in 6-well plates according to the manufacturer's instructions. Colonies were photographed and counted under a microscope with a digital camera.

2.6. Transwell assay

The transwell system was used to explore cell's migration. A total of 7500 cells suspended with 150 μL of FBS-free medium were added into the inside compartment of the transwell insert. After 48-h incubation, the migrated cells were stained with 0.1% crystal violet and quantified under a light microscope, with at least three individual fields per insert.

2.7. Immunofluorescence assays

Cells were permeabilized with Methanol:Acetone (1:1), washed with PBS and incubated in primary anti-Myc antibody for 2 h. After washing and application of the secondary FITC-conjugated secondary antibody, slides were stained using Hoechst 33258 dye and analyzed by fluorescence microscopy (LEICA).

2.8. Subcellular fractionation analysis

NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Pittsburgh, PA) were used to get cytoplasmic extract and nuclear extract, respectively, according to the manufacturer's recommendation.

2.9. Immunoprecipitation (IP) and Western blot assays

For IP assay, cells were lysed with IP buffer. The cell lysates were incubated with the primary antibody and then immunocomplexes were bound to protein-G Sepharose 4B (Pharmacia, Sweden). After extensive washing, the precipitates were analyzed by Western blot. Western blot analysis was performed as described [13]. The antibodies used for IP and Western blot analysis were listed in Table S2.

2.10. Statistical analysis

For statistical analyses, the difference between two groups was analyzed by 2-tailed Student *t* test using GraphPad Prism software. In all cases, a *P* value of less than 0.05 was considered significant.

3. Results

3.1. Fbx4 is down-regulated in HCC tissues

To examine whether Fbx4 is down-regulated in HCC tissues, we performed qRT-PCR to measure Fbx4 mRNA level in 40 pairs of HCC tumor and adjacent non-tumor tissues. The result showed that Fbx4 mRNA level in tumor tissues was significantly lower than that in adjacent non-tumor tissues ($P = 0.0238$) (Fig. 1A). To further confirm the reduced expression of Fbx4 in HCC, 9 pairs of HCC tissues were used for Western blot assay to analyze Fbx4 protein level. Consistent with the lower Fbx4 mRNA level, lower Fbx4 protein level was detected in 7 of 9 HCC tumor tissues, as compared to their matched non-tumor tissues (Fig. 1B).

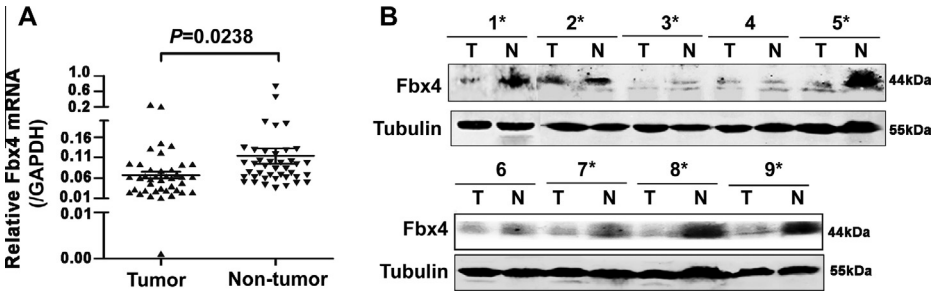

Fig. 1. Fbx4 is down-regulated in HCC. (A) Fbx4 mRNA expression was analyzed in 40 pairs of HCC and adjacent non-tumor tissues by real-time qRT-PCR using GAPDH for normalization. Horizontal bars represent median expression levels. Significant differences were determined using Student's *t* tests; (B) Western blot assay was used to detect the protein level of Fbx4 in 9 pairs of HCC and adjacent non-tumor samples.

3.2. Identification of novel Fbx4 splicing variants

To date, only two Fbx4 splicing variants created by alternative splicing are identified. Variant 1, the original transcript identified, contains 7 exons and encodes an isoform (Fbx4α) of 387 amino acids. Variant 2 contains an altered C-terminus, characterized by 7 amino acids encoded by a read through into intron 5, with the entire exon 6-encoded sequence being replaced. To further explore the complexity of Fbx4 splicing variation, we designed primers crossing the entire coding sequence of Fbx4 variant 1 and performed RT-PCR to assess Fbx4 mRNA pools in 4 liver cancer cell lines (SNU182, SNU449, HepG2, SK-Hep1) and disease-free normal liver tissues. Interestingly, the resolution of PCR products on agarose gel revealed that, besides the expected full-length Fbx4 variant 1, the primers of Fbx4 also produced several other DNA bands, which were smaller than expected size (Fig. 2A). Similar DNA bands of Fbx4 PCR products were also found in 4 esophagus carcinoma cell lines (KYSE450, KYSE510, SEG-1, BIC-1) and 1 gastric cancer cell line (NUGC3). To identify these unknown DNA fragments, the PCR products were then cloned into T-vector and randomly picked up for sequencing. These smaller bands were proved to be novel alternative splicing variants of Fbx4 gene (Fig. 2B), and were named Fbx4 variant 3, Fbx4 variant 4 and Fbx4 variant 5. All variants were derivatives of the full-length variant 1 (Fig. 2C). The variant 3 has a 168–245nt deletion of exon1 and produces an isoform of 351 amino acids (named Fbx4γ).The variant 4 has a loss of the whole exon 6 and produces an isoform similar to Fbx4 isoform 2 (named Fbx4δ).The variant 5 lacks the whole exon 2 and leads to a reading frame shift, which makes it infertile to translate into protein. These data suggest that more isoforms of Fbx4 exist than previously identified via the alternative splicing.

3.3. Tumor tissues express more Fbx4 variants

To test whether tumor cells have a more complex splicing variation of Fbx4 than normal cells, clone-sequencing analysis was conducted in normal liver tissue, normal esophagus tissue and 3 tumor cell lines: HepG2, BIC-1 and NUGC3. A total of 30 clones from each specimen were randomly picked up and sequenced. Our data showed that compared to the normal tissues, cancer cell lines indeed accommodated less wild-type Fbx4 variant 1 ($P < 0.0001$). In NUGC3 cells, even no Fbx4 variant 1 clone was detected (Table 1). When we searched human EST database to examine the presence of these variants, we could find the sequences of Fbx4 variant 4 and 5, but not that of variant 3. Interestingly, the novel splicing variants of Fbx4 existed in all three cancer cell lines as well as in the two normal human tissues, but with abundant differences. Among the 3 novel Fbx4 variants, the non-protein-coding variant 5 was the most abundant splicing variant in tumor cells (46.7 – 76.7% of all transcripts tested), much higher than that in normal tissues. Although there are no big differences in fraction of variant 3 and 4 between the normal human tissues and tumor cell lines, it is worthwhile to note that in NUGC3 cells, due to lack of the full length Fbx4 variant 1, Fbx4 variant 3 and variant 4 present as the only protein coding Fbx4 transcription variants. Semi-quantitative RT-PCR analysis of 7 pairs of HCC tumor tissues and the adjacent non-tumor tissues further confirmed that some HCC tumor tissues indeed expressed more Fbx4 variants (Fig. 2D).

3.4. Fbx4 isoforms exhibit oncogene functions

To directly address the potential role of Fbx4 isoforms, SK-Hep1 and NIH3T3 cells were transfected with either empty vector or C-terminal Myc-tagged Fbx4 expressing vector. Malignant characteristics of cells expressing Fbx4γ, Fbx4δ and Fbx4β were verified and compared with control cells in terms of their clonogenicity, wound healing ability and migration ability. As shown in Fig. 3, contrary to Fbx4α, whose overexpression could decrease both size and number of colonies formed by transfected cells, overexpression of Fbx4γ, Fbx4δ and Fbx4β in SK-Hep1 and NIH3T3 cells facilitated cell growth in soft agar, a characteristic of neoplastic transformation ($P < 0.05$ from control of Fbx4α, Fig. 3A). In addition, ectopic expression of Fbx4γ, Fbx4δ and Fbx4β in both cells remarkably enhanced cell migration ability as determined by transwell assay, which showed that migrated cells are dramatically increased in these isoforms transfected cells ($P < 0.05$ or $P < 0.01$ from control of Fbx4α, Fig. 3B). The promoting effect of these isoforms on cell migration was further confirmed by wound-healing assay ($P < 0.05$ or $P < 0.01$ from control of Fbx4α, Fig. 3C). Taken together, these data suggest that Fbx4γ, Fbx4δ and Fbx4β represent oncogenic potential in this context.

3.5. Different subcellular localization of Fbx4 isoforms

It has been reported that different isoforms of F-box protein may have different subcellular distribution with altered functions [15,16]. To test whether these novel Fbx4 isoforms exhibit different subcellular localizations, Myc-tagged Fbx4 isoforms were transfected into NIH3T3 cells. Indirect immunofluorescence staining showed that Fbx4α tends to be mainly localized in cytoplasm, while Fbx4γ, Fbx4δ and Fbx4β existed in both cytoplasm and nucleus (Fig. 4A). The subcellular localizations of these isoforms were further confirmed by Western blot analysis (Fig. 4B). The different subcellular localizations of these Fbx4 isoforms suggest that they may have different biological roles in the regulation of substrate degradation.

3.6. Differential abilities of Fbx4 isoforms on the regulation of cyclin D1 degradation

Based on the understanding of Fbx4 protein structure (Fig. 2C), Fbx4γ lacks parts of both D domain and F-box domain; Fbx4δ and Fbx4β both lack part of G domain. We postulated that these novel

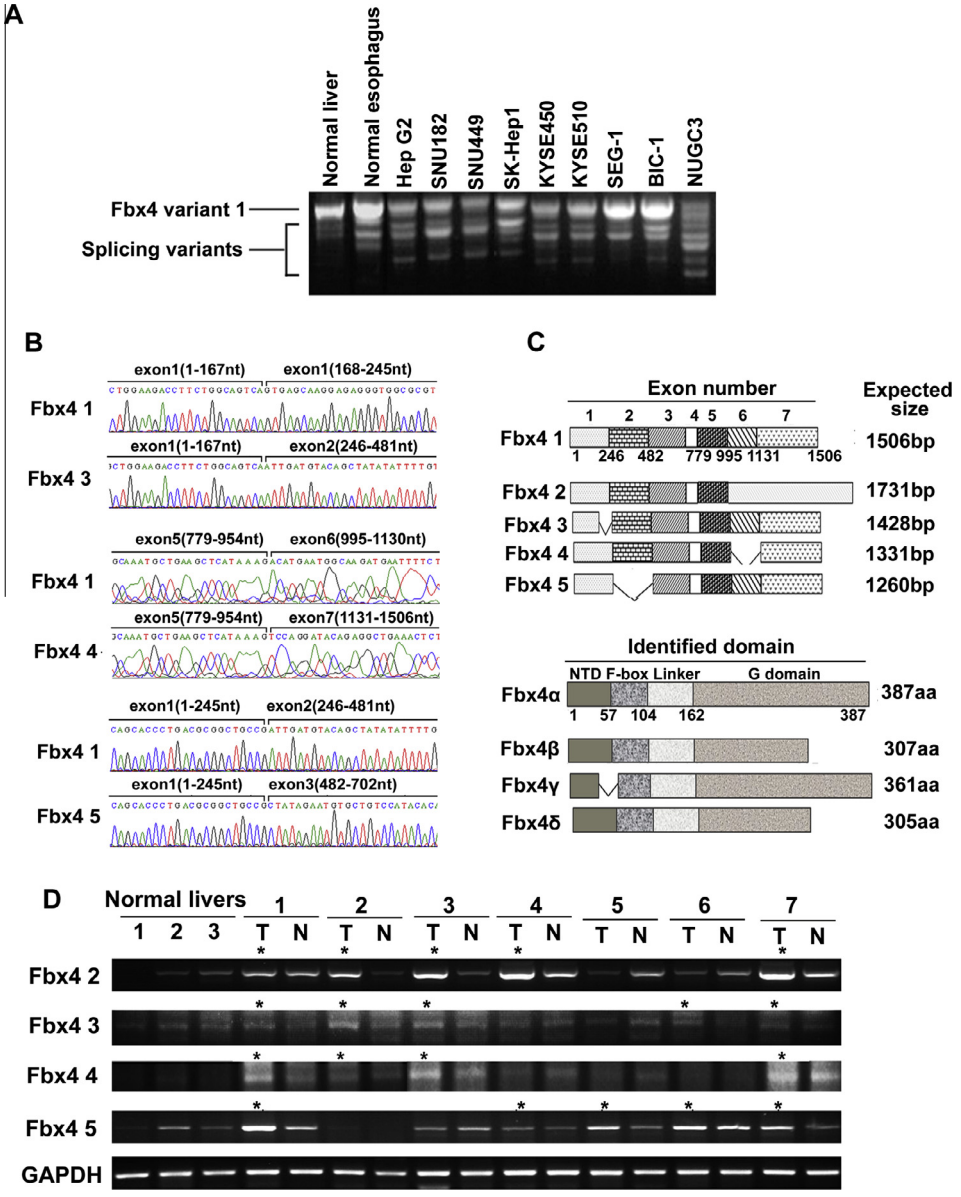

Fig. 2. Identification of novel Fbx4 splicing variants. (A) The novel Fbx4 splicing variants were identified by RT-PCR with primers crossing the entire CDS of Fbx4. (B) The sequencing results of the novel Fbx4 variants are presented in the sense direction. (C) Schematic diagrams of the identified Fbx4 variants. (D) Expression of Fbx4 variants in 7 pairs of HCC and adjacent non-tumor samples, and 3 normal liver tissues detected by using semi-quantitative RT-PCR. *, expression in tumor tissue is higher than that in corresponding non-tumor tissue.

Table 1
Distribution of Fbx4 variants in normal human tissues and cancer cell lines.

Variant	Normal liver tissue	Normal esophagus tissue	HepG2	BIC-1	NUGC3
Fbx4 1	20 (66.7%)	18 (60.0%)	10 (33.4%)	11 (36.7%)	0
Fbx4 3	1 (3.3%)	0	1 (3.3%)	1 (3.3%)	6 (20.0%)
Fbx4 4	6 (20.0%)	5 (16.7%)	4 (13.3%)	4 (13.3%)	1 (3.3%)
Fbx4 5	3 (10.0%)	7 (23.3%)	15 (50.0%)	14 (46.7%)	23 (76.7%)
Total	30	30	30	30	30

isoforms of Fbx4 should reduce the SCF^{Fbx4} activity, and therefore increase the accumulation of downstream substrates. The influence of these Fbx4 isoforms on the level of endogenous cyclin D1 and Pin2/Trf1, two known Fbx4 substrates, were then analyzed by Western blot assay. As shown in Fig. 4C, ectopic expression of Fbx4γ, Fbx4δ and Fbx4β all significantly enhanced the expression of cyclin D1 protein, while Pin2/Trf1 was barely affected. Immunoprecipitation analysis revealed that Fbx4γ, Fbx4δ and Fbx4β were still capable to bind to cyclin D1, but not as powerful as Fbx4α. (Fig. 4D). Consistent with the weak ability of these isoforms to bind to cyclin D1, turnover assay demonstrated that overexpression of Fbx4γ, Fbx4δ or Fbx4β significantly extended the half-life of endogenous cyclin D1 protein (Fig. 4E), indicating that these isoforms appear to up-regulate cyclin D1 expression by reducing its degradation.

4. Discussion

Abnormal cyclin D1 degradation has been implicated as a causative factor leading to its overexpression in human tumors [5,17]; however the underlying mechanisms remain ambiguous. Aberrant proteolytic degradation was initially thought as a result of the cyclin D1 Thr286 residue mutation or deletion, which renders cyclin D1 protein more stable [17,18]. However, this cancer-derived cyclin D1 mutation is rare in cancer, implying the involvement of

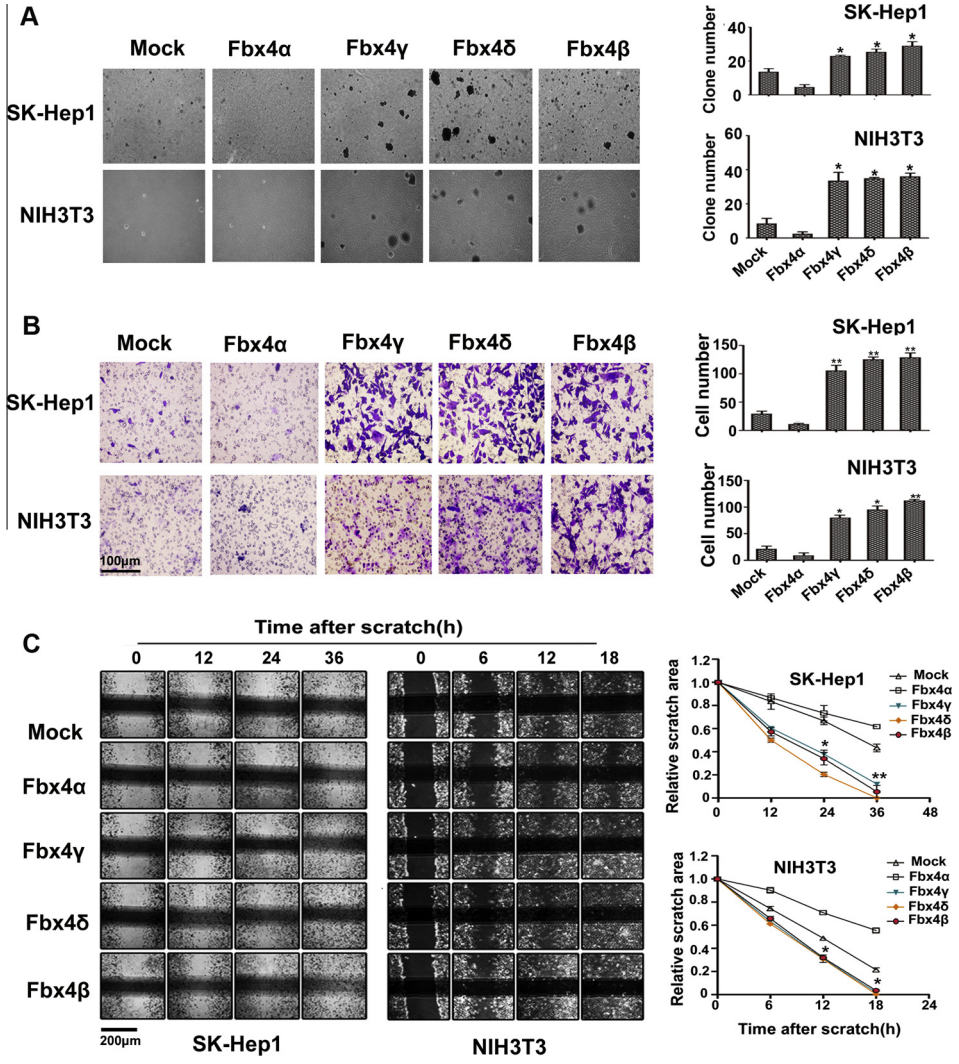

Fig. 3. Fbx4 isoforms exhibit oncogene activities. (A) Representative colony formation and corresponding histogram of SK-Hep1 and NIH3T3 cells transfected with Fbx4 isoforms. (B) Representative transwell migration and corresponding histogram of SK-Hep1 and NIH3T3 cells transfected with Fbx4 isoforms. (C) Representative scratch-wound assay and corresponding histogram of SK-Hep1 and NIH3T3 cells transfected with Fbx4 isoforms. Data are presented as mean ± SD from at least 3 independent experiments. The significance was calculated with two-tailed Student *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$ from control of Fbx4α.

other mechanisms for the abnormal cyclin D1 degradation frequently found in various human malignancies. $SCF^{Fbx4/\alpha B\text{-crystallin}}$ is a recently identified E3 ligase complex that directs ubiquitin-mediated proteolysis of cyclin D1 [8]. Herein, we identified several previously unknown Fbx4 isoforms produced by alternative splicing. These isoforms showed less capability for cyclin D1 binding and degradation. Importantly, we demonstrated that these isforms had obvious oncogenic properties of promoting tumor cell growth and metastasis. Therefore, alternative splicing of Fbx4 may add another level of complexity to the regulation of cyclin D1 in tumor development.

Alternative splicing is a major regulatory mechanism to control gene usage and increase coding capacity, and deregulation of this process is known to underlie the pathogenesis of diverse human diseases including tumor formation [19]. Alternative splicing has been assigned as a regulatory mechanism for the production of F-box proteins such as Skp2, Fbw7 andβ-TrCP, which are responsible for the regulation of proteins that control diverse cellular signaling events [14,15,20]. In this study, we are the first to provide evidence that not only does Fbx4 expression decrease in HCC tissues, but also Fbx4β, Fbx4γ and Fbx4δ are the dominant isoforms expressed in cancer. The changes of alternative splicing pattern of Fbx4 in cancer suggest that these new splice-variants may be putative oncogenes involved in carcinogenesis. This notion is further supported by data from the functional characterization *in vitro*, which showed an increased clonogenicity, wound healing ability and migration ability in NIH3T3 and Sk-Hep1 cells over-expressing these isoforms.

The integrity of Fbx4 domains is indispensable for its dimerization and subsequent activation of Fbx4 in SCF complex and Fbx4-mediated substrate degradation [14,21,22]. The N-terminal conserved D domain (residue1-57) of Fbx4 protein is important for interaction with Cul1 and also for Fbx4 dimerization and ubiquitination activity. In addition, a loop connecting the linker domain (residue 105–162) to the C-terminal G domain (residue 163–387) is crucial for dimerization, and the head-to-tail dimerization configuration of Fbx4 protein likely plays a role in stabilizing optimal orientation of the F-box domain (residue 58–104) and G domain and thus exposing the substrate-binding site in the G domain [9]. Our sequencing analyses demonstrate that Fbx4β is divergent from Fbx4α in the C-terminal and has an incomplete substrate-binding domain; Fbx4γ suffers a 168–245nt deletion of exon1 and produces a protein lacking parts of both D domain and F-box domain; Fbx4δ is similar to Fbx4β and lacks part of G domain. Thus, it is

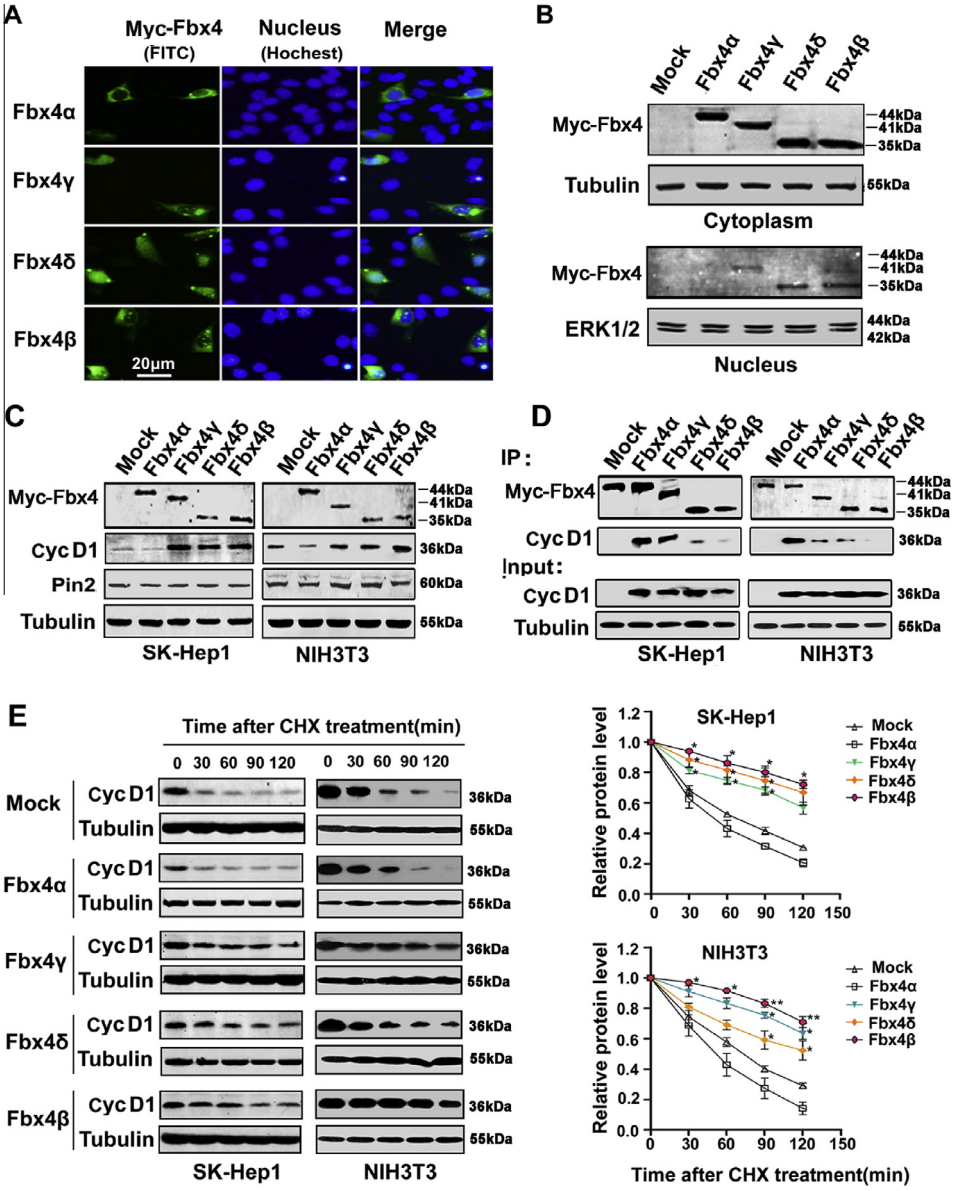

Fig. 4. The Fbx4 isoforms have different subcellular localization and reduced activity toward cyclin D1degradation. (A) Immunofluorescence was performed using NIH3T3 cells transfected with the indicated Fbx4 isoforms. (B) Nuclear fraction and Fbx4 immunoblot of NIH3T3 cells transfected with the indicated Fbx4 isoforms. (C) Endogenous cyclin D1and Pin2 were detected by Western blot assay. (D) The ability of Fbx4 binding to cyclin D1 in SK-Hep1 and NIH 3T3 cells was detected by immunoprecipitation analysis. The samples labeled "input" were typically 10% of the samples employed in binding experiments. (E) Representative immunoblot of cyclin D1 and corresponding histogram in SK-Hep1 and NIH3T3 cells incubated with Cycloheximide (CHX, 50 μg/mL). Data are presented as mean ± SD from at least 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ from control of Fbx4α.

possible that the structure defect of these Fbx4 isoforms may lead to failure of Fbx4 dimerization, substrate binding, and inactivation of the SCF^{Fbx4} ubiquitin ligase. In consist with this notion, ectopic expression of Fbx4β, Fbx4γ and Fbx4δ in NIH3T3 and Sk-Hep1 cells can disrupt endogenous cyclin D1 turnover and increase cyclin D1 expression at protein level. However, no significant increase of Pin2/Trf1 was observed by overexpression of Fbx4β, Fbx4γ or Fbx4δ, which further support the notion that Fbx4 recognizes its two substrates, Pin2/Trf1 and cyclin D1, in two very different manners [4,5,10]. Since cyclin D1 overexpression has been recognized as one of the most common mechanisms contributing to tumorigenesis in a variety of human malignancies, the up-regulation of cyclin D1 induced by these Fbx4 isoforms may at least in part contribute to their oncongenic roles. However, additional studies are required to determine the molecular details of these Fbx4 isoforms on the cyclin D1 degradation.

Recent studies have provided evidences that different isoforms of F-box protein may have different subcellular distribution with altered functions [15,16]. We found that Fbx4α is mainly localized in cytoplasm, while Fbx4β, Fbx4γ and Fbx4δ existed in both cytoplasm and nucleus. Phosphorylation of cyclin D1 at T286 triggers CRM1-dependent nuclear export of protein to the cytoplasm, where it is recognized and degraded by $SCF^{Fbx4/\alpha B\text{-crystallin}}$ ligase. Since the degradation of cyclin D1 takes place in cytoplasm, the nuclear distribution of Fbx4β, Fbx4γ or Fbx4δ may be negatively involved in the down-regulation of cytoplasmic cyclin D1. Therefore, in addition to the dimerization disturbing hypothesis, the different subcellular distribution characteristic of these Fbx4

isoforms may provide another explanation for their abated capability for cyclin D1 degradation. Further study should investigate the mechanism of Fbx4 isoform-specific localization, which will help to establish the significance of these isoforms in the regulation of substrates degradation.

In conclusion, we identified and characterized novel Fbx4 isoforms, which are produced by alternative splicing of Fbx4 pre-mRNA. These novel isoforms attenuate Fbx4-dependent cyclin D1 degradation and display obvious oncogenic properties. Our study, together with those of other groups, broadens current understanding of F-box protein regulation and emphasizes the importance of alternative splicing profiling in cancer. Future studies are required to figure out why abnormal splicing pattern of Fbx4 happens in cancer. Exploration of functions and mechanisms of these isoforms may be helpful to develop potential biomarkers and drugs for the prognosis and therapy of cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.129.

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