Fbxw7 contributes to tumor suppression by targeting multiple proteins for ubiquitin-dependent degradation

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(Received February 2, 2006/Revised March 31, 2006/Accepted April 10, 2006/Online publication June 13, 2006)

Fbxw7 (also known as Sel-10, hCdc4 or hAgo) is the F-box protein component of a Skp1–Cul1–F-box protein (SCF) ubiquitin ligase. Fbxw7 contributes to the ubiquitin-mediated degradation of cyclin E, c-Myc, Aurora-A, Notch and c-Jun, all of which appear to function as cell-cycle promoters and oncogenic proteins. Loss of Fbxw7 results in elevated expression of its substrates, which may lead to oncogenesis. However, it remains largely unclear which accumulating substrate is most related to cancer development in Fbxw7-mutant cancer cells. In the present study, we examined the abundance of cyclin E, c-Myc and Aurora-A in seven cancer cell lines, which harbor wild-type (three lines) or mutant (four lines) Fbxw7. Although these three substrates accumulated in the Fbxw7-mutant cells, the extent of increase in the expression of these proteins varied in each line. Forced expression of Fbxw7 reduced the levels of cyclin E, c-Myc and Aurora-A in the Fbxw7-mutant cells. In contrast, a decrease in the expression of cyclin E, c-Myc or Aurora-A by RNA interference significantly suppressed the rate of proliferation and anchorage-independent growth of the Fbxw7-mutant cells. These findings thus suggest that the loss of Fbxw7 results in accumulation of cyclin E, c-Myc and Aurora-A, all of which appear to be required for growth promotion of cancer cells. Fbxw7 seems to regulate the levels of multiple targets to suppress cancer development. (*Cancer Sci* 2006; 97: 729–736)

The ubiquitin-proteasome pathway plays important roles in many cell functions by determining the abundance of regulatory proteins. Dysregulation of the proteolytic system may result in uncontrolled proliferation and genomic instability, leading to cancer development.[1] The ubiquitin ligase component of the enzyme cascade that mediates ubiquitin–protein conjugation is responsible for determination of target specificity.[2] Two major classes of ubiquitin ligases, the Skp1–Cul1–F-box protein (SCF) complexes[3–5] and the anaphase promoting complex/cyclosome (APC/C),[6,7] play a central role in cell cycle regulation.[8,9] The SCF complex consists of four components: the invariable subunits Skp1, Cul1 and Rbx1 (also known as Roc1 or Hrt1) and a variable F-box protein that serves as a receptor for target proteins and thereby determines target specificity. Among the many F-box proteins that have been identified, Skp2 and Fbxw7 have been well characterized and shown to control the abundance of proteins important in cell cycle regulation. Whereas Skp2 targets mainly cyclin-dependent kinase inhibitors p21,[10] p27[11–13] and p57[14] for degradation, Fbxw7 contributes to degradation of cyclin E,[15–17] c-Myc,[18,19] Aurora-A,[20] Notch[21–24] and c-Jun,[25] all of which appear to function as cell cycle activators and oncogenic proteins.

Given that Fbxw7 is responsible for the degradation of the above-mentioned oncoproteins, Fbxw7 is thought to function as a tumor suppressor. Indeed, mutations in the *Fbxw7* gene were found in ovarian cancer,[26] breast cancer,[17,27] endometrial cancer,[28,29] lymphoma[16] and colorectal cancer.[30] In animal models, $Fbxw7^{+/-}$ mice have greater susceptibility to radiation-induced tumorigenesis, but most tumors retain and express the wild-type allele, indicating that *Fbxw7* is a haploinsufficient tumor suppressor gene.[20] However, it has been largely unclear how the decrease in Fbxw7 function results in cancer development. Dysregulation of cyclin E is considered a major factor in tumorigenesis because elevated levels of cyclin E have been associated with a variety of malignancies and constitutive expression of cyclin E leads to genomic instability.[31] Furthermore, knockdown of cyclin E expression in the cancer lines in which Fbxw7 was also knocked out significantly reduced the extent of chromosomal instability.[30]

Another important substrate of Fbxw7 whose dysregulation might be responsible for cancer development is c-Myc. We and others showed that Fbxw7 interacts with and promotes the degradation of c-Myc in a manner that is dependent on phosphorylation of threonine-58, a mutation of which stabilizes the protein strongly.[18,19] Accumulation of c-Myc is also apparent in mouse $Fbxw7^{-/-}$ cells[18] as well as in lymphomas that have arisen from $Fbxw7^{+/-}$ mice,[20] both of which do not exhibit accumulation of cyclin E.[20,32] Therefore, the mechanism that is responsible for cancer development in Fbxw7-mutant cells remains controversial.

By genetic studies, we and others have demonstrated that among the many targets of Skp2, p27 seems to be the major one: prominent cellular phenotypes apparent in $Skp2^{-/-}$ mice–including nuclear enlargement, polyploidy and an increased number of centrosomes that are likely due to overreplication of chromosomes and centrosomes[33] – disappear in $Skp2^{-/-}$, $p27^{-/-}$ double mutant mice.[34,35] By analogy to the genetic study for Skp2 and p27,[33,34,36] we depleted accumulating

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doi: 10.1111/j.1349-7006.2006.00239.x

substrates such as cyclin E, c-Myc and Aurora-A by RNA interference (RNAi) in cancer cells that lack functional Fbxw7, and evaluated the biological consequences of the knockdown. Our results indicate that all of these substrates are required for efficient proliferation, as well as anchorage-independent growth of Fbxw7-mutant cancer cells. Therefore, Fbxw7 seems to suppress cancer development by mediating ubiquitin-dependent proteolysis of multiple oncogenic proteins.

Materials and Methods

Cell culture

The T47D and SUM149PT cell lines were obtained from the University of Michigan Breast Cell/Tissue Bank (Ann Arbor, MI, USA). MDAH2774 and OVCAR3 were obtained from the American Type Culture Collection (Manassas, VA, USA). SKOV3, an ovarian cancer-derived cell line, was a kind gift of H. Kobayashi (Graduate School of Medical Sciences, Kyushu University, Kyushu, Japan). CCRF-CEM was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). SUM149PT cells were cultured under an atmosphere of 5% CO_2 at 37°C in F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Invitrogen). T47D, MDAH2774, SKOV3, OVCAR3, Jurkat and CCRF-CEM cells were cultured under the same conditions in RPMI-1640 (Sigma, St Louis, MO, USA) supplemented with 10% FBS. For serum deprivation and stimulation experiments, T47D and SUM149PT cells were deprived of serum for 48 h by culture in MCDB105 medium and were then stimulated for various times by incubation with Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 30% FBS.

Antibodies

Mouse monoclonal antibody to cyclin E (sc-247) and rabbit polyclonal antibody to c-Myc (sc-764) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies to Aurora-A kinase (Anti-IAK1/Aurora-A kinase), GSK-3β and Hsp90 were from BD Biosciences (San Jose, CA, USA). Mouse monoclonal antibodies to FLAG (M2) and hemaglutinin (HA; HA11) were from Sigma and BabCO (Richmond, CA, USA), respectively.

Immunofluorescence staining

SUM149PT cells grown on glass cover slips were transfected with the use of FuGENE6 (Roche Applied Science, Indianapolis, IN, USA). Immunofluorescence staining was carried out with the use of mouse monoclonal antibodies to c-Myc, rabbit polyclonal antibodies to HA, Alexa 546-labeled goat polyclonal antibodies to mouse IgG and Alexa 488-labeled goat polyclonal antibodies to rabbit IgG (Molecular Probes, Eugene, OR, USA) as described previously.[37]

RNA interference

The pMX-puro II vector was constructed by deletion of the U3 portion of the 3′ long-terminal repeat of pMX-puro (kindly provided by T. Kitamura, University of Tokyo, Tokyo, Japan).[38–40] The mouse U6 gene promoter, followed by DNA corresponding to a short hairpin RNA (shRNA) sequence, was subcloned into the *Not*I and *Xho*I sites of pMX-puro II, yielding pMX-puro II-U6/siRNA. The DNA for the shRNA encoded a 19–22-nucleotide hairpin sequence specific to the mRNA target, with a loop sequence (3′-TTCAAGAGA-5′) separating the two complementary domains, and contained a tract of five T nucleotides to terminate transcription. The hairpin sequences specific for human cyclin E (GenBank accession no. NM_001238.1), human c-Myc (GenBank accession no. NM_002467.3) and Aurora-A kinase (GenBank accession no. BC002499.2) mRNAs corresponded to nucleotides 1255–1273 (Cyclin E_RNAi1), 532–553 (Cyclin E_RNAi2), 589–610 (Cyclin E_RNAi3), 725–744 (c-Myc_RNAi1), 1831–1851 (c-Myc_RNAi2), 382–402 (c-Myc_RNAi3), 283–301 (Aurora-A_RNAi1) and 853–873 (Aurora-A_RNAi2) of the coding region. The resulting vectors were used to transfect Plat-E cells and to thereby generate recombinant retroviruses. MDAH2774 cells stably expressing mouse ecotropic retrovirus receptor were infected with the recombinant retroviruses. Two days after infection, cells were cultured in the presence of puromycin at 10 μg/mL. After 6 days, cells were harvested and subjected to immunoblotting, cell proliferation assay and soft agar assay.

Cell proliferation assay

Proliferation of cells was monitored using a colorimetric assay based on cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenases according to the manufacturer's instructions (Dojin Laboratories, Tokyo, Japan). Briefly, cells were seeded into 96-well plates at an initial density of 1×10^3 cells/well. Every 24 h, 10 μL of WST-8 was added and incubated for an additional 2 h. After the incubation, absorbance was measured at 450 nm and 620 nm using a microplate reader.

Soft agar assay

The cells were trypsinized, suspended in medium containing 0.33% agar and 10% FBS, and plated onto a bottom layer containing 0.5% agar. The cells were plated at a density of 1×10^4 cells/6 cm dish, and the number of colonies >0.1 mm in diameter in triplicate were counted 3 weeks later.[41]

Production of recombinant proteins

Saccharomyces cerevisiae Uba1, human UbcH, and human ubiquitin 5A were expressed in and purified from *Escherichia coli* as described previously.[40] Recombinant baculoviruses were generated using the BacPAK (BD Biosciences) or Bac-To-BacHT (Invitrogen) baculovirus systems. The recombinant SCF^{Fbxw7} complex was purified as described previously.[14] His_6-Aurora-A was purified similarly.

Assay of ubiquitylation *in vitro*

The ability of the purified recombinant SCF^{Fbxw7} complex to ubiquitylate Aurora A was assayed by incubation of His_6-Aurora-A and the complex, as described previously.[14] The reaction mixtures were subjected to immunoblot analysis with antibodies to Aurora-A.

Results

Accumulation of various substrates in Fbxw7-deficient cancer cell lines

To investigate the role of Fbxw7 in cancer cells, we compared the levels of cyclin E, c-Myc and Aurora-A in

doi: 10.1111/j.1349-7006.2006.00239.x

Table 1. Information of genetic alterations in the cell lines used in this study

Cell line	Genetic alteration
T47D	*p53* mutation
SUM149PT	*Fbxw7* mutation
OVCAR3	*p53* mutation
MDAH2774	*Fbxw7* mutation, *p53* mutation
SKOV3	*Fbxw7* mutation, *p53* mutation, *erbB2* overexpression, *Bcl-2* amplification
Jurkat	*p53* mutation
CCRF-CEM	*Fbxw7* mutation, *p53* mutation, $p16^{INK4}$ mutation

seven cancer cell lines: T47D and SUM149PT (breast cancer lines); OVCAR3, MDAH2774, and SKOV3 (ovarian cancer lines); and Jurkat and CCRF-CEM (T-cell lymphoma lines). T47D, OVCAR3 and Jurkat cells retained functional Fbxw7, whereas SUM149PT and MDAH2774 harbored nonsense mutations and SKOV3 and CCRF-CEM contained missense mutations in the WD40 repeats[16,17] (Fig. 1A). Other genetic alterations in these cell lines are summarized in Table 1. Immunoblot analysis of asynchronous cells revealed that the abundance of cyclin E, c-Myc and Aurora-A in Fbxw7-deficient cells (SUM149PT, MDAH2774, SKOV3 and CCRF-CEM) was significantly greater than that in cells that preserved functional Fbxw7 (T47D, OVCAR3 and Jurkat) (Fig. 1B). However, the extent of accumulation seemed to be variable in each cancer cell line. For example, ovarian cancer lines MDAH2774 and SKOV3, both of which do not have functional Fbxw7, exhibited a different pattern of accumulation: elevation of cyclin E and c-Myc expression was more pronounced in MDAH2774 than in SKOV3, whereas that of Aurora-A was more prominent in SKOV3 than in MDAH2774. The difference in the extent of elevation may be attributable to differences in the intracellular environment as well as in the other regulatory factors, including protein kinases or other ubiquitin ligases.[1]

We examined the turnover of c-Myc in these breast cancer cell lines by immunoblot analysis after consecutive serum deprivation and serum stimulation. In T47D cells, the expression of c-Myc was maximal 1.5 h after exposure to serum and declined gradually thereafter, being virtually undetectable after 9 h. In contrast, the abundance of c-Myc in SUM149PT cells, which was much higher than that in T47D cells, increased with time after exposure to serum, reaching a maximum at 9 h and declining only slightly at 10.5 h (Fig. 1C). These results suggest that loss of Fbxw7 results in not only high levels of c-Myc but also sustained elevation of its expression.

It is well established that cyclin E and c-Myc are the substrates of Fbxw7 and undergo ubiquitylation through a Fbxw7-mediated pathway. In contrast, there is no direct evidence showing that Aurora-A is a substrate of Fbxw7, although Aurora-A accumulates in Fbxw7-mutant tumors.[20] Therefore, we investigated whether Fbxw7 enhances the ubiquitylation of Aurora-A using an *in vitro* ubiquitylation assay. Ubiquitylation of Aurora-A was observed only when E1 (Uba1), E2 (UbcH5A), E3 (SCF^{Fbxw7}), ubiquitin and ATP were included in the reaction mixture (Fig. 2A). These data support the idea that, at least *in vitro*, Aurora-A is ubiquitylated by SCF^{Fbxw7}. Furthermore, we treated cells with the proteasome inhibitor MG132 and compared the abundance of substrates in the absence or presence of the inhibitor. In control cells with wild-type Fbxw7 (Jurkat), treatment with MG132 increased the levels of cyclin E, c-Myc and Aurora-A (Fig. 2B). In contrast, these proteins in Fbxw7-mutant lines were relatively insensitive to this inhibitor, suggesting that the accumulation of substrates is attributable to a defect in the upstream process, which is likely to be protein ubiquitylation mediated by Fbxw7. Taken together, we conclude that Fbxw7 substrates, including cyclin E, c-Myc and Aurora-A, are degraded through the ubiquitin-proteasome system.

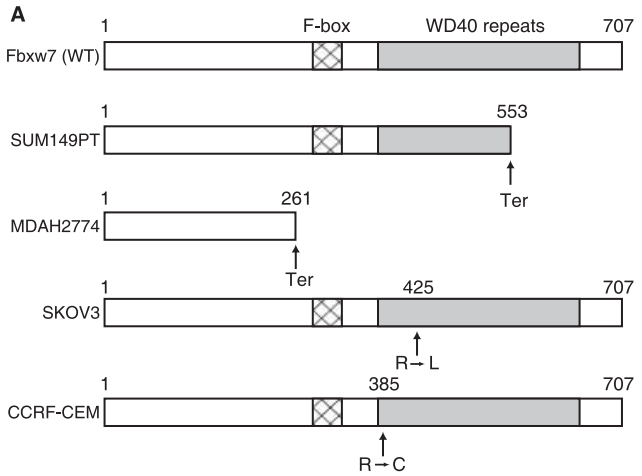
Mutation of Fbxw7 causes c-Myc accumulation in cancer cell lines

To determine whether loss of Fbxw7 might account for elevated levels of cyclin E, c-Myc and Aurora-A, we examined the effect of restoration of Fbxw7 in the Fbxw7-mutant cell lines. We transfected SUM149PT with a vector for HA-tagged Fbxw7 and carried out immunofluorescence staining with antibodies to c-Myc and to HA. In most cells in which Fbxw7 expression was restored (HA-positive cells), the abundance of c-Myc was greatly reduced compared with that in HA-negative cells (Fig. 3A). These data suggest that the deficiency of Fbxw7 in SUM149PT cells results in the accumulation of c-Myc, which may contribute to carcinogenesis.

Furthermore, we also reconstituted wild-type Fbxw7 in MDAH2774 and SKOV3 Fbxw7-mutant ovarian cancer cell lines by transfection of the vector for HA-tagged Fbxw7, and carried out immunoblot analysis. The expression levels of cyclin E, c-Myc and Aurora-A in transfected cells were decreased markedly compared to those in non-transfected cells (Fig. 3B). These results suggest that mutations of Fbxw7 are directly responsible for the elevated expressions of cyclin E, c-Myc and Aurora-A in the Fbxw7-mutant breast and ovarian cancer cell lines.

Decrease in expression of the substrates by RNA interference significantly reduces the growth rate of Fbxw7-mutant cells

Given that cyclin E, c-Myc and Aurora-A are well-known cell cycle regulators that are integral for cellular proliferation, elevated levels of these proteins in Fbxw7-mutant cells might be responsible for oncogenesis. To investigate which accumulating protein is most related to cellular proliferation, we tried to decrease the expression of cyclin E, c-Myc and Aurora-A using RNAi in MDAH2774 cells. Cells infected with a retroviral vector encoding a shRNA specific for cyclin E, c-Myc, or Aurora-A mRNA exhibited a significant decrease in the abundance of these proteins compared with that apparent in cells infected with a control vector (Fig. 4A,C,E). Depletion of cyclin E, c-Myc or Aurora-A by RNAi also



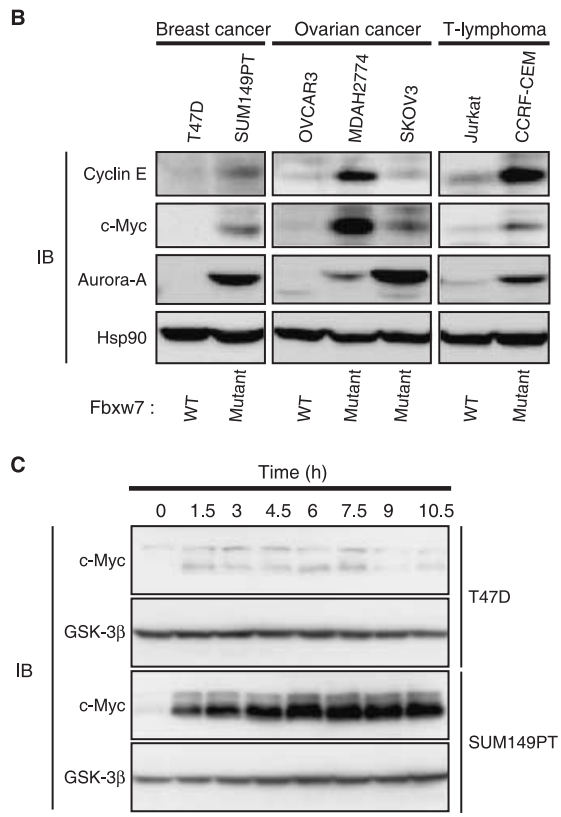
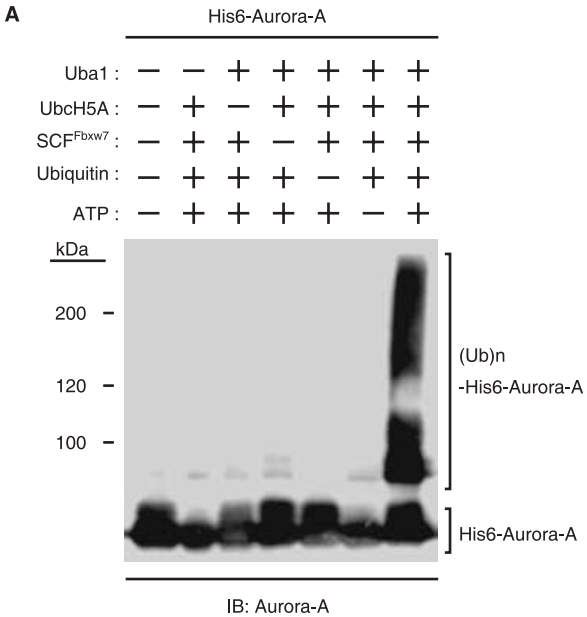


Fig. 1. Elevated expression of cyclin E, c-Myc and Aurora-A in Fbxw7-mutant cancer cell lines. (A) The structure of wild-type (WT) and mutant Fbxw7 in cancer cell lines. The SUM149PT breast cancer cell line harbors a mutation that eliminates the last four of seven WD40 repeats. In the MDAH2774 ovarian cancer cell line, a splice acceptor site is disrupted, giving rise to a protein that lacks the F-box domain and all seven WD40 repeats. SKOV3 and CCRE-CEM cell lines possess amino acid changes in the WD40 repeats as indicated. (B) Immunoblot analysis of Fbxw7-intact (WT) and Fbxw7-mutant cell lines for the abundance of cyclin E, c-Myc and Aurora-A. The expression of Hsp90 was examined as a loading control. (C) SUM149PT and T47D cells were deprived of serum for 48 h and then stimulated by reexposure to serum for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to c-Myc or to GSK-3β.



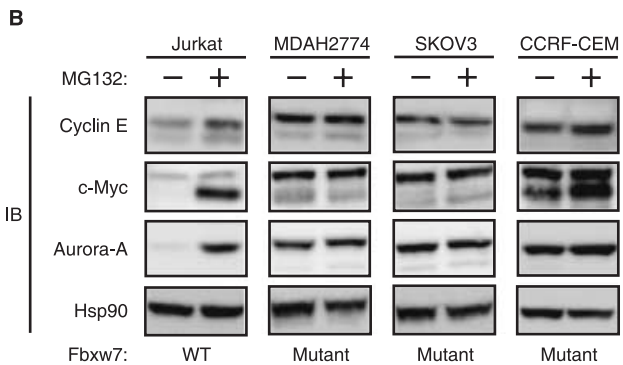


Fig. 2. Fbxw7 mediates ubiquitin-dependent degradation of target proteins. (A) Ubiquitylation of Aurora-A by the recombinant SCF^{Fbxw7} complex *in vitro*. Recombinant SCF^{Fbxw7} was assayed for ubiquitylation activity with His_6-Aurora-A as substrate in the absence or presence of the indicated reaction mixture components. The reaction mixtures were then subjected to immunoblot analysis with antibodies to c-Myc. The positions of unmodified His_6-Aurora-A and of His_6-Aurora-A conjugated with ubiquitin ($[Ub]_n$) are indicated. (B) Cells were incubated with or without the proteasome inhibitor MG132 for 6 h. Cell lysates were subjected to immunoblot analysis with the antibodies indicated. WT, wild type.

resulted in a marked decrease in the rate of proliferation monitored by WST-8 assay (Fig. 4B,D,F). These findings suggest that any one of cyclin E, c-Myc or Aurora-A is required for efficient proliferation of the Fbxw7-mutant cells.

Anchorage-independent growth also requires accumulation of substrates in Fbxw7-mutant cells

We then tested whether dysregulated expression of cyclin E, c-Myc or Aurora-A is required for the anchorage-independent growth of MDAH2774 cells that are cultured in soft agar. MDAH2774 cells that were infected with the mock retroviral vector showed efficient growth in soft agar (Fig. 5A). In

doi: 10.1111/j.1349-7006.2006.00239.x

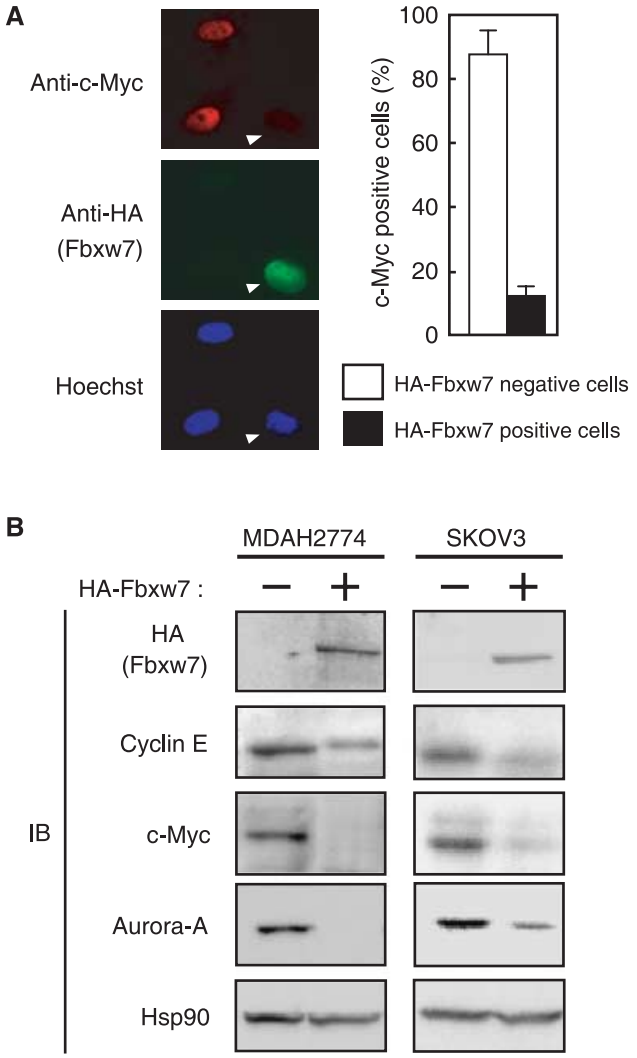


Fig. 3. Downregulation of cyclin E, c-Myc and Aurora-A by introduction of wild-type Fbxw7 in the Fbxw7-mutant cells. (A) Twenty-four hours after transfection with a vector for hemaglutinin (HA)-Fbxw7, SUM149PT cells were stained with antibodies to c-Myc and to HA, and with Hoechst dye (left panels), as indicated. Arrowhead shows a cell in which expression of Fbxw7 was restored. The percentage of cells positive for c-Myc among HA-negative or HA-positive cells was determined (right panel); data are mean ± SD of at least 300 cells positive or negative for HA expression. (B) HA-tagged Fbxw7 or mock vector was introduced in MDAH2774 and SKOV3 ovarian cancer cell lines. Immunoblot analysis was performed as indicated.

contrast, RNAi-directed suppression of cyclin E (Fig. 5B–D), c-Myc (Fig. 5E–G) or Aurora-A (Fig. 5H and I) resulted in a significant decrease in colony formation of MDAH2774 cells in soft agar (Fig. 5J). These results suggest that accumulation of any of these substrates is essential for the anchorage-independent growth of MDAH2774 cells.

Our data suggest that the deficiency of Fbxw7 in cancer cells results in the accumulation of cyclin E, c-Myc and Aurora-A, all of which may contribute to cancer development. Therefore, Fbxw7 seems to regulate multiple proteins that promote cell proliferation and oncogenesis by ubiquitin-mediated degradation.

Discussion

Fbxw7 was first discovered as a negative regulator of the lin-12 (Notch) signaling pathway in *Caenorhabditis elegans* by genetic screening.[21] We and others have generated mice that are deficient in Fbxw7 and found that the embryos die *in utero* at embryonic day 10.5, manifesting marked abnormalities in vascular development.[32,42] Notch accumulates in *Fbxw7*$^{-/-}$ embryos, resulting in the increased expression of Hey1, a transcriptional repressor that acts downstream of Notch and is implicated in vascular development.[32] Thus, Fbxw7 plays a critical role in mammalian vascular development by regulating Notch stability during embryogenesis.

Fbxw7 participates in not only development but also tumor suppression, given that Fbxw7 is responsible for the degradation of cyclin E, c-Myc, Aurora-A, Notch and c-Jun, all of which are known as growth promoters whose dysregulation may induce oncogenesis. How does the decrease in Fbxw7 function result in cancer development? Although dysregulation of cyclin E has been considered a major factor in accelerated growth of cells that lack functional Fbxw7, recent studies do not always support this notion. Expression of cyclin E is not always elevated in cancer cells in which Fbxw7 is mutated[20,27] (our unpublished observations). Furthermore, expression of cyclin E was unaffected in *Fbxw7*$^{-/-}$ embryos,[20,32] whereas the abundance of cyclin E was increased only in the placenta.[42] Radiation-induced lymphomas are observed frequently in *Fbxw7*$^{+/-}$ mice, but the levels of cyclin E are not elevated.[20] It therefore seems likely that Fbxw7 contributes to cyclin E proteolysis in a context-dependent manner.

Another critical target of Fbxw7 whose dysregulation might be responsible for cancer development is c-Myc. Given that the expression level of c-Myc is increased in many malignant tumors and many c-*Myc* mutations affect the stability of the encoded protein,[43] its turnover is thought to be a critical determinant of carcinogenesis. c-Myc is ubiquitylated and degraded by the proteasome. The region of c-Myc that signals its ubiquitylation (the degron) overlaps with the transactivation domain, in which two highly conserved sequence elements, Myc box (MB) 1 and MB2, have been implicated in the proteolysis of c-Myc. In particular, phosphorylation of Thr-58 and Ser-62 in MB1 is an important determinant of c-Myc stability.[43] Growth factors control c-Myc stability mainly through the phosphorylation of these sites. Consistent with the effect of phosphorylation on c-Myc stability, these two residues are mutated frequently in human tumors.[43] Fbxw7 interacts with and promotes the degradation of c-Myc in a manner that is dependent on phosphorylation of MB1.[18,19] In the present study, we demonstrated that c-Myc expression is not only elevated but also abnormally extended in SUM149PT (Fbxw7-mutant breast cancer line) compared with that in T47D (Fbxw7-intact breast cancer line).

Elevated expression not only of cyclin E and c-Myc but also of other substrates of Fbxw7 may contribute to cancer development. Increased levels of Aurora-A have been described in many human cancers,[44] and lead to cells passing through mitosis without cytokinesis, producing tetraploid progeny, which gives rise to aneuploid cells in the subsequent cell division cycle particularly in the absence of the tumor suppressor p53. Dysregulation of wild-type Notch, Notch ligands and

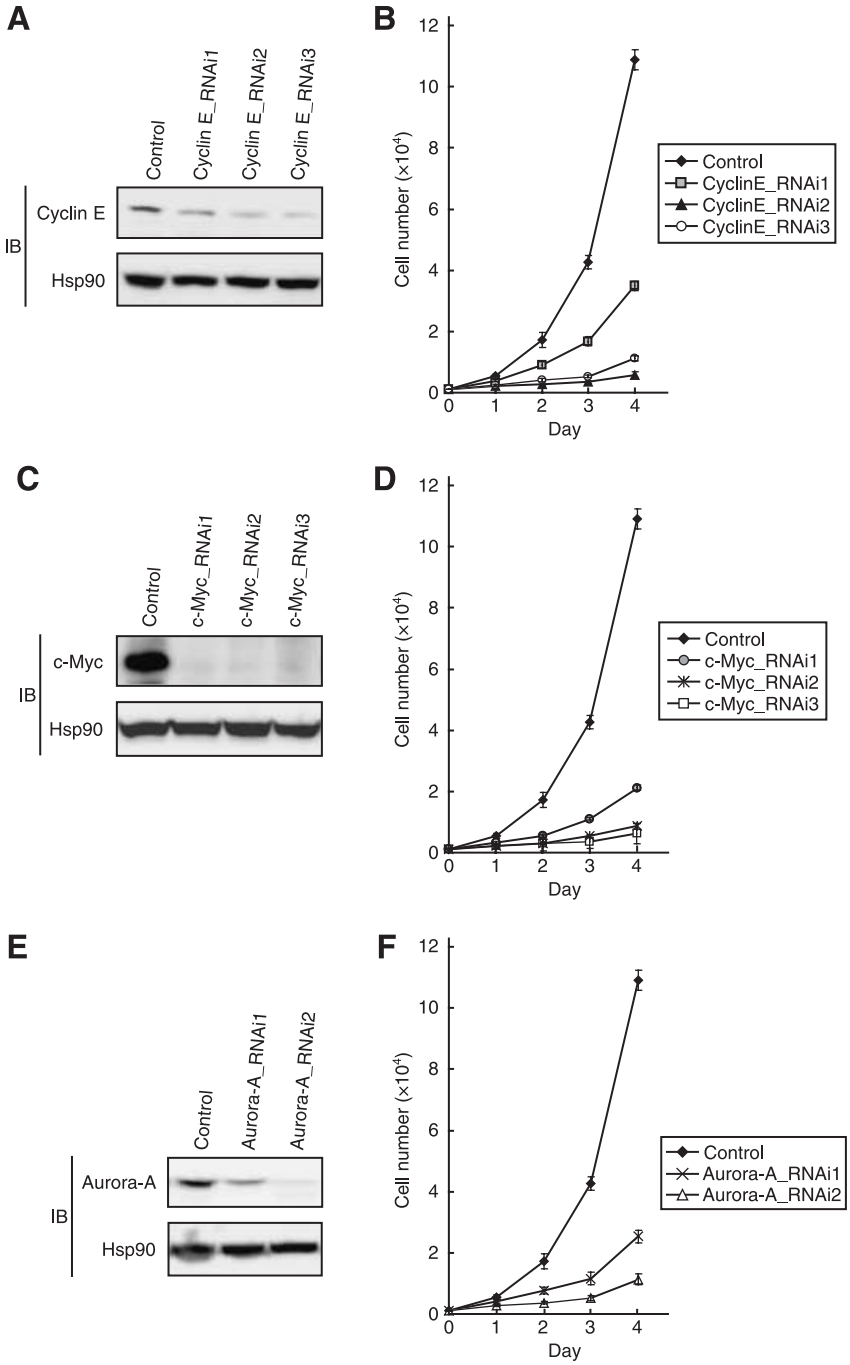


Fig. 4. RNA interference-mediated knockdown of cyclin E, c-Myc or Aurora-A reduces the rate of cell proliferation in Fbxw7-mutant cells. (A,C,E) Lysates from MDAH2774 cells that were infected with a retroviral vector alone (control) or those encoding short hairpin RNA (shRNA) targeting (A) cyclin E, (C) c-Myc or (E) Aurora-A were subjected to immunoblot analysis with antibodies to the indicated proteins. Hsp90 was used as a loading control. (B,D,F) The proliferation rate of MDAH2774 cells that were infected with a retroviral vector alone (control) or those encoding shRNA targeting (B) cyclin E, (D) c-Myc or (F) Aurora-A were monitored with the WST-8 assay. These experiments were performed three times. Cell number was estimated from the absorbance value. RNAi, RNA interference.

downstream targets has also been detected in many human malignancies.[45] Truncated Notch proteins exhibit transforming activity both *in vitro* and in animal models.[46–49] *Notch4* was originally identified as *Int3*, a protooncogene that is a frequent target for the integration of mouse mammary tumor virus in mammary carcinomas.[50] The c-Jun oncoprotein is a major component of transcription factor AP-1, the constitutive activation of which is apparent in various types of human tumor cells, suggesting that AP-1 plays an important role in human oncogenesis.[51] Mutation of the human *Fbxw7* gene may thus result in impaired degradation of multiple substrates and their consequent accumulation, which might then cooperatively contribute to carcinogenesis. However, our strategy to reduce the expression of each Fbxw7 substrate in Fbxw7-deficient cells might have a potential problem in that excessive removal of an essential factor to drive the cell cycle under physiological levels results in inhibition of cellular proliferation regardless of the status of Fbxw7. Indeed, some reports showed that cellular proliferation as well as anchorage-independent colony formation in Fbxw7-normal cells was suppressed by RNAi-mediated suppression of cyclin E,[52] c-Myc[53] and Aurora-A,[54,55] although further investigation

doi: 10.1111/j.1349-7006.2006.00239.x

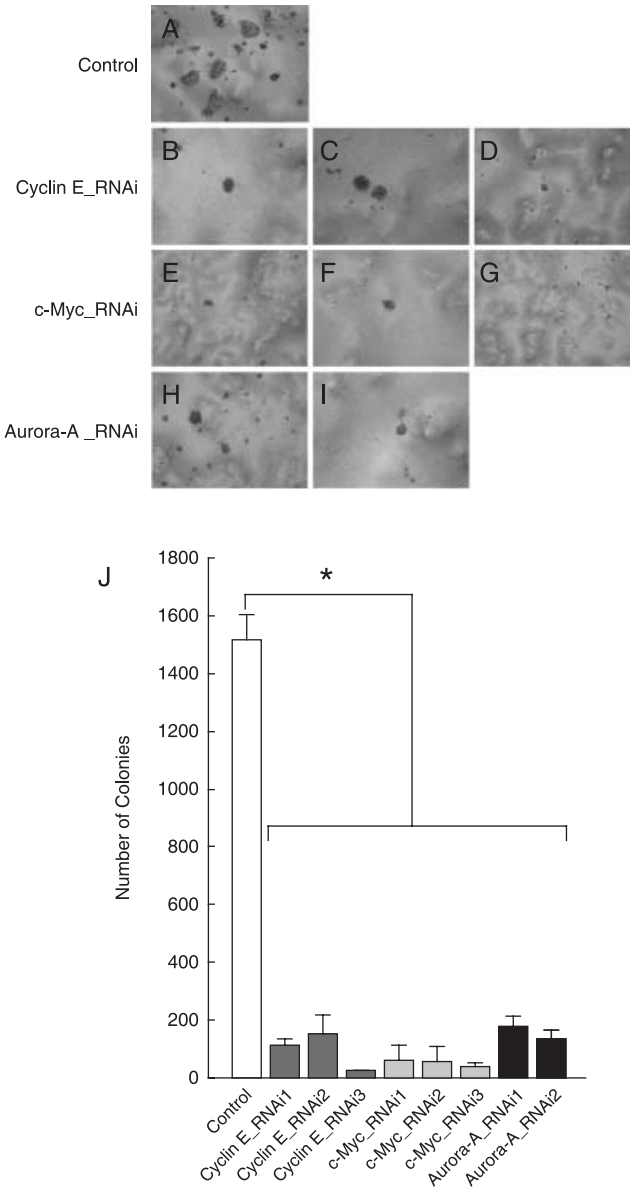


Fig. 5. Downregulation of Cyclin E, c-Myc or Aurora-A was sufficient to suppress colony formation in a Fbxw7 mutant ovarian cell. (A–I) Anchorage-independent growth of MDAH2774 cells that were infected with (A) a retroviral vector alone (control) or those encoding short hairpin RNA (shRNA) targeting (B–D) cyclin E, (E–G) c-Myc or (H,I) Aurora-A were monitored in soft agar. The cells were cultured for 3 weeks. Representative images are shown. (J) Statistical analysis for the colony formation of MDAH2774 cells in soft agar. The total number of colonies with diameter >0.1 mm are shown. *$P < 0.005$. RNAi, RNA interference.

remains to be performed. Given that Fbxw7 targets multiple oncoproteins for degradation, introduction of Fbxw7 into cancer cells may be a powerful tool for cancer treatment.

Acknowledgments

We thank T. Sato, I. Onoyama and Y. Fukui for valuable discussions and N. Nishimura, K. Shinohara and Y. Yoshimura for technical assistance. We also thank A. Ohta and M. Kimura for help in preparation of the manuscript.

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doi: 10.1111/j.1349-7006.2006.00239.x