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Molecules and Cells

c-Myc Stimulates Cell Invasion by Inhibiting FBX8 Function

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c-Myc is a cellular onco-protein and a transcriptional activator important for cell growth, cell division, and tumorigenesis. Despite all that is known of its function, the mechanism of how c-Myc contributes to tumorigenesis is unclear. To gain insight into the mechanism through which c-Myc protein exerts its oncogenic activity, we performed large-scale, tandem repeat affinity purification and identified the F box only protein 8 (FBX8), an F-box and Sec7 domain-containing protein, as a novel Myc-binding protein. The c-Myc/FBX8 interaction was mediated by the c-Myc box II (MBII) region. We also confirmed that Myc protein overexpression in 293T cells affected FBX8 cellular translocation and led to recovery from FBX8-mediated inhibition of ADP-ribosylation factor 6 (ARF6) function during cell invasion. Together, these results suggest that FBX8 is a novel c-Myc binding protein and that c-Myc induces cell invasive activity through the inhibition of FBX8 effects on ARF6 function during cell invasion.

INTRODUCTION

c-Myc is a transcription factor and proto-oncogene that plays an important role in cell growth, proliferation, and division (Dang, 1999; Grandori et al., 2000; Lutz et al., 2002; Mateyak et al., 1997). Aberrant overexpression of this gene results in cancer, such as Burkitt's lymphoma and malignant tumors in the prostate, colon, and breast.

The c-Myc onco-protein has 439 amino acids and two major functional domains. The basic-helix-loop-helix leucine zipper (bHLH/LZip) domain is located in the c-terminal region of c-Myc, and it mediates binding to the DNA consensus site, CACGTG (Enhancer Box sequences; E boxes), and protein-protein interactions (Adhikary and Eilers, 2005; Amati et al., 1992; Luscher and Larsson, 1999; Pelengaris et al., 2002). A transcription activation domain (TAD) is located in the N-terminal region of c-Myc. The transcription activation domain includes the Myc box I (MBI) and Myc box II (MBII) elements. MBI regulates c-Myc stability through phosphorylation on the threonine 58 and serine 62 residues. MBII is required for transcriptional activation, transformation, and cell growth, and these functions are mediated by association with the TRRAP or TIP48/TIP49 proteins (Dai et al., 2007; Kim et al., 2003; Kuttler et al., 2001; McMahon et al., 1998; von der Lehr et al., 2003; Wood et al., 2000).

FBX8 is an F-box- and Sec7 domain-containing protein, the function of which had been largely unknown. However, recent research revealed that FBX8 has E3 ligase activity mediating the ubiquitination of the GTP-binding protein ARF6; further, it was shown that ubiquitination of ARF6 does not induce the degradation of ARF6 but inhibits ARF6 function (Yano et al., 2008). ARF6 is a small GTPase and one of the Ras superfamily proteins (Chavrier et al., 1999). ARF6 was shown to regulate membrane trafficking and actin cytoskeleton remodeling through the activation of Rac1 GTPase and/or lipid metabolism (Boshans et al., 2000; Santy et al., 2005). In addition, the ARF6-GTPase cycle can affect the invasive potential of tumor cells (Hashimoto et al., 2004; Tague et al., 2004).

FBX8 overexpression was found to inhibit ARF6-mediated cell invasion activity in breast cancer cells, and breast cancer cells, which lack invasion activity, were found to have reduced levels of FBX8 protein (Yano et al., 2008).

To understand the role of c-Myc in tumorigenesis, we performed large-scale, tandem repeat affinity purification using 293T cells stably expressing c-Myc to find new c-Myc binding proteins. c-Myc protein overexpression in 293T cells was found to affect FBX8 cellular translocation, and it also induced recovery from the FBX8-mediated inhibition of ARF6 function during cell invasion. Based on our data, we suggest that the cell invasive activity of c-Myc is mediated by inhibition of FBX8 tumor suppressor functions.

MATERIALS AND METHODS

Plasmids

The plasmids for the mammalian wild-type and deletion mutants of FBX8 (SFB-FBX8), wild-type USP11 (SFB-USP11), and ARF6 (SFB-ARF6) were generated by cloning PCR fragments into the S-Flag-Streptavidin binding peptide-tagged mammalian expression plasmid. The plasmids encoding full length, deletion, and point mutants of c-Myc (Myc-WT, -F1, -F2, -F3, -F4, -F5, -F1D1, -F1D2, -F1D3, -F1D4, -ΔMBI, -ΔMBII, -W135E, or -F138C) were generated by cloning PCR frag-

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ments into pcDNA3βm-1 (Pharmacia; USA). SFB-RAP80 (Kim et al., 2007) and HA-Ub (Sobhian et al., 2007) plasmids were described previously. ARF6 was cloned into the pENTR3C vector (*Eco*RI/*Xho*I), and His-ARF6 was generated using the GATEWAY system (Invitrogen, USA).

Cell culture and transient transfection

293T, U2OS, Hela, HepG2, HLE, PLC, and Huh7 cells were purchased from American Type Culture Collection (USA) and maintained in DMEM medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 (v/v). The cells (3×10^6 cells) were plated on a 100 mm plate and transfected with the plasmids using the Fugene6 reagent (Roche, Germany). The total amount of transfected DNA was normalized using the pcDNA3.1 plasmid as a carrier.

siRNA

The sequence of c-Myc siRNA is 5-CAGAAAUGUCCUGAGCAAUTT was described previously (Hu et al., 2009). The sequence of control siRNA is UUCAAUAAAUUCUUGAGGUUU. siRNAs were transfected into cells using oligofectamine (Invitrogen) according to the manufacturer's instructions.

Stable cell line establishment and affinity purification of S-Flag-SBP-tagged Myc-containing complexes

The establishment of stable cell lines was described previously (Kim et al., 2007). To construct a stable cell line, 293T cells were transfected with wild-type Myc (SFB-Myc) or the SFB plasmid as a control. After 48 h, the cells were split to a 10:1 ratio and cultured in medium containing 10 μg/ml of puromycin for three weeks. Individual puromycin-resistant colonies were isolated, and overexpression of the Myc constructs was confirmed by Western blotting. 293T cells stably expressing SFB-Myc proteins were lysed with 4 ml NETN buffer on ice for 10 min. Crude lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 min, and supernatants were incubated with 300 μl streptavidin-conjugated beads (Amersham Bioscience, USA). The immunocomplexes were washed three times with NETN buffer, and bead-bound proteins were eluted with 500 μl NETN buffer containing 2 mg/ml biotin (Sigma, USA). The eluted supernatant was incubated with 60 μl S protein beads (Novagen, Germany). The immunocomplexes were washed three times with NETN buffer and subjected to SDS-PAGE, and the binding proteins were analyzed by a mass spectrometer.

Antibodies and immunoprecipitation

Anti-Myc and anti-Flag (Roche, Germany), anti-HA, anti-GST, anti-ubiquitin (Sigma, USA), and anti-ARF6 antibodies (Thermo Scientific) were obtained from commercial sources. Rabbit polyclonal anti-FBX8 antibody was generated by injecting a mix of two peptides (C-KEQEGFINLEM, C-KMSKREFIRNTR) into a rabbit. For immunoprecipitation, 293T cells (3×10^6 cells) in a 100 mm plate were transfected with each expression plasmid, as indicated. After 48 h, cells were lysed in NETN buffer for 20 min on ice. Crude lysates were cleared by centrifugation at 14,000 rpm at 4°C for 5 min, and supernatants were incubated with protein A- or G-agarose-conjugated primary antibodies. The immunocomplexes were washed three times with NETN buffer and subjected to SDS-PAGE. Western blotting was performed using the antibodies indicated.

GST pull-down assay

Insect cells were infected with baculovirus encoding Flag-Myc, GST, GST-FBX8, or GST-Fbw7. Cell lysates were subjected to a GST pull down assay and immunoblotted with the indicated antibodies. The amounts of Flag-Myc, GST, GST-FBX8, or GST-Fbw7 in these lysates were analyzed by immunoblotting with the indicated antibodies.

ARF6 activitys

ARF6 activity was measured by pulling down the GTP-bound form using GST-GGA (Golgi-localizing γ-adaptin ear homology domain, Arf-binding protein) according to the manufacturer's instructions (Thermo Scientific).

***In vitro* invasion assays**

The cell invasion assay was performed using 24-well transwells (8 μm pore size, Corning Life Sciences) coated with 60 μl (1 mg/ml) of matrigel (BD Sciences). Cells were starved in serum-free media overnight, trypsinized, and washed three times in DMEM containing 1% FBS. Twenty thousand cells in 1% FBS-DMEM were seeded into the upper chamber, and 600 μl of DMEM containing 10% or 1% FBS were placed in the lower chamber. After 16 h incubation, the matrigel and cells remaining in the upper chamber were removed. Cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. For the quantitative assay, the cells that had invaded through the matrigel were stained with 4 μg/ml calcein-AM (Molecular Probes) in PBS for 30 min at 37°C and scanned for fluorescence with the Victor 3 multiplate reader (Perkin-Elmer life and Analytical Sciences, USA); fluorescent cells were counted. All experiments were performed in duplicate and were repeated two times.

Immunofluorescence staining

The immunofluorescence staining was described previously (Kim et al., 2007). Cells grown on coverslips were fixed with 3% paraformaldehyde at room temperature for 15 min. Next, the cells were permeabilized with PBS containing 0.5% Triton X-100 at room temperature for 5 min and blocked with PBS containing 5% goat serum at room temperature for 30 min. The coverslips were incubated with primary antibodies at room temperature for 20 min. After washing with PBS, cells were incubated with secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-rabbit IgG, or rhodamine-conjugated goat anti-mouse IgG (Jackson Immuno-Reserach Laboratories, Inc.), at room temperature for 20 min. 4, 6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. After a final wash with PBS, coverslips were mounted with glycerin containing p-phenylenediamine. All images were obtained with a fluorescence microscope.

RESULTS

Identification of FBX8 as a Myc-binding protein

To identify new Myc-binding proteins using the tandem repeat affinity purification, we first generated 293T cells stably expressing SFB-Myc, the Myc protein triply tagged at the N-terminal with S, Flag epitope, and streptavidin-binding peptide (SBP) (SFB tag) (Fig. 1A). After sequential affinity chromatography with streptavidin agarose and S-agarose beads and after mass spectrometry analysis using the cell lysates prepared from cells expressing SFB-Myc, we identified several putative Myc binding proteins (Table. 1). Among the proteins found in the purified SFB-Myc complexes, we decided to investigate FBX8 further, because FBX8 contains an F-box domain, suggesting involvement in ubiquitination. Binding between FBX8 and c-Myc was verified by overexpression in 293T cells, with

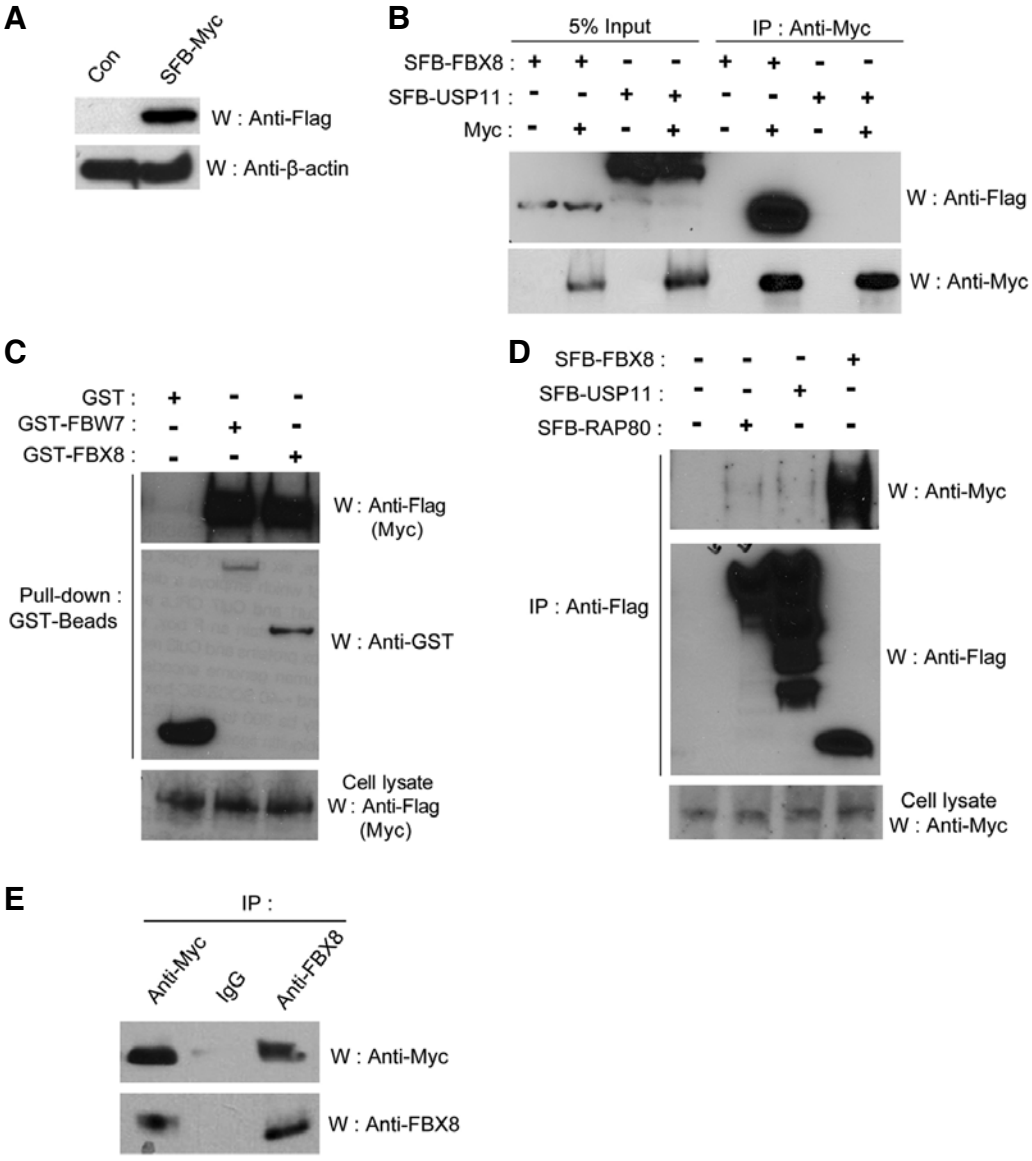

Fig. 1. Identification of FBX8 as a novel c-Myc-binding protein. (A) Establishment of human embryo kidney 293T cell lines stably expressing SFB-Myc. Cell extracts prepared from 293T cells stably expressing mock or SFB-Myc fusion protein were subjected to Western blotting analysis using the anti-Flag antibody. Mock: mock transfected. (B) Overexpressed SFB-FBX8 binds to overexpressed Myc in 293T cells. 293T cells (3×10^6/100 mm dish) were transfected with 4.5 μg of the Myc expression plasmid, with or without the expression plasmid for S-Flag-tagged FBX8 or USP11, as indicated. After 48 h, cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibody and immunoblotting (W) with the anti-Flag antibody (upper panel). The levels of immunoprecipitated Myc were analyzed by Western blot with an anti-Myc antibody (bottom panel). (C) Exogenous Myc and FBX8 associate in insect cells. Insect cells were infected with baculovirus encoding Flag-Myc and baculovirus encoding GST, GST-FBX8, or GST-Fbw7. Cell lysates were subjected to GST pull-down and immunoblotted with the indicated antibodies. (D) Overexpressed FBX8 binds to endogenous c-Myc in 293T cells. 293T cells (3×10^6/100 mm dish) were transfected with 4.5 μg of the S-Flag-tagged FBX8, USP11, or RAP80 expression plasmid. After 48 h, cell lysates were subjected to immunoprecipitation with an anti-Flag antibody and immunoblotted with the anti-Myc antibody (upper panel). The level of immunoprecipitated protein was analyzed by Western blot with the anti-Flag antibody (middle panel). The amount of endogenous c-Myc in each lysate is shown in the bottom panel. (E) Interaction of FBX8 with c-Myc at endogenous levels. Cell lysates were immunoprecipitated with anti-Myc, -IgG, or -FBX8 antibody and immunoblotted with anti-Myc antibody (upper panel) or anti-FBX8 antibody (bottom panel).

ubiquitin specific peptidase 11 (USP11) as a negative control. As shown in Fig. 1B, FBX8 strongly coimmunoprecipitated with c-Myc, but USP11 did not. To confirm the interaction between FBX8 and c-Myc, GST pull-down assays were performed using the GST protein, GST-FBX8, or GST-FBW7 fusion protein coexpressed with Flag-Myc in insect cell lysates. Full-length c-Myc bound specifically to GST-FBX8 and GST-FBW7 but not to GST alone (Fig. 1C). In addition, overexpressed FBX8 specifically associated with endogenous c-Myc in 293T cells, but overexpressed USP11 or RAP80 did not (Fig. 1D). Finally, we

Table 1. List of proteins associated with SFB-Myc identified by mass spectrometry analysis

Protein name	Number of peptides obtained per experiment
Galanin receptor 3	6
Myc	4
Leucine-rich repeat-containing protein 59	3
F-box protein 8	1
GTP Cyclohydrolase 1	1
Mitochodrial phosphate carrier protein	1

confirmed the binding between FBX8 and c-Myc at endogenous levels (Fig. 1E). These data indicate that FBX8 is a *bona fide* c-Myc-binding partner.

The Myc box II region of c-Myc is required for interaction with FBX8

Next, we identified which regions of c-Myc are involved in the association with FBX8. Full length c-Myc and various deletion mutants (Fig. 2A) were tested for interaction with full-length FBX8 in coexpressing 293T cells. The Myc-F1 and -F2 deletions largely abolished binding to FBX8 (Fig. 2B). These two mutants lack the c-Myc transcription activation domain (TAD), which contains two conserved regions important for c-Myc function: Myc box I (MBI) and Myc box II (MBII). Therefore, we investigated whether or not these two regions have the ability to associate with FBX8. Several mutants with deletions in the TAD, including deletions of MBI or MBII, were tested for their ability to bind to FBX8. Deletion of MBI, including serial deletions to amino acid 91 and an MBI deletion mutant, did not affect c-Myc binding to FBX8 (Figs. 2C and 2D). Since the Myc F1 deletion in conjunction with the deletion of the entire TAD domain abolished c-Myc binding to FBX8, it seemed likely that MBII would be the region essential for binding to FBX8. As anticipated, MBII deletion and MBII point mutations caused a loss of the ability to bind FBX8 (Fig. 2D), indicating that the MBII region of c-Myc is used for FBX8 binding.

c-Myc affects the subcellular localization of FBX8

To investigate the effects of c-Myc binding on FBX8 cellular distribution, 293T cells cotransfected with SFB-FBX8 and Myc-WT, Myc-F1, or Myc-ΔMBII were immunostained and observed. When only FBX8 was expressed in 293 T cells, most of the FBX8 protein was detected in the cytoplasm. However, after cotransfection with the wild-type c-Myc expression plasmid, FBX8 protein colocalized with c-Myc in the nucleus. However, the FBX8 protein was not detected in the nucleus when cotransfected with c-Myc deletion mutants lacking the transcription activation domain or Myc box II region (Fig. 3). These data

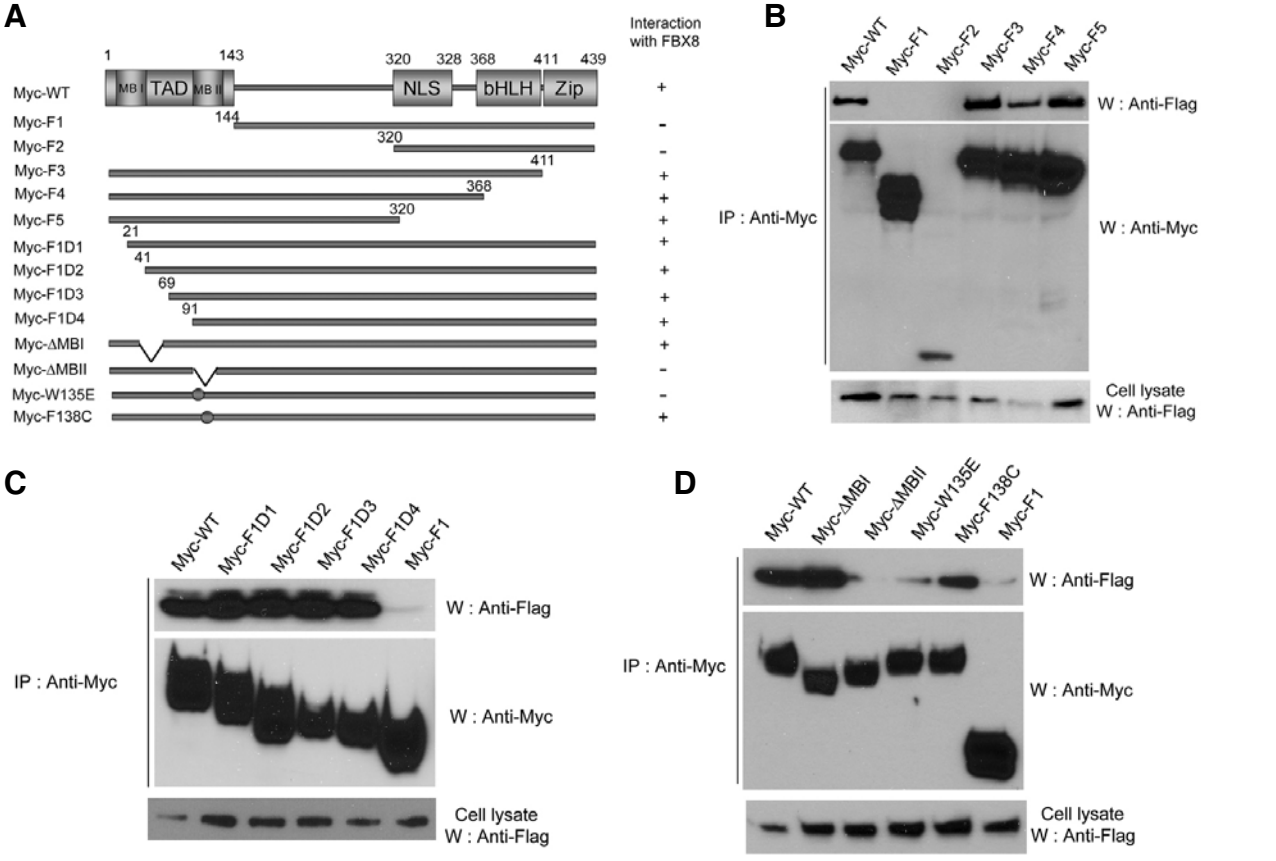

Fig. 2. Identification of the FBX8-binding region of c-Myc. (A) Diagram of wild-type and deletion mutants of c-Myc. (B-D) 293T cells were transfected with plasmids encoding SFB-FBX8 and wild-type or mutant c-Myc. Cell lysates were subjected to immunoprecipitation with the anti-Myc antibody and immunoblotted with the anti-Flag antibody (upper panel). The levels of immunoprecipitated wild-type and mutant c-Myc were analyzed by Western blot with anti-Myc antibody (middle panel). The amount of SFB-FBX8 was analyzed by immunoblotting with anti-Flag and is shown in the bottom panels.

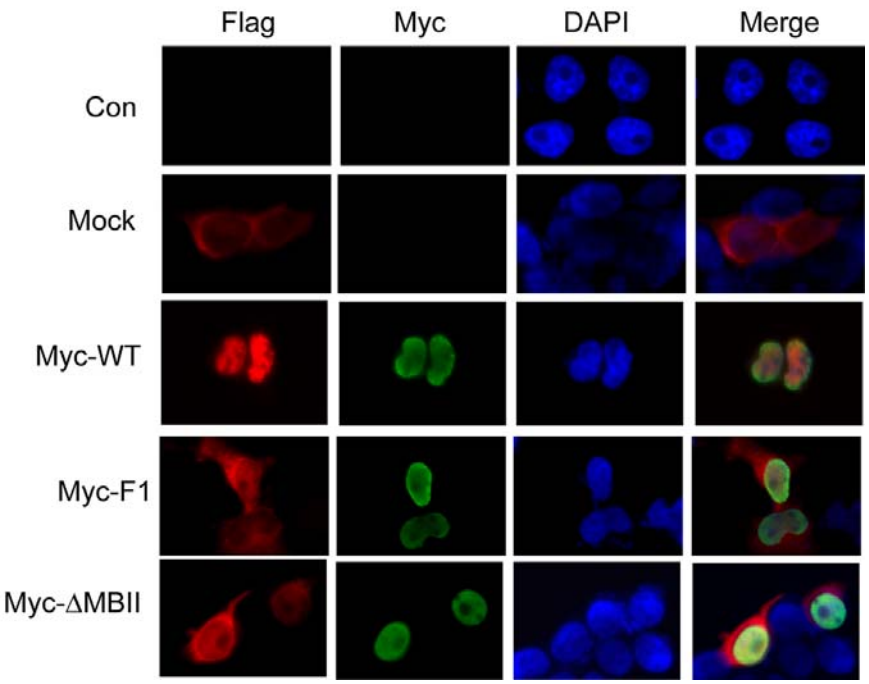

Fig. 3. c-Myc affects the subcellular localization of FBX8. 293T cells were transfected with plasmids encoding SFB-FBX8 olny (Mock) and Myc-WT, Myc-F1, or Myc-ΔMBII. Con: control plasmid transfec-ted into the 293T cells. Immunofluorescent assays using transfected cells were performed with anti-Flag and -Myc antibodies. DAPI was used as an indicator for the nucleus.

indicate that c-Myc affects the nuclear localization of FBX8 through binding with its Myc box II region.

c-Myc releases the FBX8-mediated inhibition of ARF6 activity

Since c-Myc affects the cellular localization of FBX8, we hypothesized that c-Myc protein could allow recovery from FBX8 inhibition of ARF6-mediated cell invasion activity. ARF6 is active when bound to GTP and inactive when bound to GDP, in a manner similar to that of other small members of the G-protein family. Active ARF6 binds specifically to the protein-binding domain (PBD) of GGA3, a downstream effector (Hall et al., 2008). Therefore, we examined the effects of Myc overexpression on ARF6 activity using the GST-GGA pull down assay. Overexpression of c-Myc in 293T cells increased ARF6 activity, showing increased binding to GGA protein, but the TAD-deleted mutant c-Myc (Myc F1) did not (Fig. 4A). In addition, we confirmed that the presence of c-Myc protein induces recovery from the FBX8-mediated suppression of ARF6 activity (Fig. 4B). We also analyzed the effect of endogenous c-Myc activity on FBX8-mediated ARF6 suppression using siRNA-mediated c-Myc knockdown Hela cells. Downregulation of c-Myc in Hela cells transfected with c-Myc siRNA inhibits ARF6 activity by reducing the association of ARF6 protein with GGA (Fig. 4C). These data indicate that c-Myc is a very important molecule for the activation of ARF6. Next, we measured *in vitro* cell invasive activity by quantitative assay (Fig. 4D). The cells that invaded through the matrigel were stained with calcein-AM in PBS, and fluorescent cells were counted. Following ARF6 expression, invasion increased slightly, and ARF6-mediated cell invasive activity decreased when co-transfected with both ARF6 and FBX8 expression plasmids. In addition, the presence of c-Myc protein induces recovery from FBX8-mediated inhibition of cell invasive activity (Fig. 4D). We also obtained similar results using a matrigel barrier for cell invasive activity (Fig. 4E). We also examined whether or not c-Myc inhibits the FBX8-mediated ubiquitination of ARF6. Figure 4F shows that coexpression of FBX8 and ARF6 increases ARF6 ubiquitination, and FBX8-mediated ubiquitination of ARF6 is reduced in the presence of c-Myc protein. These data indicate that c-Myc has the ability to inhibit FBX8-mediated ubiquitination of ARF6, suggesting a mechanism by which c-Myc protein may contribute to the acquisition of invasive activity of certain cancer cells. Our *in vitro* data show that FBX8 likely inhibits cell invasion, and they suggest the possibility that FBX8 expression would be reduced in highly invasive cancer cell lines compared to that in non-invasive cancer cell lines. Therefore, we checked the expression levels of FBX8 and c-Myc in several hepatocellular carcinoma cell lines that have different cell invasive activities (Masumoto et al., 1999) by Western blotting with anti-FBX8 or anti-Myc antibodies. As shown in Fig. 4G, the expression levels of c-Myc were not different, but the expression level of FBX8 was very low in several invasive hepatocellular carcinomas including HepG2, HLE, and PLC compared to the non-invasive Huh7 cell line.

DISCUSSION

In this paper, we identify FBX8 as a novel, c-Myc binding protein using the tandem repeat affinity purification method and provide the novel mechanism of c-Myc protein influence on cell invasion by regulating FBX8 on the activation of the ARF6 protein.

The oncogenic activity of Myc depends on the increase of its stability by mutating in the MBI region and association with its MBII region. Our data also demonstrate that c-Myc binds to FBX8 in its MBII region, showing that the interaction between c-Myc and FBX8 is reduced when we use the the MBII region deletion mutants and tumor-derived c-Myc mutations located in the c-Myc box II (P138C and W135E), which are less potent than wild-type c-Myc in promoting cell transformation (Kuttler et al., 2001). This association translocates FBX8 proteins into the nucleus from the cytoplasm and suggests that cytoplasmic ARF6 is released via the inhibitory effects of FBX8-mediated

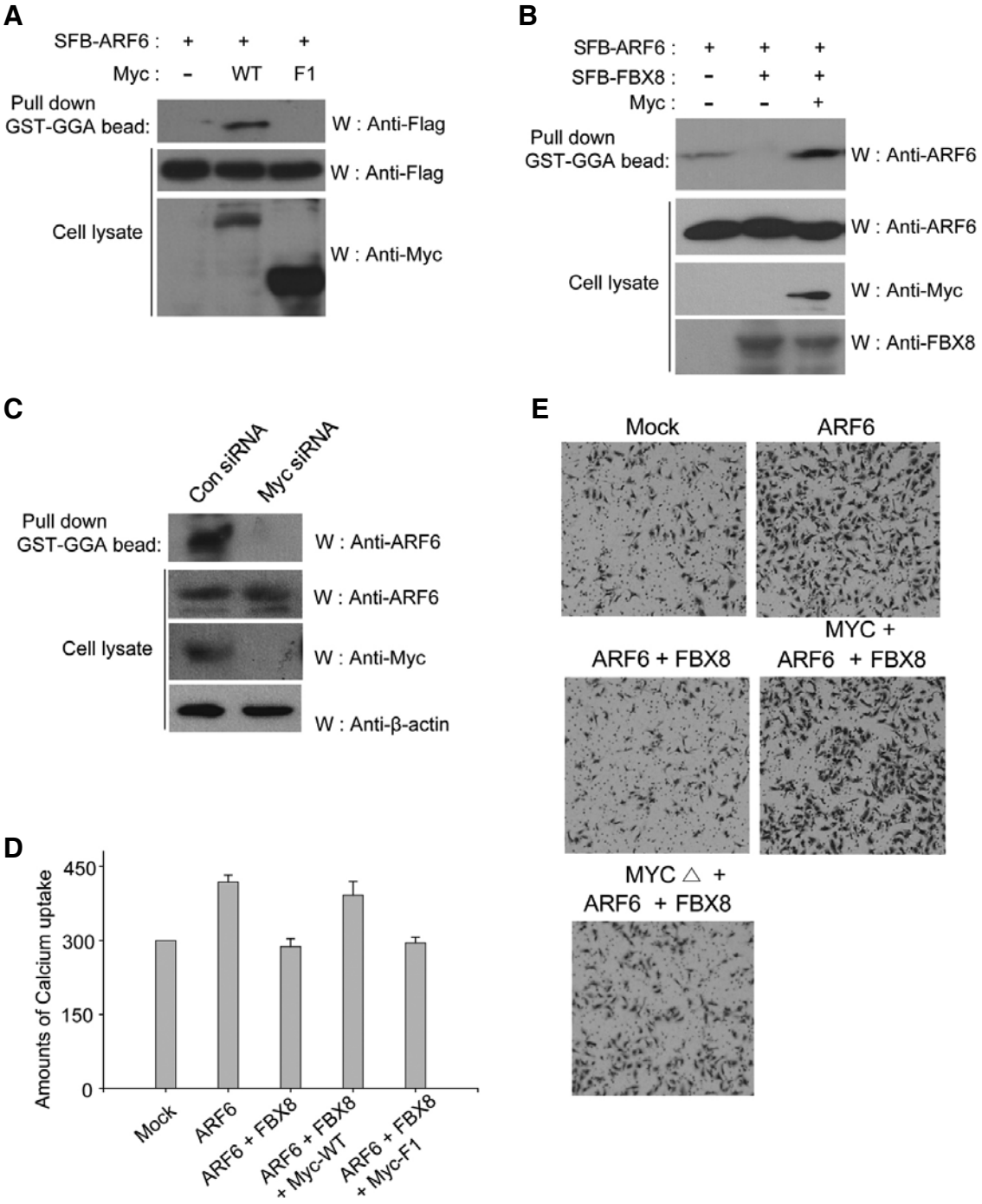

Fig. 4. (continued)

ubiquitination, and it subsequently stimulates ARF6 activation of cell invasion. The ability of c-Myc to inhibit a specific protein's activity by translocating from the cytoplasm into the nucleus may be a novel mechanism of a Myc protein on its cell invasive activity. It remains to be elucidated if the binding between FBX8 and c-Myc is a consistent or inducible event; however, this association may be mediated by several proteins because many proteins bind to the MBII region.

We show that hepatocellular carcinomas have similar c-Myc expression levels, but these cells have FBX8 expression levels that differ from their invasive activities (Fig. 4G). This result is consistent with results from a recent paper (Yano et al., 2008), which shows that the expression level of FBX8 protein is impaired in the highly invasive breast cancer cells even though the amount of FBX8 mRNAs in these cells remains unchanged. Therefore, FBX8 expression levels may be an important factor that contributes to malignant cancer development in different cancer cells. Our results also indicate that c-Myc is also an important factor for cell invasive activity to translocate FBX8 from the cytoplasm into the nucleus. Furthermore, it is interest-

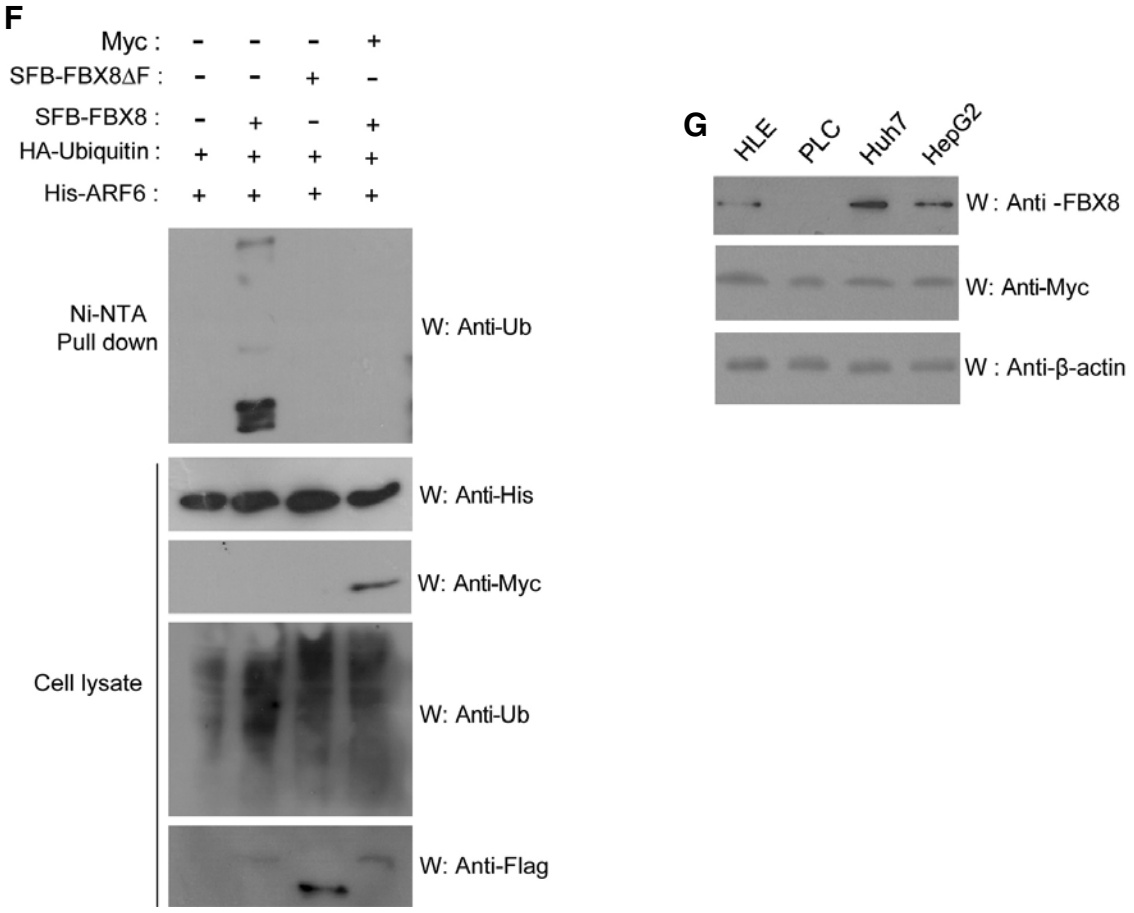

Fig. 4. c-Myc protein releases the FBX8-mediated inhibition of ARF6 function. (A) c-Myc overexpression increases ARF6 activity. 293T cells were transfected with the Flag-ARF6 expression plasmid with/without Myc-WT or Myc-F1 deletion mutant expression plasmid, as indicated in the figure. After 48 h, the GST pull down assay was performed on the transfected cell lysates using GST-GGA beads and analyzed by Western blot using anti-Flag antibody (upper panel). The expression levels of ARF and wild-type c-Myc or the Myc-F1 deletion mutant were detected using the anti-Flag antibody (middle panel) or the anti-Myc antibody (bottom panel). (B) c-Myc releases FBX8-mediated inhibition of ARF6 activity. 293T cells were transfected with ARF6, FBX8, and the c-Myc wild-type expression plasmid, as indicated. After 48 h, transfected cell lysates were subjected to a GST pull down assay using the GST-GGA beads and analyzed by Western blot using the anti-ARF6 antibody (first panel). The expression levels of ARF6, wild-type c-Myc, or FBX8 were detected using the anti-ARF6 antibody (second panel), anti-Myc antibody (third panel), or anti-FBX8 antibody (fourth panel). (C) Downregulation of c-Myc inhibits ARF6 activity. Hela cells were transfected with control or c-Myc siRNA. After 48 h, transfected cell lysates were subjected to a GST pull down assay using the GST-GGA beads. The expression levels of ARF6, wild-type c-Myc, or FBX8 were detected using the anti-ARF6 antibody (second panel), anti-Myc antibody (third panel), or the anti-FBX8 antibody (fourth panel). (D, E) c-Myc expression promotes recovery from FBX8-mediated inhibition of cell invasion. Invasion assays using the transfected U2OS cells were performed using the quantitative assay (D) or the matrigel assay (E). U2OS cells were transfected with ARF6, FBX8, c-Myc wild-type, and/or Myc F1 deletion mutant expression plasmid as indicated. After 16 h, the quantitative assay (D) and the matrigel assay (E) were used to measure the invasive activity of the transfected cells. The quantitative assay was performed in duplicate, and the results shown are the average of two independent experiments. S.D. is shown on each bar. (F) c-Myc reduces the FBX8-mediated ubiquitination of ARF6. 293T cells were transfected with ARF6, FBX8, wild-type c-Myc, and/or ubiquitin expression plasmid as indicated. FBX8ΔF lacks the F-box domain required for E3 ligase activity. After 48 h, a Ni-NTA pull down assay was performed on the cell lysates, followed with Western blot analysis using the anti-HA antibody (first panel) to detect ubiquitinated ARF6. The expression levels of ARF6, FBX8, or wild-type c-Myc were detected using the anti-His antibody (second panel), anti-Myc antibody (third panel), anti-Ub antibody (fourth panel), and anti-Flag antibody (fifth panel). (G) c-Myc and FBX8 protein expression levels in hepatocellular carcinomas. The lysates of various hepatocellular carcinoma cell lines were subjected to immunoblotting with the indicated antibodies.

ing to analyze the mutation in the MBII region, causes hepatocellular carcinomas to reduce the binding between FBX8 and c-Myc.

In conclusion, we show the novel mechanism of the c-Myc protein for its cell invasive activity by recovering FBX8-mediated inhibition of the ARF6 functions. This identification of FBX8 as a new c-Myc binding protein provides new implications for the interplay of c-Myc, FBX8, and ARF6 in cell invasion and it provides insight into the protein networks involved in cancer development in the some cancer cells.

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