

I κ B Kinase Promotes Tumorigenesis through Inhibition of Forkhead FOXO3a

Mickey C.-T. Hu,^{1,3,*} Dung-Fang Lee,^{1,3,4}

Weiya Xia,^{1,4} Leonard S. Golfman,¹

Fu Ou-Yang,¹ Jer-Yen Yang,¹ Yiyu Zou,¹

Shilai Bao,¹ Norihisa Hanada,¹ Hitomi Saso,¹

Ryuji Kobayashi,² and Mien-Chie Hung^{1,3,*}

¹Department of Molecular and Cellular Oncology

²Department of Molecular Pathology

M.D. Anderson Cancer Center

³Graduate School of Biomedical Sciences

The University of Texas

Houston, Texas 77030

Summary

Nuclear exclusion of the forkhead transcription factor FOXO3a by protein kinase Akt contributes to cell survival. We investigated the pathological relationship between phosphorylated-Akt (Akt-p) and FOXO3a in primary tumors. Surprisingly, FOXO3a was found to be excluded from the nuclei of some tumors lacking Akt-p, suggesting an Akt-independent mechanism of regulating FOXO3a localization. We provide evidence for such a mechanism by showing that I κ B kinase (IKK) physically interacts with, phosphorylates, and inhibits FOXO3a independent of Akt and causes proteolysis of FOXO3a via the Ub-dependent proteasome pathway. Cytoplasmic FOXO3a correlates with expression of IKK β or Akt-p in many tumors and associates with poor survival in breast cancer. Further, constitutive expression of IKK β promotes cell proliferation and tumorigenesis that can be overridden by FOXO3a. These results suggest the negative regulation of FOXO factors by IKK as a key mechanism for promoting cell growth and tumorigenesis.

Introduction

Constitutive activation of cell survival signaling is a general mechanism underlying tumor development and resistance to therapy and constitutes a major clinical problem in cancer (Hanahan and Weinberg, 2000). One of the key survival signaling mechanisms is the PtdIns-3-kinase (PI3K)/Akt pathway that can be activated by oncogenes, and the catalytic subunit of PI3K and Akt have been aberrantly expressed or constitutively activated in several tumor malignancies (reviewed in Vivanco and Sawyers, 2002; Nicholson and Anderson, 2002). Upon stimulation with growth factors, their receptors trigger PI3K to increase production of PtdIns-3,4,5-triphosphate that binds to the pleckstrin homology domain of Akt, resulting in recruitment of Akt to the plasma membrane. A conformational change of Akt follows that enables residues Thr308 and Ser473 of Akt to be phosphorylated by upstream kinases (Vivanco and Sawyers,

2002). Hence Akt becomes activated and in turn phosphorylates a number of downstream critical targets that regulate cell death such as Bad and caspase-9 or protein synthesis such as the mammalian target of rapamycin (mTOR) in translation initiation or other metabolic functions (Nicholson and Anderson, 2002).

In addition to translational modification, Akt regulates transcription through modulation of the activity of Forkhead FOXO transcription factors FOXO1 (FKHR), FOXO3a (FKHRL1), and FOXO4 (AFX) by phosphorylating them at three conserved serine/threonine residues (Thr32, Ser253, and Ser315 of FOXO3a) (reviewed in Burgering and Kops, 2002; Tran et al., 2003). This leads to the release of FOXO transcription factors from the DNA and translocation to the cytoplasm, where 14-3-3 protein binds to the phosphorylated FOXO factors and retains them in the cytoplasm presumably by masking their nuclear localization signal and preventing their re-entry into the nucleus. However, in the absence of growth or survival signal stimulation, Akt is inactivated in the quiescent cells; this inactivation results in retention of FOXO factors in the nucleus and thereby upregulates expression of specific target genes that modulate the metabolic state (Carlsson and Mahlapuu, 2002), control cell cycle progression (e.g., cyclin-dependent kinase inhibitor p27kip1 [Medema et al., 2000] and Rb2 [P130] [Kops et al., 2002]), regulate the mitotic program (e.g., cyclin B and Polo-like kinase [Alvarez et al., 2001]), or induce cellular apoptosis (e.g., Fas ligand [Brunet et al., 1999] and Bim [Burgering and Kops, 2002]). Therefore, FOXO transcription factors play a pivotal role in regulating cell proliferation and survival.

Recent studies in *C. elegans* (DAF-16), *Drosophila* (dFOXO), and mammals reveal that the Akt/FOXO signaling pathway is largely conserved in metazoans. In invertebrates, this pathway plays a critical role in regulating body size and life span (Burgering and Kops, 2002; Puig et al., 2003). In mammalian cells, activation of FOXO transcription factors can induce either cell cycle arrest or apoptosis probably depending on the physiological conditions and cell types (Burgering and Kops, 2002; Tran et al., 2003); and therefore, it has been postulated that FOXO factors may inhibit cell transformation or tumorigenesis. For instance, it has been shown that FOXO3a and FOXO1 are sequestered in the cytoplasm and become inactivated in certain tumor cell lines deficient in PTEN, a tumor suppressor that blocks the PI3K/Akt signaling pathway (Medema et al., 2000; Nakamura et al., 2000; Vazquez and Sellers, 2000). While re-expression of PTEN in the PTEN-negative tumor cells induces cellular apoptosis or upregulates p27kip1 that results in cell cycle arrest in G1 phase, the same phenotypes can also be exerted by overexpression of FOXO factors in these cells (Nakamura et al., 2000), suggesting that activation of FOXO factors may play a role in PTEN-mediated tumor suppression. Thus, nuclear exclusion of FOXO factors regulated by the PI3K/Akt pathway not only plays a critical role in survival and antiapoptosis of normal cells but contributes to the pathogenesis and development of cancer (Nicholson and Anderson, 2002).

*Correspondence: michu@mdanderson.org (M.C.-T.H.), mhung@mdanderson.org (M.-C.H.)

[†]These authors contributed equally to this work.

However, the pathological correlation between nuclear exclusion of FOXO factors and phosphorylated-Akt (Akt-p, activated Akt) in primary tumors has not been reported and little is known about the role and significance of FOXO factors in tumorigenesis.

In an attempt to investigate the correlation between expression of FOXO3a and Akt-p in human primary breast tumors, we unexpectedly found that in some tumor specimens with negative Akt-p, FOXO3a was still excluded from the nucleus, suggesting that other mechanisms may retain FOXO3a in the cytoplasm of tumor tissues. For instance, the I κ B kinase (IKK) signaling pathway has been established as a key survival and antiapoptotic mechanism, and aberrant expression of IKK has been implicated in constitutive activation of NF- κ B in human breast cancer cell lines and primary tumors (reviewed in Karin et al., 2002). Thus, it seems to be plausible that the IKK/NF- κ B pathway may be involved in regulation of FOXO factors and leading to cell survival. The primary form of NF- κ B is a heterodimer of p50 and p65 (RelA) and is retained as a latent form in the cytoplasm of resting cells by I κ B, an inhibitor of NF- κ B. NF- κ B is activated by stimulation of an IKK complex, containing IKK α / β / γ , which phosphorylates I κ B and triggers its ubiquitination (Ub)-dependent degradation through the proteasome pathway (Karin et al., 2002; Karin and Ben-Neriah, 2000). This leads to nuclear translocation of activated NF- κ B and upregulation of its target genes. It has been suggested that inhibition of NF- κ B activation plays an important role in the tumor necrosis factor (TNF)-induced apoptosis. The activity of IKK is often activated constitutively, which results in the sustained activation of members of the NF- κ B family during the development of breast carcinoma (reviewed by Karin et al., 2002). This elevated NF- κ B activity may lead to aberrant upregulation of certain tumorigenic or angiogenic factors, chemokines, adhesion proteins, and inhibitors of apoptosis. Strikingly, we have recently found that the level of nuclear FOXO3a inversely correlates with expression of IKK β in the human breast tumor specimens and positively correlates with the survival rate in breast cancer. We have further demonstrated that IKK inhibits FOXO3a activity, physically interacts with and phosphorylates FOXO3a independent of Akt, and causes proteolysis of FOXO3a via the Ub-dependent proteasome pathway. Downregulation of FOXO3a by IKK can induce cell proliferation and tumorigenesis in breast cancer cells. Our results suggest that negative regulation of FOXO factors by IKK is a key mechanism for promoting tumor cell growth and tumorigenesis.

Results

Nuclear Exclusion of FOXO3a Correlates with Expression of IKK β and Akt-p in Tumors and Normal Tissues and Is Associated with Poor Survival in Breast Cancer

To determine the physiological or pathological relationship between expression of FOXO3a and Akt-p in vivo, we examined the level of FOXO3a and Akt-p proteins in 131 human primary breast tumor specimens by immunohistochemical (IHC) staining and the survival data of the corresponding patients. As expected, FOXO3a was

mainly localized in the cytoplasm of tumor tissues with a high level of Akt-p (Figure 1A, case 1) while FOXO3a was largely located in the nucleus of many tumors with negative Akt-p (Case 2). However, in a significant number (23 cases) of tumor specimens lacking detectable Akt-p, FOXO3a was still predominantly confined in the cytoplasm (case 3), suggesting that other mechanisms may contribute to nuclear exclusion of FOXO3a in these tumors. To examine a potential involvement of IKK β (a major catalytic subunit of IKK) in breast tumors, we found that FOXO3a was excluded from the nucleus when the tumors expressed IKK β but without detectable Akt-p (case 3), while FOXO3a was located in the nucleus of tumors with negative IKK β and Akt-p (case 2). Among 131 tumor specimens examined, 113 tumors were FOXO3a positive and cytoplasmic localization of FOXO3a was detected in the majority (90%) of these 113 tumors with positive IKK β and Akt-p (Figure 1B). However, FOXO3a was found in the cytoplasm only in a small fraction (30%) of these 113 tumor specimens with negative IKK β and Akt-p, suggesting that cytoplasmic FOXO3a correlates with the expression of IKK β and Akt-p. Moreover, we validated the positive staining of IKK β and Akt-p by using two different Ab against IKK β , and Ab specific to Akt-p (Thr308) and Akt-p (Ser473) on randomly selected primary breast tumor specimens. Both Thr308 and Ser473 are known to be phosphorylated in activated Akt. The strong correlative staining between Akt-p (Thr308) and Akt-p (Ser473) (Figure 1C) further strengthens the significance of Figures 1A and 1B.

To further test whether this correlation is a general phenomenon in multiple tumors and normal tissues, we examined a number of human primary tumor specimens and normal tissues by IHC. Our results showed that the same functional relationship between cytoplasmic FOXO3a and positive IKK β and Akt-p occurred not only in other tumor entities, but also in normal tissues (Figures 1D and 1E), suggesting that the correlation between cytoplasmic FOXO3a localization and positive IKK β and Akt-p may be a general phenomenon. We compared the expression of Akt-p, IKK β , and FOXO3a in tumor tissues with the patient survival follow-up and found that cytoplasmic distribution ($N < C$) of FOXO3a was strikingly linked to a poor survival in patients with breast cancer (Figure 2A). However, negative IKK β , Akt-p, and a greater nuclear localization ($N \geq C$) of FOXO3a were significantly associated with an increase of overall survival (Figures 2B–2D). These results suggest that cytoplasmic localization of FOXO3a and expression of IKK β and Akt-p negatively correlate with the survival rate in breast cancer.

IKK Represses FOXO3a Transactivation Activity and Nuclear Translocation Independent of Akt

The correlation between cytoplasmic FOXO3a and IKK β in both normal and tumor tissues promoted us to examine the regulatory role of IKK on FOXO3a activity. We tested the effect of IKK on the FOXO3a-dependent transcriptional activation of a promoter containing the FOXO-responsive elements (FRE) that drive a luciferase (luc) reporter gene. Cotransfection of FOXO3a together with IKK α or IKK β into 293T cells resulted in strong inhibition of FOXO3a activity, whereas cotransfection of

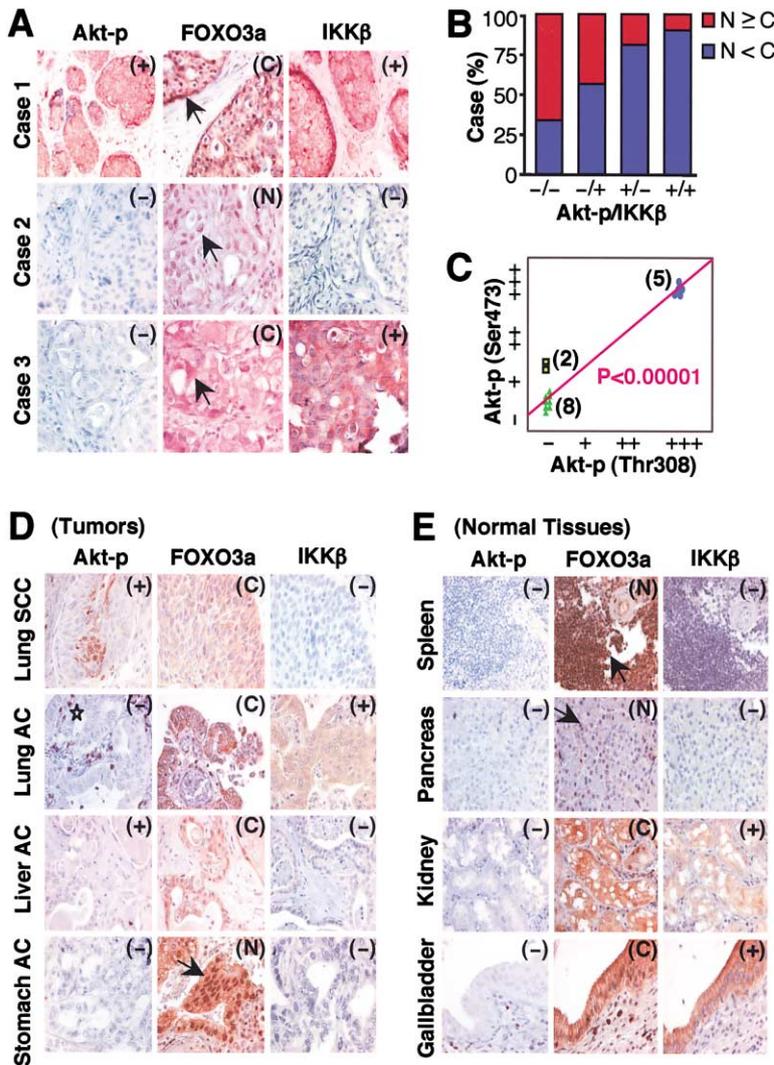


Figure 1. Nuclear Exclusion of FOXO3a Correlates with Expression of IKKβ and Akt-p in Many Types of Tumors and Normal Tissues (A) Primary human breast tumor specimens were stained with antibodies (Ab) specific to Akt-p (Thr308) or Akt-p (Ser473), FOXO3a, and IKKβ.

(B) An average (%) of FOXO3a localization in the nucleus or cytoplasm was determined.

(C) A correlation analysis between Akt-p (Thr308) and Akt-p (Ser473) IHC staining signals was performed by Spearman rank correlation.

(D) Human primary tumor specimens were stained with Ab specific to Akt-p (Thr308), FOXO3a, and IKKβ. AC and SCC denote adenocarcinoma and squamous cell carcinoma, respectively. An asterisk marks a macrophage.

(E) Human normal tissues were stained with Ab as indicated.

FOXO3a with either IKK γ or a dominant-negative IKK β (nIKK β) did not affect FOXO3a activity (Figure 3A). To rule out the possibility that the repression of FOXO3a activity was through an Akt-dependent pathway, we tested a mutant FOXO3a with all three Akt recognition sites mutated (FOXO3a-A3) in the cotransfection experiments and showed that FOXO3a-A3 activity was still suppressed by IKK in the same fashion as the wild-type (wt) FOXO3a (Figure 3A), suggesting that the IKK-mediated inhibition of FOXO3a function is not Akt dependent. To further support this notion, we compared FOXO3a activity in between human breast cancer MDA-MB-453 (453) and 453-nAkt (expressing a dominant-negative Akt) cell lines and showed similar inhibitory effects on FOXO3a by IKK in 453 and 453-nAkt cells (Figure 3B), supporting that the IKK-mediated inhibition of FOXO3a is Akt independent and may be a general phenomenon in multiple cell types. We further showed that this IKK-mediated inhibition of FOXO3a was dependent on the dose of IKK α/β but not nIKK α/β (Figure 3C). To determine whether IKK could regulate FOXO3a activity on the native promoters, we examined the effects of various IKK constructs on FOXO3a by cotrans-

fection with a luc reporter under the control of p27Kip1 promoter, which is a transcriptional target of FOXO3a. Consistent with the FRE-luc results, FOXO3a activity was significantly reduced by IKK on the p27Kip1 promoter (Figure 3D). A similar inhibitory pattern of FOXO3a by IKK was observed on the cotransfection experiments with a luc reporter driven by the promoter of Fas-ligand (FasL) (Figure 3E), supporting that IKK can inhibit the native FOXO3a-targeted promoters.

To understand the molecular mechanism for the IKK-mediated inhibition of FOXO3a activity, we investigated whether the subcellular distribution of FOXO3a is regulated by IKK. We cotransfected the FOXO3a-GFP vector with the various IKK constructs into 293T cells and examined the subcellular localization of FOXO3a-GFP. While FOXO3a-GFP protein was localized primarily within the nuclei of cells cotransfected with a control vector or IKK γ , FOXO3a-GFP was largely excluded from the nucleus and detected in the cytoplasm when it was cotransfected with IKK α or IKK β (Figure 3F). This IKK α - and IKK β -dependent nuclear exclusion of FOXO3a-GFP was further verified by an average of >500 GFP-positive cells, and a parallel comparison with the effect of Akt

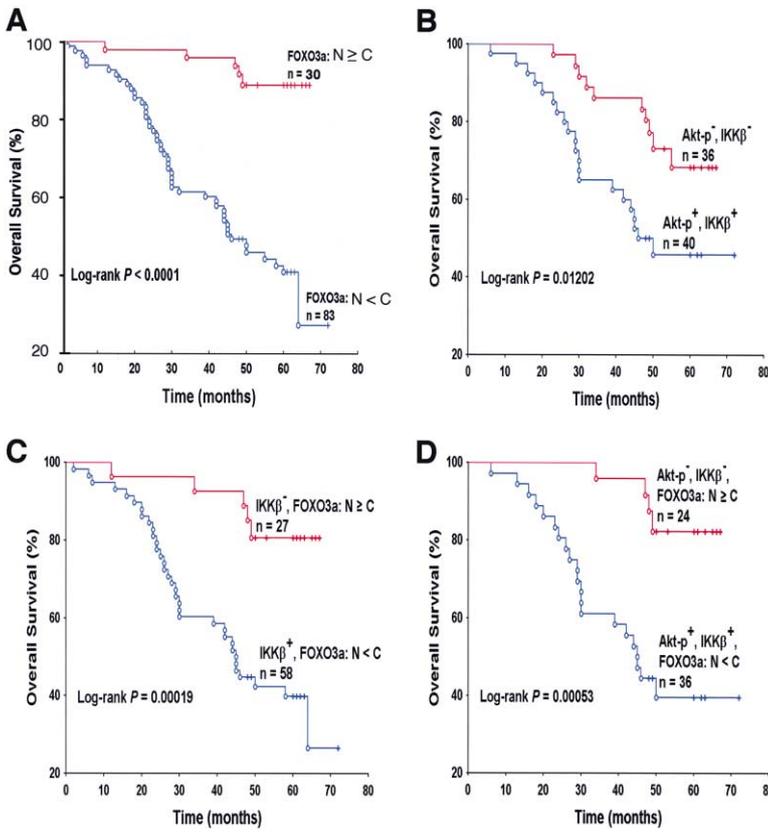


Figure 2. Cytoplasmic FOXO3a and Expression of IKK β and Akt-p Are Associated with Poor Survival in Breast Cancer

(A) The Kaplan-Meier overall survival curve shows that the predominant cytoplasmic FOXO3a group ($N < C$, blue) was linked with a strong reduction of overall survival time as compared to that of the nuclear FOXO3a ($N \geq C$, red) group.

(B) The survival curves show that the Akt-p and IKK β positive group (blue) was associated with a decreased survival.

(C) The survival curves indicated that the detection of positive IKK β and predominant cytoplasmic FOXO3a (blue) was linked with a strong reduction of overall survival.

(D) A similar survival correlation was obtained as in (C) when comparing the three parameters of Akt-p and IKK β and FOXO3a.

by cotransfection with a constitutively active Akt (Akt-DD) (Figure 3G).

IKK Interacts with and Phosphorylates FOXO3a In Vivo and In Vitro

To elucidate the mechanism by which IKK can regulate the cellular localization and activity of FOXO3a, we examined if endogenous FOXO3a is physically associated with IKK β by reciprocal coimmunoprecipitation (IP) followed by an immunoblotting (IB) analysis with lysates of 453 and SKOV3-ip1 (ip1) cells using antibodies (Ab) against FOXO3a and IKK β . Our results showed that endogenous FOXO3a was specifically associated with endogenous IKK β in vivo (Figure 4A). To examine if this association is inducible upon activation of endogenous IKK β by TNF α treatment, we performed the co-IP experiments with lysates of MCF-7 cells treated or untreated with TNF α plus or minus proteasome inhibitor MG132. We found that the binding between endogenous IKK β and FOXO3a appeared to be enhanced by MG132 treatment, and this association was slightly increased by TNF α treatment (Figure 4B).

To further confirm this association with the tag-specific Ab, we cotransfected FOXO3a-Myc with Flag-IKK α or -IKK β into cells and examined their interactions by reciprocal IP/IB using anti-Myc and -Flag and showed that the exogenously expressed FOXO3a-Myc specifically interacted with Flag-IKK α and -IKK β in vivo (Figure 4C). To verify a direct binding between FOXO3a and IKK in vitro, we performed the glutathione S-transferase (GST) pulldown assays with the GST-FOXO3a fusion

proteins and showed that IKK interacted with the carboxyl-portion of FOXO3a specifically but not the N-terminal domain of FOXO3a (Figure 4D).

Given this physical association, we examined whether IKK could phosphorylate FOXO3a in vivo. We cotransfected HA-FOXO3a with the indicated IKK vectors into cells, followed by orthophosphate (^{32}P i) labeling, and performed IP with an anti-HA. Indeed, HA-FOXO3a was phosphorylated in vivo and phosphorylation of HA-FOXO3a was significantly enhanced by IKK β or IKK α (to a lesser degree) but not by NIKK β , supporting that FOXO3a is a target for IKK (Figure 4E). To address if IKK could phosphorylate FOXO3a in vitro, we produced several overlapping fragments of FOXO3a (FO3a) fused to GST and used these proteins as substrates in an IKK β -immunocomplex kinase assay and showed that the C-terminal portion Gst-FO3a (301–673) but not the N-terminal portion Gst-FO3a (1–300) was phosphorylated by IKK β and the intensity of phosphorylation was comparable to that of Gst-I κ B α , a known substrate of IKK β (Figure 4F). Further analysis indicated that most phosphorylation was detected on the fragment FO3a (626–673) and the rest of fragments were not phosphorylated significantly (Figure 4G), suggesting that the primary phosphorylation site(s) may locate within the region of residues 626–673. To narrow down the potential phosphorylation sites, we synthesized peptides corresponding to the candidate serine residues among fragments FO3a (301–346), FO3a (579–625), and FO3a (626–673) and a peptide consisting of several serine residues within the region of FO3a (393–439) (Figure 4G) as a control and used them as substrates in the IKK β -kinase

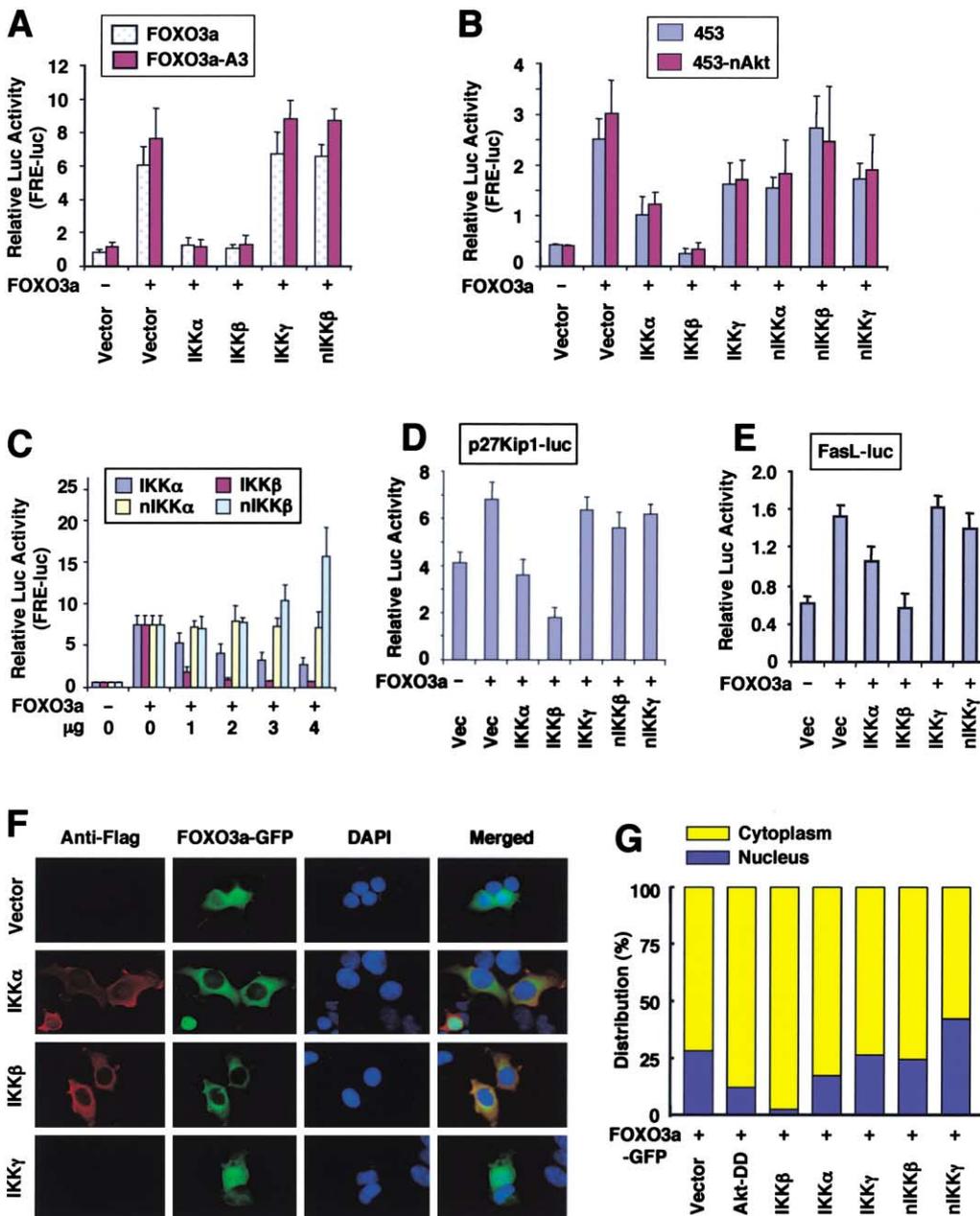


Figure 3. IKK Inhibits FOXO3a Transactivation Activity and Its Nuclear Translocation

- (A) Lysates of 293T cotransfected with FRE-luc, pRL-TK, and FOXO3a plus the marked IKK constructs were subjected to luciferase (luc) assays. The relative luc activity was normalized.
 (B) Lysates of 453 or 453-nAkt cotransfected with the indicated DNA vectors were subjected to luc assays.
 (C) Lysates of 293T cotransfected with FRE-luc and FOXO3a plus different doses of IKK α/β or nIKK α/β were subjected to luc assays.
 (D) Lysates of 293T cotransfected with p27Kip1-luc and FOXO3a plus the indicated IKK constructs were subjected to luc assays.
 (E) Lysates of 293T cotransfected with FasL-luc and FOXO3a plus the various IKK constructs were subjected to luc assays.
 (F) 293T cotransfected with FOXO3a-GFP plus Flag-IKK α or Flag-IKK β or HA-IKK γ were subjected to GFP or anti-Flag immunofluorescence.
 (G) An average of GFP-positive cells in the nucleus or cytoplasm was determined.

assays. The results indicated that the major phosphorylation occurred on the peptide 625–648 (Figure 4H), which is consistent with the primary phosphorylation detected in the fragment FO3a (626–673) (Figure 4G). Since Ser644 in the segment of FO3a (626–673) is conserved with the putative IKK phosphorylation sequences (Karin and Ben-Neriah, 2000), we mutated Ser644 into

alanine (S644A) and found that phosphorylation of the mutant protein was abrogated (Figure 4I), suggesting that Ser644 is the predominant site of IKK β phosphorylation on FOXO3a. However, we could not rule out certain minor target sites matching the putative IKK recognition sequences for phosphorylation in FOXO3a (Figure 4J) that might be phosphorylated by IKK β .

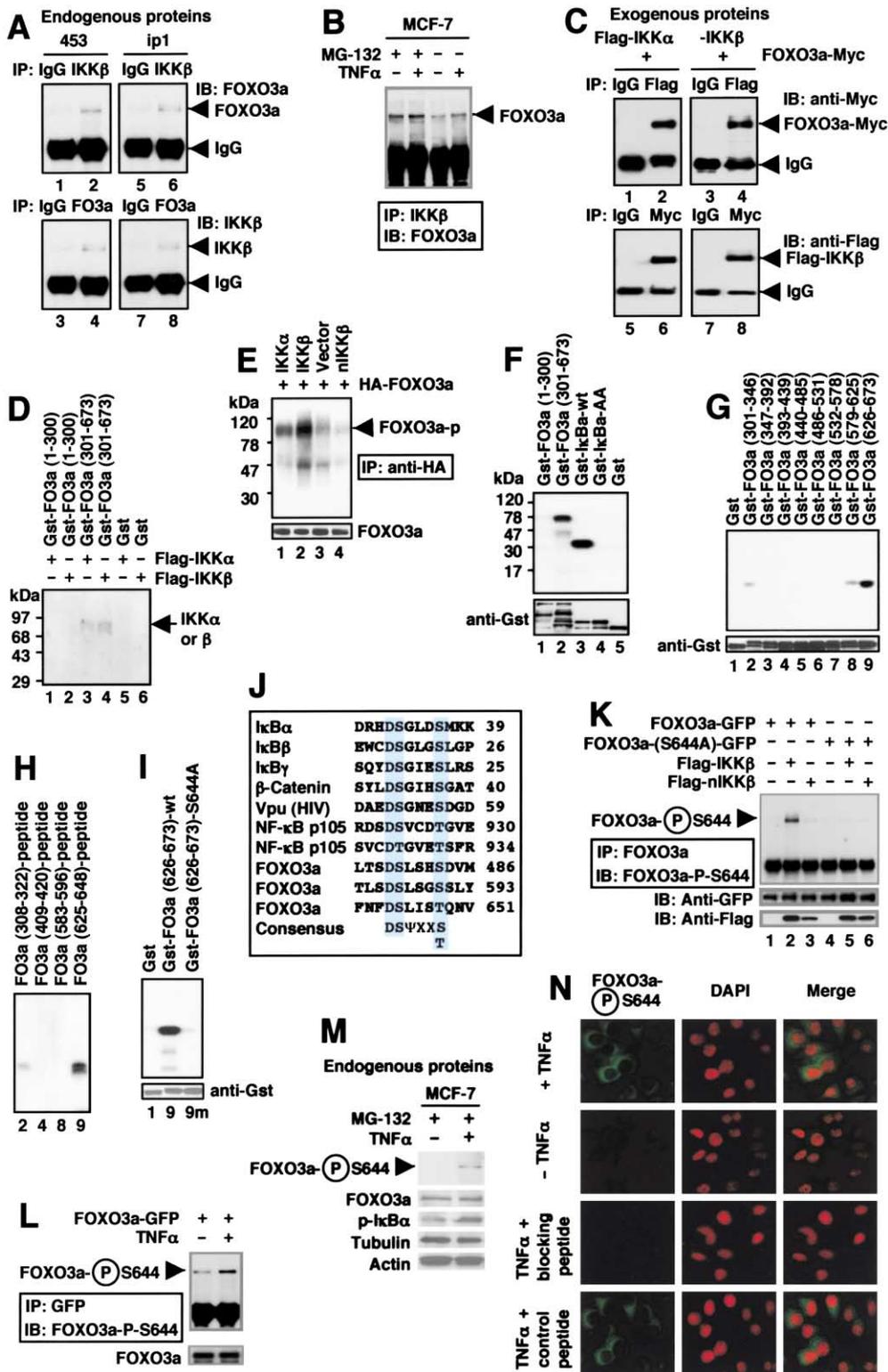


Figure 4. IKK Interacts with and Phosphorylates FOXO3a In Vivo and In Vitro

(A) Lysates of 453 and ip1 were subjected to reciprocal IP/IB (anti-FOXO3a/anti-IKK β).
 (B) Lysates of MCF-7 treated or untreated with TNF α plus or minus MG132 were subjected to IP/IB (anti-IKK β /anti-FOXO3a).
 (C) Lysates of 293T cotransfected with FOXO3a-Myc plus Flag-IKK α or -IKK β were analyzed by IP/IB.
 (D) Lysates of 293T transfected with Flag-IKK α or -IKK β were incubated with GST-FOXO3a (FO3a) proteins, followed by IB.
 (E) Lysates of 293T cotransfected with FOXO3a plus the indicated IKK and labeled with [³²P]orthophosphate were subjected to IP (anti-HA) and autoradiograph.

To confirm that S644 is the site of IKK-catalyzed phosphorylation of the endogenous FOXO3a protein in response to TNF α , we raised an Ab against a phosphopeptide corresponding to the phosphorylation site of S644 and used this Ab for IB analysis. We first demonstrated that this Ab recognized FOXO3a-GFP in 293T cells cotransfected with FOXO3a-GFP and IKK β but did not recognize FOXO3a-GFP in cells cotransfected with FOXO3a-GFP and nIKK β (Figure 4K). Neither could this Ab recognize FOXO3a-(S644A)-GFP in which S644 had been converted to alanine (S644A) (Figure 4K), indicating that this Ab recognizes FOXO3a specifically when it is phosphorylated at S644 (FOXO3a-P-S644). Moreover, this Ab recognized FOXO3a-GFP with TNF α treatment more significantly than that without treatment (Figure 4L), suggesting that this Ab can recognize the TNF α -induced phosphorylation of FOXO3a. We next examined whether this Ab could detect endogenous FOXO3a-P-S644 in response to TNF α . We found that treatment of MCF-7 cells with TNF α stimulated phosphorylation of a 100 kDa protein that could also be recognized by other anti-FOXO3a Ab, whereas phosphorylation at S644 of FOXO3a was not detectable in MCF-7 without TNF α treatment (Figure 4M). We further tested if this phospho-Ab could assess nuclear exclusion of endogenous FOXO3a-P-S644 in response to TNF α by immunofluorescence. Indeed, we found that endogenous FOXO3a recognized by this Ab was predominantly excluded from the nucleus and detected in the cytoplasm of MCF-7 treated with TNF α , while FOXO3a was not detected with this Ab without TNF α treatment. These results are consistent with our previous data on the IKK-dependent nuclear exclusion of FOXO3a-GFP (Figure 3F). Moreover, the binding of this Ab to endogenous FOXO3a-P-S644 in response to TNF α was abrogated with a blocking peptide, whereas the signal of immunofluorescence with this Ab (green) was largely unaffected in the presence of a control peptide (Figure 4N), suggesting that the binding of this phospho-Ab to the endogenous FOXO3a protein is specific to the phosphorylated state of S644 residue. Taken together, our results strongly suggest that phosphorylation of the endogenous FOXO3a at S644 is induced by endogenous IKK in response to its physiological stimulation.

IKK Induces Ubiquitination and Degradation of FOXO3a through the Proteasome Pathway that Is Dependent on Ser644 of FOXO3a

To further address the mechanism for the IKK-mediated FOXO3a inactivation, we investigated the level of FOXO3a protein in whole lysates prepared from cells cotransfected with HA-FOXO3a plus the various IKK

constructs or a control vector. Strikingly, we observed not only a significant decrease of HA-FOXO3a in the cells cotransfected with HA-FOXO3a and IKK β , but an evident high M.W. band (>150 kDa) that could be detected with either an anti-HA or anti-FOXO3a (Figure 5A). A similar high M.W. band (to a lesser degree) was also detected in cells cotransfected with HA-FOXO3a and IKK α but not in the samples with mutant nIKK α or β . Since Ub is a well-known protein modification that induces protein degradation through the proteasome pathway, we examined if the IKK-induced protein modification of FOXO3a was through Ub. To this end, we cotransfected cells with FOXO3a-Myc together with HA-Ub plus the various IKK vectors and examined the HA-tagged protein patterns of FOXO3a. After IP with an anti-Myc, a characteristic pattern of polyUb on Myc-FOXO3a was readily detected by IB with an anti-HA on the samples cotransfected with IKK β or IKK α (to a lesser degree) but not with IKK γ or nIKK $\alpha/\beta/\gamma$ (Figure 5B), suggesting that Ub of FOXO3a is dependent on the IKK kinase activity. In addition, we tested if sumoylation was involved in the IKK-mediated modification of FOXO3a by cotransfecting cells with Myc-FOXO3a and HA-SUMO1 vector with or without IKK β . The results showed that no HA-tagged high M.W. band of FOXO3a was detected by IB with an anti-HA after IP with an anti-Myc, suggesting that the IKK-mediated FOXO3a modification may be independent of sumoylation (Figure 5B). As a control, we detected a band at \sim 12 kDa of SUMO1 by IB (not shown). Furthermore, this IKK-mediated Ub of FOXO3a was supported by the facts that the IKK-dependent decrease of FOXO3a activity could be significantly recovered by MG132 treatment (Figure 5C) and the IKK-induced degradation of HA-FOXO3a could be blocked by multiple proteasome inhibitors with different modes of action (Figure 5D). To ascertain that endogenous FOXO3a is also ubiquitinated in an IKK-dependent manner, we examined endogenous FOXO3a in lysates prepared from wt or IKK β -deficient ($-/-$) mouse embryonic fibroblasts (MEF) treated or untreated with TNF α and showed that a high M.W. band of FOXO3a (Ub-FOXO3a) was mainly detected in the wt MEF with TNF α treatment, whereas this band was not evident in IKK β ($-/-$) MEF in the presence of TNF α (Figure 5E), suggesting that TNF α induces Ub of endogenous FOXO3a in an IKK β -dependent manner.

To substantiate whether the level of Ub of endogenous FOXO3a observed in response to IKK β activation is effective to functionally degrade FOXO3a in cells, we performed a timecourse of the TNF α -induced effects on FOXO3a total protein level plus or minus MG132 and showed that endogenous FOXO3a was significantly removed from cells in the absence of MG132 after TNF α

(F-I) Lysates of 293T transfected with HA-IKK β were IP with an anti-HA, and kinase assays were performed with the various FOXO3a Gst-ubiquitin proteins (F), the Gst-FOXO3a sequential fusion fragments (G), synthetic peptides (H), and wt or the S644A mutant Gst-FOXO3a (626-673) fragment (I).

(J) The putative IKK consensus sequences for phosphorylation (S, serine; T, threonine; ψ , hydrophobic amino acid; and X, any amino acid).

(K) Lysates of 293T cotransfected with the indicated vectors were analyzed by IP/IB (anti-FOXO3a/anti-FOXO3a-P-S644).

(L) Lysates of 293T transfected with FOXO3a-GFP plus or minus TNF α were analyzed by IP/IB (anti-GFP/anti-FOXO3a-P-S644).

(M) Lysates of MCF-7 treated or untreated with TNF α plus MG132 were subjected to IB.

(N) MCF-7 treated or untreated with TNF α plus MG132 were stained with anti-FOXO3a-P-S644 with or without a blocking or control peptide and followed by immunofluorescence.

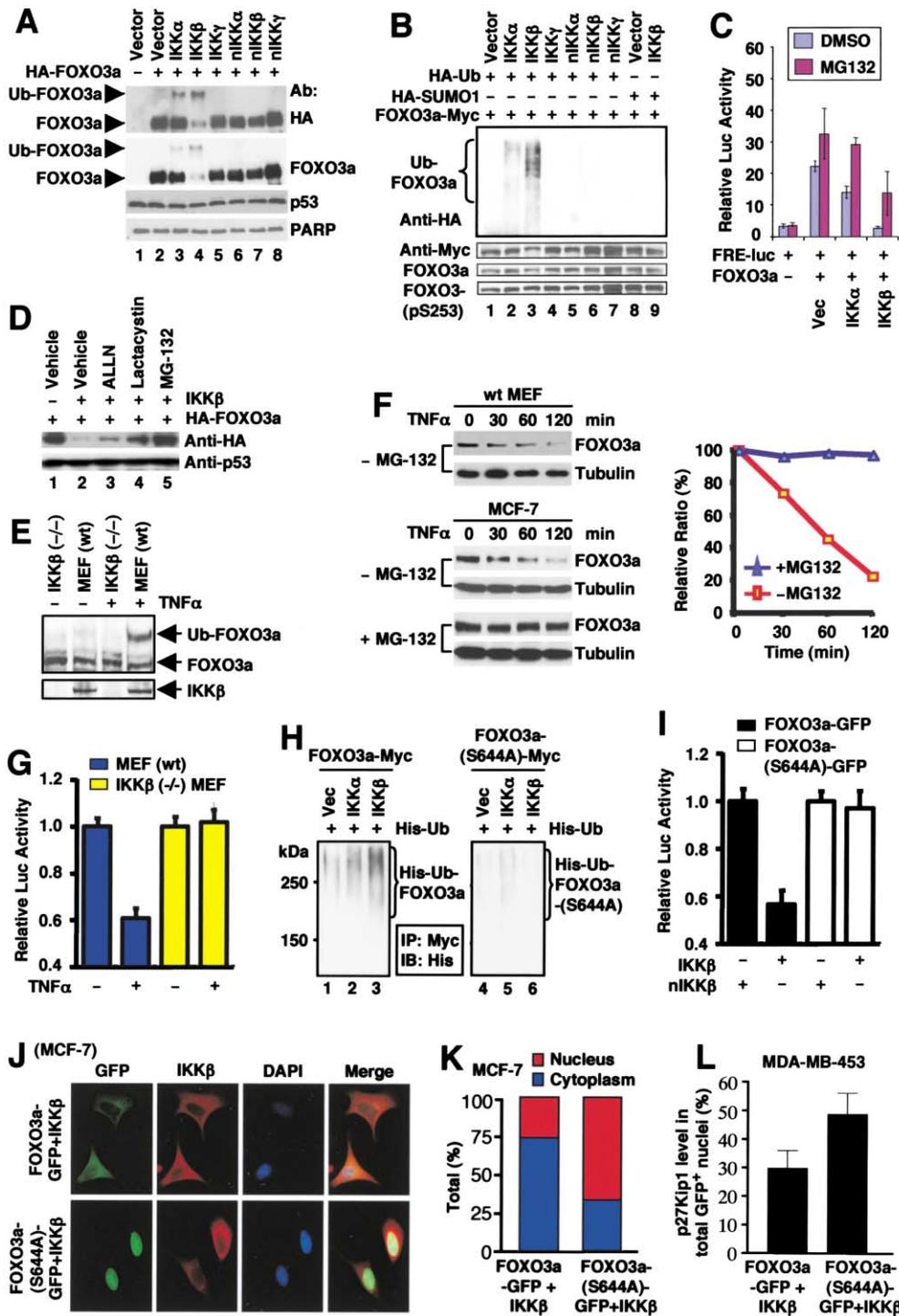


Figure 5. IKK Induces Ubiquitination and Degradation of FOXO3a via the Proteasome Pathway that Is Dependent on Ser644 of FOXO3a

(A) Lysates of 293T cotransfected with HA-FOXO3a plus the indicated IKK vectors were analyzed by IB.
 (B) Lysates of 293T cotransfected with HA-Ub or HA-SUMO1 plus FOXO3a-Myc and the various IKK vectors were IP/IB (anti-Myc/anti-HA).
 (C) Lysates of 293T cotransfected with the marked vectors with or without MG132 were subjected to luc assays.
 (D) Lysates of 293T cotransfected with HA-FOXO3a and IKK β , treated with the indicated proteasome inhibitors, were analyzed by IB.
 (E) Lysates of the wt or IKK β (-/-) MEF, treated or untreated with TNF α , were analyzed by IB.
 (F) Lysates of MCF-7 untreated or treated with TNF α for various time periods plus or minus MG132 were subjected to IB.
 (G) Lysates of the wt or IKK β (-/-) MEF cotransfected with FRE-luc and pRL-TK, with or without TNF α , were subjected to luc assays.
 (H) Lysates of 293T cotransfected with the indicated vectors were subjected to IP/IB (anti-Myc/anti-His).

treatment for 60 min, while the majority of FOXO3a was not degraded in the presence of MG132 after TNF α treatment (Figure 5F). We further compared the ability of IKK β to inhibit endogenous FOXO3a activity in response to TNF α in MEF by using the luc assays with or without TNF α treatment and showed that FOXO3a activity was significantly suppressed in wt MEF but not in IKK β (-/-) MEF upon TNF α treatment (Figure 5G), indicating that IKK β is required for the TNF α -mediated repression of FOXO3a activity. Taken together, these results indicate that the degree of Ub of endogenous FOXO3a in response to IKK β activation is sufficient to functionally remove FOXO3a from cells, and normal activation of IKK by TNF α leads to an inactivation of endogenous FOXO3a in MEF in an IKK β -dependent manner.

To determine if phosphorylation of Ser644 on FOXO3a was required by this IKK-mediated Ub of FOXO3a, we cotransfected cells with IKK and His-Ub plus FOXO3a-Myc or mutant FOXO3a-(S644A)-Myc and examined the His-tagged protein patterns of FOXO3a. A characteristic pattern of polyUb on FOXO3a-Myc was readily detected by IP/IB (anti-Myc/anti-His) on the samples cotransfected with IKK and FOXO3a-Myc but not with FOXO3a-(S644A)-Myc (Figure 5H), suggesting that the IKK-induced Ub of FOXO3a depends on phosphorylation of Ser644. To test if the IKK-mediated inhibition of FOXO3a function is Ser644 dependent, we compared the FOXO3a activity and nuclear translocation between FOXO3a-GFP and FOXO3a-(S644A)-GFP in the cotransfection experiments and showed that the activity and nuclear localization of FOXO3a-(S644A)-GFP was no longer negatively regulated by IKK β (Figures 5I–5K) in cells even with a high level of IKK β . We further verified that the IKK β -mediated inhibition of FOXO3a activity was dependent on Ser644 by transfecting 453 cells with IKK β plus FOXO3a-GFP or FOXO3a-(S644A)-GFP and assessing expression of p27Kip1 by immunofluorescence with an anti-p27Kip1. The p27Kip1 expression was enhanced in the FOXO3a-(S644A)-GFP-transfected cells as compared to that in the FOXO3a-GFP-transfected cells (Figure 5L), confirming that Ser644 is required for the IKK β -mediated repression of FOXO3a.

IKK β Stimulates Cell Cycle Progression, Proliferation, and Tumorigenesis that Can Be Overridden by Expression of FOXO3a

To determine whether constitutive expression of IKK β could decrease FOXO3a expression in cell lines stably expressing IKK β , we generated several 453-IKK β and 453-nIKK β stable transfectants (designated as 453- β and 453-n β , respectively). We first examined the expressions of endogenous FOXO3a, I κ B α , and p27Kip1 in the various 453-C (the vector-transfected control cell lines), 453- β , 453-n β , and 453-nAkt cell lines by IB analysis. The levels of FOXO3a and I κ B α proteins were significantly decreased in 453- β cell lines, and the p27Kip1

expression was also reduced in 453- β cells due to inhibition of FOXO3a activity (Figure 6A). Since degradation of I κ B α leads to activation of NF- κ B, which is associated with mitogenic stimulation, upregulation of cyclin D1 expression, and cell proliferation (Joyce et al., 2001), we next tested if constitutive expression of IKK β could promote cell cycle progression and proliferation. After cells were arrested with serum starvation and treated with a reversible cell cycle inhibitor mimosine to synchronize cells in G1 phase of the cell cycle, we measured the percentage of cells at S phase of the cell cycle as the rate of cell cycle entry by flow cytometry and found that the 453- β cells entered the cell cycle significantly faster than 453-C and 453-n β cells (Figure 6B). Consistently, the proliferation rates of 453- β cells were significantly greater than those of 453-C cells (Figure 6C), suggesting that constitutive expression of IKK β stimulates cell cycle progression and proliferation.

To investigate if this stimulatory effect of IKK β on cell proliferation was via repression of FOXO3a, we transfected HA-FOXO3a into 453- β 5 cells and selected two independent cell clones (453- β 5-FO5 and -FO18) with high levels of FOXO3a (FO) expression (Figure 6D). Since FOXO3a might be antagonized by IKK β in 453- β 5 cells, we examined whether FOXO3a expressed in 453- β 5-FO5 cells was functionally localized in the nucleus and recruited to the promoter of its target gene p27Kip1 using chromatin immunoprecipitation (ChIP). Indeed, the p27Kip1 promoter occupancy by FOXO3a, particularly to the region containing the putative FOXO3a binding sites, was observed in 453-C1 and enhanced in 453- β 5-FO5 but not detectable in 453- β 5 (Figure 6E), indicating that re-expression of FOXO3a constitutively in 453- β 5-FO5 can overcome the negative regulation of IKK β and enhance the physical association of FOXO3a with the promoter of its target gene. The proliferation rates of 453- β 5-FO5 and -FO18 were reduced or comparable with those of 453-C1 or -C5 (Figure 6C), implying that cell proliferation stimulated by IKK β can be overridden by constitutive expression of FOXO3a. In addition, the proliferation rates of 453-C1-FOPool were also significantly decreased (Figure 6C), suggesting that expression of FOXO3a may exert a general inhibitory effect on cell growth. Taken together, our results suggest that a mechanism by which IKK β stimulates cell cycle progression and proliferation is through suppression of FOXO3a activity.

Since IKK β can stimulate cell proliferation and its expression contributes to a prediction of poor survival for the breast cancer patients (Figures 2B–2D), we tested if constitutive expression of IKK β in breast cancer cells can enhance tumorigenesis in vivo. The 453- β , -n β , and -C cells were injected into the mammary fat pads of nude mice. In agreement with wt 453, injection of 453-C1, -C2, -n β 2, and -n β 4 did not initiate or maintain tumorigenesis in mice (Figure 6F). However, inoculation of

(I) Lysates of 453 cotransfected with the marked vectors were subjected to luc assays.

(J) MCF-7 were cotransfected with the vectors as marked and subjected to immunofluorescence.

(K) An average of ~200 GFP-positive cells in the nucleus or cytoplasm was determined.

(L) 453 cotransfected with the indicated vectors were subjected to immunofluorescence. An average of p27Kip1-positive nuclei in the total GFP-positive cells was determined.

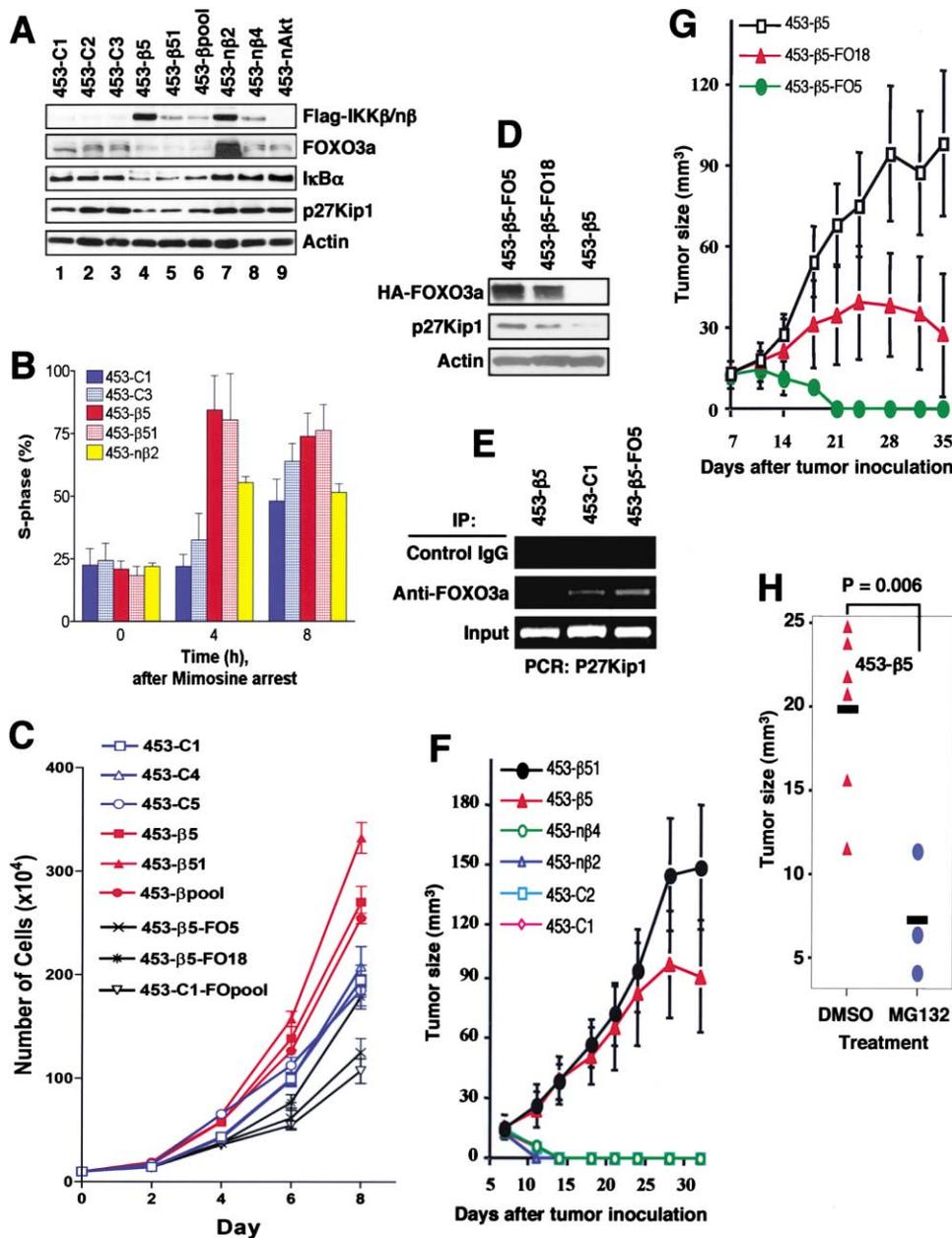


Figure 6. IKK β Stimulates Cell Cycle Progression, Proliferation, and Tumorigenesis that Can Be Overridden by Re-expression of FOXO3a
 (A) Lysates of 453 lines as marked were analyzed by IB.
 (B) The various 453 lines were arrested, synchronized, and followed by cell cycle analysis.
 (C) Cells were synchronized and their proliferation rates were determined (pool, pooled clones; FO, cell line stably transfected with FOXO3a).
 (D) Lysates of the indicated 453 lines were analyzed by IB.
 (E) Association of FOXO3a with the endogenous p27Kip1 promoter in vivo was shown by the ChIP assays with the marked 453 lines.
 (F) The various 453 lines were injected into the mammary fat pads of nude mice.
 (G) Similar mouse models were performed with 453- β 5, 453- β 5-FO5, and -FO18.
 (H) 453- β 5 cells were pretreated with MG132 DMSO before injection into nude mice, and MG132 or DMSO was injected into the tumor-bearing mice twice/week.

the 453- β 5 and - β 51 in nude mice induced mammary tumors consistently (Figure 6F), suggesting that constitutive expression of IKK β can promote tumorigenesis in vivo. To determine if this promoting effect of IKK β on tumorigenesis is via inhibition of FOXO3a, we performed the tumorigenesis experiments in vivo with the 453- β 5-FO cell lines. Strikingly, the tumor formation from 453- β 5-FO5 or -FO18 in nude mice was reduced (Figure 6G),

indicating that tumorigenesis induced by IKK β can be suppressed by re-expression of FOXO3a. Since the IKK β -mediated repression of FOXO3a could be recovered by MG132 treatment (Figures 5C and 5D), as an alternative approach to confirm the inhibitory role of FOXO3a in tumorigenesis, we further investigated if the IKK β -induced tumorigenesis could be suppressed by MG132. As shown in Figure 6H, inoculation of MG132

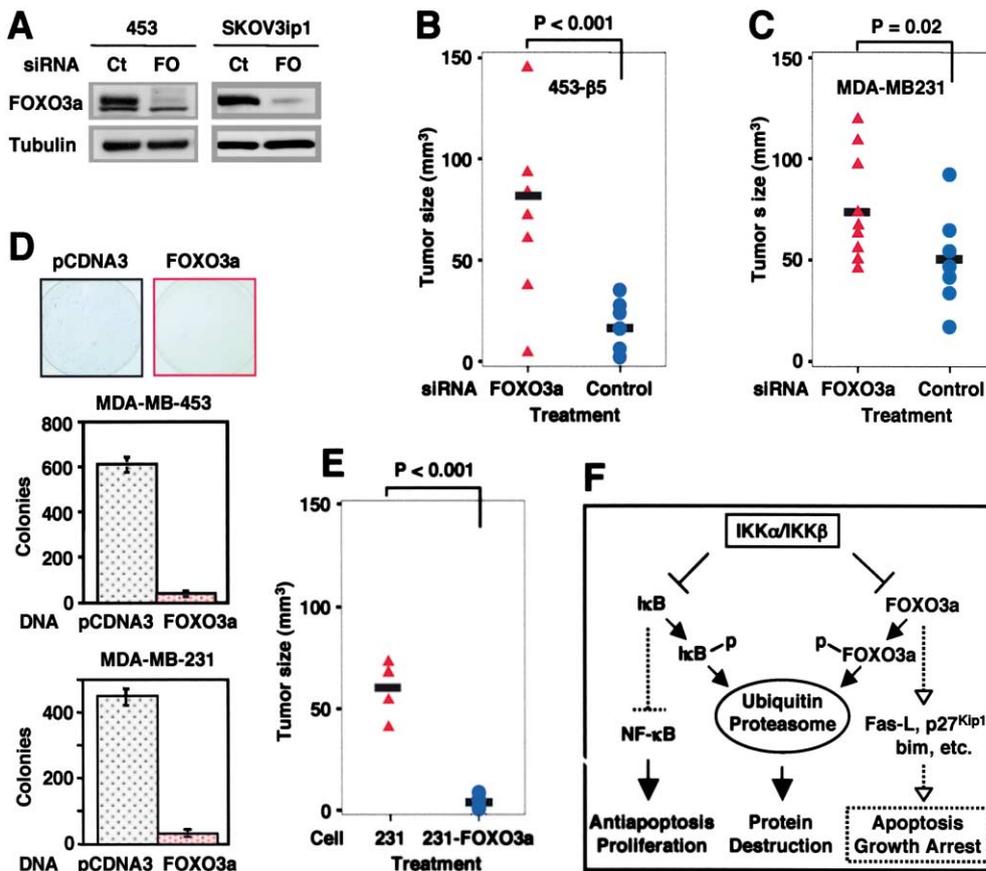


Figure 7. Downregulation of Endogenous FOXO3a Contributes to Tumorigenesis whereas Ectopic Expression of FOXO3a Leads to Tumor Suppression

(A) Lysates of 453 and ip1 transfected with FOXO3a-siRNA or control RNA were analyzed by IB.
 (B) 453-β5 cells transfected with FOXO3a-siRNA or control RNA were injected into the mammary fat pads of nude mice.
 (C) 231 cells transfected with FOXO3a-siRNA or control RNA were injected into the mammary fat pads of nude mice.
 (D) 453 and 231 were transfected with a FOXO3a or control vector, followed by G418 selection for stable clones.
 (E) 231 or 231-FOXO3a cells were injected into the mammary fat pads of nude mice.
 (F) A diagram depicts the IKK-mediated cooperative antiapoptosis signaling pathways (dashed lines, normal regulations of IκB and FOXO3a on cellular targets in the absence of IKK activity).

into the 453-β5-bearing mice significantly suppressed tumorigenesis, suggesting that a mechanism underlying IKKβ-mediated tumorigenesis in vivo may be through inhibition of FOXO3a.

To examine whether IKKβ-mediated tumorigenesis is due to inhibition of FOXO3a function, we carried out siRNA-mediated gene silencing to confirm if IKKβ-mediated tumorigenesis could be enhanced by further downregulation of residual FOXO3a in 453-β5 cells. We first determined if FOXO3a-siRNA could affect endogenous FOXO3a expression by IB with an anti-FOXO3a after cells were transfected with FOXO3a-siRNA or control siRNA. Indeed, FOXO3a-siRNA specifically decreased FOXO3a expression in 453 and ip1 cells (Figure 7A). We then tested whether downregulation of FOXO3a can affect tumorigenesis by transfecting 453-β5 with FOXO3a-siRNA or control siRNA and then injecting the transfected cells into the mammary fat pads of nude mice. Strikingly, FOXO3a-siRNA in 453-β5 augmented tumor growth in nude mice significantly (Figure 7B), suggesting that 453-β5-induced tumorigenesis is through downregulation of FOXO3a.

To further determine whether FOXO3a has a general suppression effect for tumorigenesis, we transfected MDA-MB-231 (231) cells with FOXO3a-siRNA or control siRNA and injected the transfected cells into the mammary fat pads of nude mice. We found that FOXO3a-siRNA in 231 also increased tumorigenesis in nude mice (Figure 7C), suggesting that downregulation of FOXO3a can enhance tumorigenesis in vivo. In contrast, ectopic expression of FOXO3a in 453 and 231 by DNA transfection inhibited colony formation in vitro (Figure 7D); and the ability of tumorigenesis of the FOXO3a-transfected 231 (designated as 231-FOXO3a) in nude mice was impaired (Figure 7E). Taken together, our results strongly suggest that FOXO3a has a general suppression effect for tumorigenesis in vivo.

Discussion

Nuclear exclusion of FOXO3a in human primary tumors lacking Akt activity has not been elucidated, and its pathological or clinical significance has not been reported. Our results indicate a new mechanism for an

Akt-independent inhibition of FOXO3a that promotes cell proliferation and tumorigenesis. Our findings point to a striking inverse correlation between cytoplasmic FOXO3a and the survival rate in breast cancer, suggesting that FOXO3a may become a novel prognostic biomarker for cancer survival. In addition, FOXO3a may be a new tool for a therapeutic or preventive intervention for breast cancer and perhaps other types of cancer.

The molecular mechanisms by which the FOXO factors inhibit tumorigenesis are not fully elucidated yet. Our results indicate that FOXO3a is a cellular target for IKK and its fate after phosphorylation by IKK appears to be similar to those of members of the I κ B family with regards to their cytoplasmic retention, Ub, and degradation. A schematic representation of the IKK-mediated phosphorylation, Ub, and proteolysis of FOXO3a and I κ B in the parallel antiapoptotic signaling pathways is proposed (Figure 7F). This model is unique and important for the following reasons. Firstly, it provides a mechanism by which a cancer cell may acquire sustained resistance to apoptosis through an orchestrated inhibition of FOXO3a and I κ B by IKK, which can lead to constitutive survival signaling. Secondly, it suggests that stimulation of cell proliferation and tumorigenesis by NF- κ B may require a concurrent inhibition of FOXO3a, whereas NF- κ B activation alone may not be sufficient to promote tumorigenesis but activate certain NF- κ B target genes, which induce the production of inflammatory cytokines or chemokines that may trigger recruitment of the immune cells to the tumors and cause their rejections. This may explain why NF- κ B can also contribute to tumor suppression under certain circumstances although NF- κ B activation has been largely associated with the development of a variety of malignancies (Deng et al., 2002; Karin et al., 2002). Thirdly, blocking NF- κ B activity has been viewed as a promising approach to inhibit tumor proliferation or increase the sensitivity of cancer cells to chemotherapeutic drugs. Intense efforts have been underway to identify small molecule antagonists for IKK or NF- κ B as promising chemotherapeutic drugs (Karin et al., 2002). Our model suggests that IKK β should be the therapeutic target of choice and the IKK β -specific antagonists may be more useful than general inhibitors of NF- κ B for inducing apoptosis of most types of tumors, with or without constitutive NF- κ B activity (Karin et al., 2002).

Based on our findings, we propose a model for an IKK-dependent repression of FOXO3a that promotes cell growth and tumorigenesis. However, we cannot rule out one possible mechanism by which overexpression of FOXO3a in cells may predominantly occupy functional IKK and abolish the IKK-mediated proteolysis of I κ B, resulting in sustained retention of the I κ B/NF- κ B complex in the cytoplasm that leads to suppression of cell proliferation and tumorigenesis. This mechanism will imply that the inhibitory effect of FOXO3a in the IKK β -mediated cell growth and tumorigenesis may be via inactivation of NF- κ B. Alternatively, the observed FOXO3a-mediated suppression of cell growth and tumorigenesis in our breast cancer animal models may be attributed to cooperative upregulation of the FOXO3a-induced proapoptotic genes and downregulation of the NF- κ B-mediated antiapoptotic genes. Further investigation of the signaling mechanism by which FOXO3a pre-

vents tumor cell survival or resistance to apoptosis is necessary for understanding the molecular basis for its tumor suppression activity or application as an anti-cancer agent for therapeutic intervention.

Experimental Procedures

Immunohistochemical Staining

IHC staining was performed as described (Deng et al., 2002). Tissue sections were incubated with anti-Akt-p (Thr308) and -Akt-p (Ser473), two anti-FOXO3a Ab, and two anti-IKK β Ab. Sections were treated with biotin-conjugated 2nd Ab and followed by avidin biotin-peroxidase complex and amino-ethyl carbazole chromogen. Kaplan-Meier survival curves were generated using scores of positive versus negative Akt-p and IKK β , positive IKK β and cytoplasmic FOXO3a versus negative IKK β plus nuclear FOXO3a, or positive Akt-p and IKK β and FOXO3a versus negative Akt-p and IKK β and nuclear FOXO3a as strata. Two-sided log-rank analysis was used to assess statistical significance, and the association between discrete variables was tested using the χ^2 test.

Plasmids, Antibodies (Ab), Luciferase (Luc), and Immunofluorescence Assays

The HA- and Myc-tagged FOXO3a expression plasmids were constructed by subcloning of wt FOXO3a containing HA-tag or Myc-tag using PCR. Plasmids of wt FOXO3a, FRE-Luc, FOXO3a-A3, p27Kip1-Luc, pFasL-Luc, and IKK were kindly provided by K. Arden, M. Greenberg, B. Burgering, C. Paya, B. Zhou, and K. Jeang. The various FOXO3a-GFP and -GST fusion vectors were constructed by subcloning of the DNA fragments of full-length or an indicated domain of FOXO3a. The S644A mutant FOXO3a DNA was generated using QuickChange site-directed mutagenesis (Stratagene) to change the Ser644 residue to Ala644 and confirmed by DNA sequencing. Ab against the phosphorylation site of FOXO3a-S644 were produced with a synthetic phosphorylated peptide DGLDFNFDpSLISTQNVVGL and purified with the phosphopeptide column at Bethyl Laboratories, Inc, Montgomery, Texas. Luc assays were performed using Dual-Luciferase Assay (Promega). For immunofluorescence, 293T were cotransfected with FOXO3a-GFP or mutant FOXO3a-(S644A)-GFP plus the indicated IKK vectors. After fixation, the cells were stained with an anti-Flag and a Texas Red-2nd Ab, and the cellular localizations of FOXO3a and IKK were determined under a fluorescent microscope.

Immunoprecipitation, Immunoblotting, Kinase, and GST Pulldown Assays

We carried out IP, IB, and kinase assays as described (Deng et al., 2002; Zhou et al., 2000). For in vivo kinase assay, 293T cells were cotransfected with the indicated vectors. After 36 hr, cells were starved in serum-free and phosphate-free medium for 12 hr and incubated in phosphate-free medium supplemented with 1 mCi/ml [³²P]orthophosphate for 3 hr. HA-FOXO3a was IP with an anti-HA, subjected to SDS-PAGE and autoradiography. GST pulldown assays were performed as described (Deng et al., 2002).

Cell Culture, Cell Cycle Analysis, and Proliferation Assay

Cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). Transient or stable transfections of cells with DNAs were performed with an optimal ratio of DNAs and liposome. Stable cell lines were isolated with G418 or puromycin selection. For colony assays, cells were transfected with DNAs. After G418 selection, the cells were stained with crystal violet, and the average number of colonies and standard deviation were calculated based on three independent replicates. For cell cycle analysis, cells were serum starved and incubated in medium containing mimosine (400 μ M) for 30 hr. Cells were harvested at 0, 4, 8 hr after replacing the medium with fresh medium with 10% FBS, stained with propidium iodide, and subjected to FACS analysis. Cell proliferation rates were measured by direct cell counting using a counting chamber with trypan blue.

ChIP Assays

The ChIP assays were carried out as described (Lin et al., 2001; Weinmann and Farnham, 2002). Briefly, cells were fixed with formaldehyde, washed, resuspended in hypotonic buffer, passed through a 25-gauge needle, sonicated, and lysates containing soluble chromatin were prepared and incubated with Ab. DNA-protein immunocomplexes were collected with protein A-agarose beads, washed, treated with RNase A, and followed by incubation with proteinase K at 65°C; the DNA samples were extracted with phenol/chloroform and precipitated with ethanol. Specific sequences of human p27Kip1 gene promoter in the DNA samples were detected by PCR with primers 5'GTCTTCTACCAGGGTCCACC3' and 5'CACTTCGATCCCCTTCACATG3'.

SiRNA

Cells were transfected with FOXO3a-siRNA (5'GAGCUCUUGGUG GAUCAUCTT3') or control luc RNA (5'GAGCUCUUGGUGGAUCAUCTT3') duplex (Dharmacon) (4 μ M/2 \times 10⁶ cells) by Lipofectamine 2000 (Invitrogen) or electroporation using Nucleofector 1 (amaxa), and lysates prepared 48 hr after transfection.

Animal Studies

We performed tumorigenesis assays in an orthotopic breast cancer mouse model (Chang et al., 1997). Cells were injected (2 \times 10⁶ cells) into the mammary fat pads of nude mice (n = 10/group), the tumor volumes were measured twice weekly, and the data were tested statistically using two-sided long-rank analysis. For MG132 treatment, cells were pretreated with MG132 (5 μ g/ml) or DMSO for 6 hr before injection into the mammary fat pads of nude mice. One week after inoculation, MG132 (5 nmole/mouse) or DMSO (100 μ l) was injected into the tumor-bearing mice twice/week. For siRNA treatment, cells were transfected with siRNA 24 hr before injection into the mammary fat pads of nude mice. The data were tested statistically using the t test.

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