Paclitaxel-Induced Nuclear Translocation of FOXO3a in Breast Cancer Cells Is Mediated by c-Jun NH_2-Terminal Kinase and Akt

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Abstract

The microtubule-targeting compound paclitaxel is often used in the treatment of endocrine-resistant or metastatic breast cancer. We have previously shown that apoptosis of breast cancer cells in response to paclitaxel is mediated by induction of FOXO3a expression, a transcription factor downstream of the phosphatidylinositol-3-kinase/Akt signaling pathway. To further investigate its mechanism of action, we treated MCF-7 cells with paclitaxel and showed a dose-dependent increase in nuclear localization of FOXO3a, which coincided with decreased Akt signaling but increased c-Jun NH_2-terminal kinase 1/2 (JNK1/2), p38, and extracellular signal-regulated kinase 1/2 (ERK1/2) activity. Flow cytometry revealed that paclitaxel-induced apoptosis of MCF-7 cells and of other paclitaxel-sensitive breast cancer cell lines was maintained in the presence of inhibitors of p38 (SB203580) or mitogen-activated protein/ERK kinase 1 signaling (PD98059) but abrogated when cells were treated with the JNK1/2 inhibitor SP600125. SP600125 reversed Akt inhibition and abolished FOXO3a nuclear accumulation in response to paclitaxel. Moreover, conditional activation of JNK mimicked paclitaxel activity and led to dephosphorylation of Akt and FOXO3a. Furthermore, mouse embryonic fibroblasts (MEF) derived from JNK1/2 knockout mice displayed very high levels of active Akt, and in contrast to wild-type MEFs, paclitaxel treatment did not alter Akt activity or elicit FOXO3a nuclear translocation. Taken together, the data show that cell death of breast cancer cells in response to paclitaxel is dependent upon JNK activation, resulting in Akt inhibition and increased FOXO3a activity. (Cancer Res 2006; 66(1): 212-20)

Introduction

Breast cancer is one of the most common forms of cancer that affect women in the western world. This condition arises as a consequence of cellular changes that increase the rate of cell division and/or decrease the rate of apoptosis. Typically, breast cancer is treated surgically as well as with antiendocrine therapies that target either the activation of the estrogen receptor (e.g., tamoxifen) or estrogen production (e.g., aromatase inhibitors; ref. 1). However, some tumors display intrinsic or acquired resistance to antiestrogen therapies (2) or metastasize to distal sites. These cancers require treatment with other chemotherapeutic agents, such as taxanes, of which paclitaxel and its chemical derivatives are the most commonly used in the clinic (3). This class of drugs binds to the tubulin microtubules of the spindle apparatus during mitosis and inhibits the segregation of sister chromatids (3, 4). The cellular responses to taxanes also involve activation of signal transduction intermediates, such as Akt (also called protein kinase B) and c-Jun NH_2-terminal kinase (JNK; reviewed in ref. 4).

The FOXO class of forkhead proteins are downstream targets of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. Activated PI3K phosphorylates phosphatydylinositol (4, 5) diphosphate (PIP_2) on the 3-position, thereby forming phosphatydylinositol (3–5) triphosphate (PIP_3). PIP_3 binds to the serine/threonine kinase Akt via its pleckstrin homology domain, which causes its translocation to the inner surface of the cell membrane. At the cell membrane, Akt becomes activated by phosphorylation, catalyzed by PDK1. FOXOs are phosphorylated by Akt on highly conserved serine and threonine residues, resulting in impaired DNA binding activity and increased binding to the chaperone protein, 14-3-3. Newly formed 14-3-3-FOXO complexes are then exported from the nucleus (reviewed in ref. 5), thereby inhibiting FOXO-dependent transcription of key target genes that promote cell cycle arrest and apoptosis, such as $p27^{Kip1}$ and *Bim* (6–9).

We previously showed that paclitaxel treatment of breast cancer cells stimulates the expression of the FOXO family member FOXO3a, which in turn increases cell death by inducing the expression of the Bcl-2 homology 3 domain (BH3)-only proapoptotic protein Bim (6). Others have shown that overexpression of Akt can increase resistance to paclitaxel (10, 11), further emphasizing the importance of PI3K/Akt/FOXO pathway in determining drug sensitivity. Other signal transduction pathways may also affect paclitaxel sensitivity. For instance, the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) become activated following paclitaxel treatment (12), and enhanced paclitaxel-induced cellular toxicity has also been observed following ERK1/2 inhibition (13). Similarly, p38 MAPK, a member of the stress response class of MAPKs, is also activated following paclitaxel treatment, and inhibition of this kinase also augments the apoptotic response (14).

Whereas the available evidence suggests that activation of the PI3K, ERK1/2, and p38 pathways may protect cells from the toxic effects of paclitaxel, the reverse seems to be true for members of the JNK family. JNK1 and JNK2 belong to the MAPK family and phosphorylate components of the activator protein transcription factor complex, such as c-Jun and ATF2, as well as TCF/ELK (15–17). In the same way that MAPK is activated by a series of

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sequential phosphorylation events by MAPKK, JNK is also activated by JNK kinase/SEK1/MAPKK through targeted phosphorylation of conserved threonine and serine residues (18). JNK kinase is in turn activated following phosphorylation by either MAPK kinase kinase 1 or 3 (MEKK1 or MEKK3), as well as by apoptosis signal-regulating kinase-1 (19). Many forms of cellular insult lead to activation of JNK, including UV light, osmotic stress, and irradiation (20–22). JNK signaling is activated following paclitaxel treatment (23–25), but unlike Akt, ERK1/2, and p38, inhibition of JNK reduces apoptosis following paclitaxel treatment (24).

We have previously reported that silencing of FOXO3a by small interfering RNA greatly reduces the induction of apoptosis caused by paclitaxel (6). The observations by others that JNK signaling is also needed for paclitaxel-induced apoptosis suggest a possible crosstalk between the PI3K-Akt-FOXO3a and JNK signaling pathways. In this report, we show that paclitaxel not only induces FOXO3a expression but also enhances its nuclear relocation through JNK-dependent inhibition of the PI3K/Akt signaling pathway.

Materials and Methods

Cell culture. The human breast carcinoma cell lines HMT 3522, MCF-7, 734 B, ZR-75-1, T47-D, CAL-51, CAMA-1, MDA-MB-231, and SKBR-7; mouse embryonic fibroblasts (MEF) derived from wild-type and *Jnk1* and *Jnk2* double knockout mice (26); and Rat-1 and RM3 (Rat-1 cells stably expressing the conditional protein kinase ΔMEKK3:ER*; ref. 27) were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 units/mL of penicillin/streptomycin in a humidified incubator in an atmosphere of 10% CO_2 at 37°C. Paclitaxel was obtained from Sigma Chemical Co. (Poole, United Kingdom), and SP600125, SB203580, and PD98059 were from Tocris (Avonmouth, United Kingdom); LY294002 and Triciribine were from Calbiochem (via VWR International Ltd., Lutterworth, United Kingdom); all were dissolved in DMSO.

Cell cycle analysis. Cell cycle analysis was done using propidium iodide staining as described (28). Briefly, cells were trypsinized, washed in PBS, and then fixed in 90% ethanol. Fixed cells were then washed twice in PBS and stained in 50 μmol/L propidium iodide containing 5 μg/mL Dnase-free RNase (Sigma, Poole, United Kingdom) for 1 hour, then analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Cowely, United Kingdom) and analyzed using Cell Quest software (Becton Dickinson).

Western blotting. Western blotting was done on whole-cell extracts prepared by lysing cells in NP40 lysis buffer [1% NP40, 100 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 30 mmol/L Naβ-glycerophosphate, and protease inhibitors ["Complete" protease inhibitor cocktail, as instructed by the manufacturer (Roche, Welyn Garden City, United Kingdom)] on ice for 15 minutes. Insoluble material was removed by centrifugation, and protein concentration was determined by Bio-Rad Dc protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). Twenty micrograms of protein were size fractionated using SDS-PAGE and electrotransferred onto Protran nitrocellulose membranes (Schliecher and Schuell, Dassel, Germany). Membranes were incubated with specific antibodies recognizing FOXO3a phosphorylated at Thr^{32} (06-953, Upstate, Dundee, United Kingdom), total FOXO3a (06-951, Upstate). Antibodies specific for Akt phosphorylated at the Ser^{473} (9271), Akt (9272), JNK1/2 phosphorylated at Thr^{183}/Tyr^{185} (9251), JNK1/29 (9252), c-Jun phosphorylated at Ser^{73} (9164), p38 phosphorylated at Thr^{180}/Tyr^{182} (9211), p38 (9212), ERK1/2 phosphorylated at $Thr^{202/204}$ (9101), total Erk1/2 (9102), and human caspase-9 (9502) were purchased from Cell Signaling Technologies (Hitchin, United Kingdom). Antibody to c-Jun (H79) was purchased from Santa Cruz (La Jolla, CA). Primary antibodies were detected using horseradish peroxidase–linked anti-mouse or anti-rabbit conjugates as appropriate (DAKO, Ely, United Kingdom) and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Amersham, United Kingdom). Nuclear and cytoplasmic lystes were prepared according to ref. (29). Briefly, cells were lysed on ice for 20 minutes in buffer containing 10 mmol/L HEPES (pH 7.4), 10 mmol/l KCl, 0.01 mmol/L EDTA, 0.1 mmol/L EGTA, 2 mmol/L DTT, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 30 mmol/L Naβ-glycerophosphate, and protease inhibitors ("Complete" protease inhibitor cocktail, as instructed by the manufacturer; Roche). Nuclei were sedimented by centrifugation, and the supernatant (cytoplasmic fraction) was retained. Nuclei were lysed in the same buffer containing 1% v/v NP40 and 400 mmol/L NaCl.

Immunofluorescence. Cells were grown on sterile, 13-mm-diameter coverslips and fixed in 4% formaldehyde before being permeabilized in 0.01% v/v Triton X-100. Coverslips were blocked in PBS containing 3% bovine serum albumin, and antibody recognizing FOXO3a or Bim (N20 Santa Cruz, La Jolla, USA) was added at 80 μg/mL. Specific staining was visualized with a secondary antibody conjugated to Alexa 488 (antirabbit) or Alexa 647 (antigoat; Molecular Probes, Eugene, OR) and analyzed on a Zeiss confocal microscope with LSM meta 510 software.

Results

Paclitaxel causes nuclear translocation of FOXO3a. We have previously shown that paclitaxel-induced apoptosis in the breast cancer cell line MCF-7 is mediated by increased expression and transcriptional activity of FOXO3a, which results in increased transcription of *Bim*, a proapoptotic gene that encodes for a BH3-only protein; specifically, paclitaxel induced an increasing FOXO3a expression. To activate the apoptotic machinery, FOXO3a has to reside in the nucleus, indicating that paclitaxel may also affect the subcellular localization of this transcription factor in breast cancer cells. To test this, we did confocal microscopy on MCF-7 cells treated with various concentrations of paclitaxel (1-10 nmol/L) at a time point (16 hours) that preceded the induction of FOXO3a expression. As shown in Fig. 1*A*, endogenous FOXO3a resided almost exclusively in the cytosol of untreated cells, whereas nuclear staining was negligible. The subcellular distribution of FOXO3a was unaltered in the presence of 1 nmol/L paclitaxel (Fig. 1*A*). However, at higher concentrations, FOXO3a became increasingly localized to the nuclei of MCF-7 cells, although some punctate perinuclear FOXO3a staining remained apparent. Also shown is the confocal microscopy result showing the expression of FOXO3a and the proapoptotic FOXO3a target Bim in cells treated with paclitaxel for 16 hours (Fig. 1*B*). The results support our hypothesis that nuclear translocation of the FOXO3a transcription factor is a necessary prerequisite for the activation of proapoptotic genes, such as *Bim*, in response to paclitaxel treatment.

Changes in cell signaling pathways occur following paclitaxel treatment and apoptosis. We next sought to identify the signal transduction pathways involved in regulating the subcellular redistribution of FOXO3a in response to paclitaxel treatment. To this end, we used total and phospho-specific antibodies to monitor the expression levels and activation status, respectively, of key members of several signal transduction pathways (Fig. 1*B*). Paclitaxel treatment of MCF-7 cells triggered transient phosphorylation of FOXO3a at the Thr^{32} residue, a known Akt phosphorylation site. The transient increase in FOXO3a phosphorylation was most pronounced 4 hours after treatment, but subsequently, the phospho-FOXO3a levels declined, reaching levels lower than those of the untreated cells by 16 hours. The levels of total FOXO3a began to increase after 16 hours and remained at an elevated level until 72 hours. The profile of phosphorylation of Akt at Ser^{473}, which reflects its activation status, mimicked that of phospho-FOXO3a, characterized by an initial but transient increase in the levels followed by a marked and persistent reduction. Total levels of Akt remained initially unchanged but increased slightly after 8 hours

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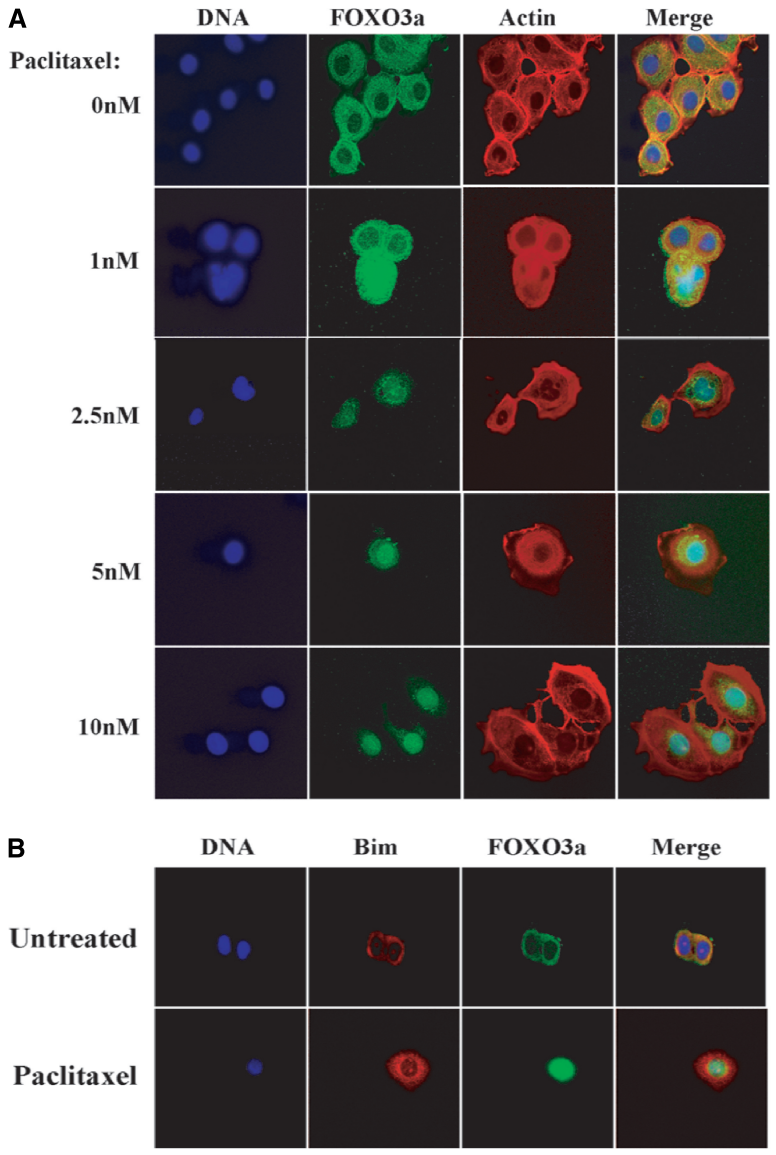

Figure 1. Paclitaxel causes FOXO3a translocation into the nucleus, which correlates with changes in signal transduction pathways and induction of FOXO3a targets. *A,* MCF-7 cells were cultured on sterile coverslips and treated for 16 hours with a range of concentrations of paclitaxel as shown, before being fixed in 4% formaldehyde. FOXO3a was visualized with a rabbit polyclonal antibody followed by the addition of ALEX488 (*green*) labeled anti-rabbit antisera. TRITC-labeled phalloidin (*red*) and DAPI (*blue*) were also applied to visualize the cytoplasm and nuclei, respectively. MCF-7 cells were treated with 10 nmol/L paclitaxel. *B,* MCF-7 cells were treated in the presence or absence of 10 nmol/L paclitaxel for 24 hours and processed as before. Cells were stained with an anti-FOXO3a rabbit polyclonal antibody and an anti-Bim goat polyclonal antibody followed by the addition of ALEX488 (*green*) and ALEX567 (*red*) labeled anti-rabbit and goat antisera, respectively. Nuclei were also stained with DAPI (*blue*). *C,* protein lysates were prepared at the times indicated, and protein expression levels were analyzed by Western blotting.

of paclitaxel treatment. The data suggest that dephosphorylation and hence nuclear accumulation of FOXO3a in response to paclitaxel treatment is likely to be a result of Akt inactivation.

Activation of JNK requires dual phosphorylation of conserved Thr^{183} and Tyr^{185} residues that can be monitored using phospho-specific antibodies. Upon paclitaxel treatment, the levels of phospho-JNK1/2 increased, which was first apparent as early as 4 hours after treatment, and this was followed by an increase in total JNK1/2 levels. The levels of activated p38 but not total p38 increased 16 hours after paclitaxel treatment and remained elevated throughout the time course. A similar profile, albeit less pronounced, was found for activated (phosphorylated at residues Thr^{202} and Tyr^{204}) and total ERK1/2. The Western blot results also showed that the expression of the FOXO3a target Bim increased from 24 hours after paclitaxel treatment and remained high. Active caspase-9 levels also increased, reaching a maxima at 48 and 72 hours after treatment, indicating that apoptosis was occurring in these cells at these times.

Activation of the JNK1/2 but not the ERK1/2 or p38 signaling pathways is required for the induction of apoptosis in paclitaxel-treated MCF-7 cells. To determine which of the kinases activated by paclitaxel affects breast cancer cell survival, we treated MCF-7 cells with SP600125, SB203580, and PD98059, which are inhibitors of JNK1/2, p38, and MAP/ERK kinase 1 (MEK1; the upstream activator of ERK1/2), respectively. Cells were analyzed by flow cytometry, and the extent of apoptosis was determined by measuring the fraction of cells with sub-G_1 DNA content. As shown in Fig. 2*A*, untreated MCF-7 cells displayed negligible apoptosis, and cells remained in exponential growth throughout the time course. Paclitaxel induced apoptosis of MCF-7 cells, and the levels gradually increased over the 72-hour treatment period. Cells also arrested in the G_2-M phase of the cell cycle upon paclitaxel treatment, in agreement with previous reports (3, 6). Treatment of MCF-7 cells with the JNK inhibitor SP600125 alone also caused accumulation of cells in the G_2-M phase of the cell

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cycle, but the fraction of cells undergoing apoptosis was far lower when compared with paclitaxel-treated cells. However, when cells were treated with a combination of SP600125 and paclitaxel, the cell cycle phase distribution profiles resembled those of cells treated with SP600125 alone. Interestingly, the percentage of cells undergoing apoptosis was dramatically lower than those with paclitaxel alone, indicating that inhibition of JNK1/2 activation protects against the cytotoxic effects of paclitaxel. Treatment of cells with the MEK1 inhibitor PD98059 or p38 inhibitor SB203580 alone did not elicit a cell death response at any time point or induce discernable changes in the cell cycle phase distributions. Furthermore, PD98059 as well as SB203580 failed to protect the cells against paclitaxel-induced apoptosis. Together, these results show that the activation of JNK but not ERK or p38 is necessary for the induction of apoptosis in response to paclitaxel treatment.

To extend these observations, we treated a panel of paclitaxel-sensitive breast cancer cell lines with either paclitaxel, SP600125, or a combination (Fig. 2*B*). Paclitaxel induced apoptosis in HMT3552, MCF-7, T47D, ZR-75-1, Cal-51, and SKBR-7, whereas addition of SP600125 markedly reduced the levels of cell death induced by paclitaxel. It is notable that SP600125 alone sometimes also elicited an apoptotic response but at a much lower level. These data indicate that JNK1/2 activation is a general requirement for paclitaxel-mediated apoptosis in breast cancer cells.

JNK signaling is required for FOXO3a activation in response to paclitaxel. Next, we investigated the consequences of JNK1/2 inhibition on the signaling intermediates activated by paclitaxel. As shown in Fig. 3, SP600125 markedly attenuated the drop in phospho-FOXO3a levels upon 16 hours of treatment with paclitaxel, this reduction was not observed in cells treated with a combination of SP600125 and paclitaxel. Treatment with SP600125 alone slightly lowered the phospho-FOXO3a levels but much less so than paclitaxel. There was little variation in total FOXO3a levels, regardless the treatment within the time course. The phosphorylation status of Akt mirrored that of FOXO3a, and SP600125 largely abolished the decline in phospho-Akt levels upon paclitaxel

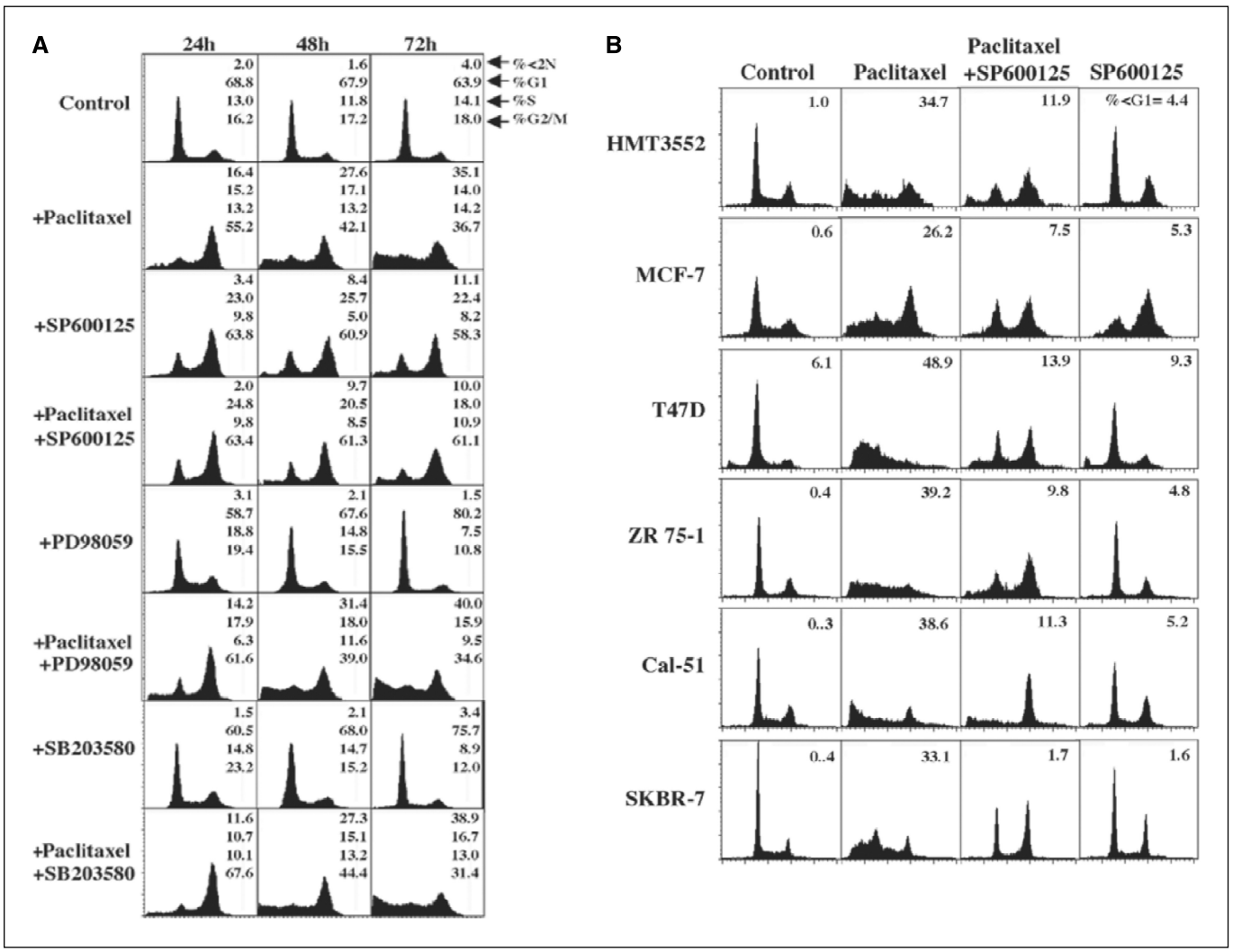

Figure 2. SP600125 inhibits apoptosis induced by paclitaxel treatment in a panel of breast cancer cell lines. *A,* MCF-7 cells were treated with either 10 nmol/L paclitaxel, one of the following chemical kinase inhibitors: 20 μmol/L SP600125, 30 μmol/L PD98059, and 30 μmol/L SB203580, or a combination of paclitaxel and a chemical inhibitor. Cells were fixed at 24, 48, and 72 hours after treatment, and cell cycle phase distribution was analyzed by flow cytometry. *B,* a panel of breast cancer cell lines (HMT3552, MCF-7, T47D, ZR-75-1, Cal-51, and SKBR-7) were treated with 10 nmol/L paclitaxel, 20 μmol/L SP600125, or a combination of 10 nmol/L paclitaxel and 20 μmol/L SP600125. Cells were fixed at 48 hours after treatment, and cell cycle phase distribution was analyzed by flow cytometry.

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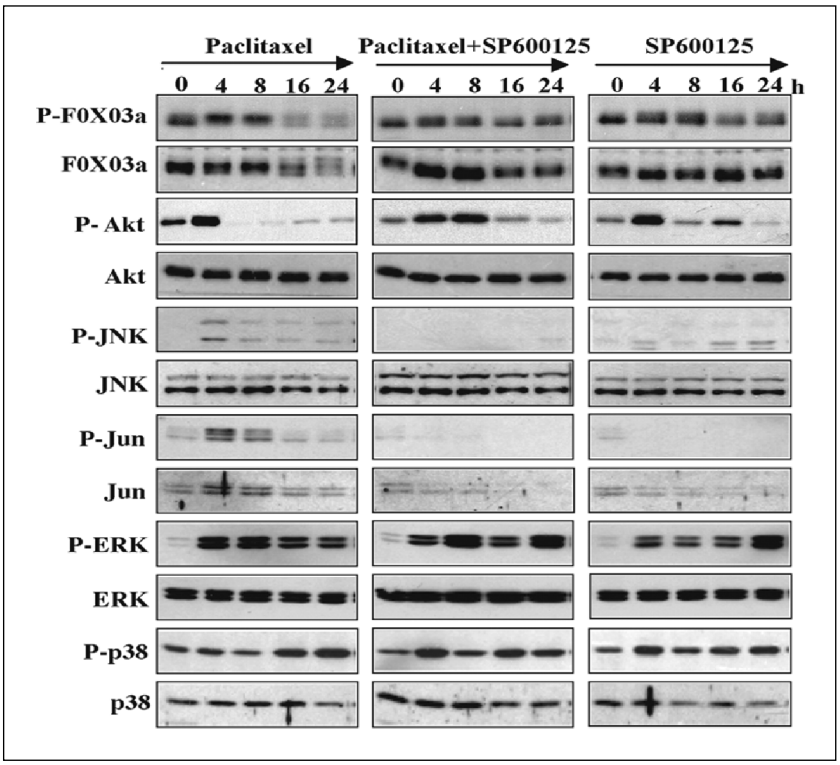

Figure 3. Changes in signal transduction pathways induced by paclitaxel in the presence and absence of JNK activity. MCF-7 cells were treated with 10 nmol/L paclitaxel, 20 μmol/L SP600125, or a combination of 10 nmol/L paclitaxel and 20 μmol/L SP600125. Protein lysates were prepared at the times indicated and analyzed by Western blotting.

treatment. Furthermore, treatment with SP600125 alone modestly inhibited Akt phosphorylation. The activation of JNK1/2 (phospho-JNK1/2) and the downstream target (phospho-c-Jun) upon paclitaxel treatment was abolished in the presence of SP600125. Interestingly, we observed a consistent, albeit very modest, increase in c-Jun levels in the paclitaxel but not paclitaxel plus SP600125-treated cells. This has been reported previously and may reflect stabilization of c-Jun by JNK1/2-dependent phosphorylation (26). The activation of ERK1/2 and p38 by phosphorylation seemed unaffected by inhibition of JNK1/2, suggesting that SP600125 does not affect the activity of these signaling molecules.

Inhibition of JNK1/2 by SP600125 prevents FOXO3a nuclear localization in MCF-7 cells and is dependent on PI3K/Akt signaling. To determine whether inhibition of JNK1/2 by SP600125 prevented apoptosis via attenuation of the nuclear translocation of FOXO3a, we did confocal microscopy on MCF-7 cells treated with paclitaxel, SP600125, or the combination for 16 hours. As shown in Fig. 4*A*, the majority of the endogenous FOXO3a remained in the cytoplasmic compartment in the untreated control cells and in the cells treated with SP600125. Interestingly, SP600125 largely abolished nuclear accumulation of FOXO3a in response to paclitaxel, indicating that the activation of JNK1/2 by paclitaxel mediates the nuclear translocation of FOXO3a. However, it was still unclear as to whether the effect of JNK on FOXO3a subcellular localization was dependent on PI3K-Akt signaling. To investigate this, we treated MCF-7 cells with either Triciribine or LY294002, specific inhibitors of Akt and PI3K, respectively, alone or in combination with SP600125, and examined the subcellular localization of FOXO3a (Fig. 4*B*). Immunocytochemistry showed that FOXO3a in control cells or those treated with SP600125 was predominantly localized within the cytoplasm. However, treatment of cells with either LY294002 and Triciribine resulted in a translocation of FOXO3a into the nucleus. Treatment with SP600125 in combination with either LY294002 or Triciribine failed to prevent the nuclear translocation of FOXO3a. Consistent with this, we also obtained data showing that SP600125 failed to revert the predominant nuclear localization of FOXO3a in a MCF-7 cell line expressing a dominant-negative Akt construct (data not shown). We also did nuclear and cytoplasmic fractionation experiments to analyze the relative amounts of FOXO3a in the nucleus and cytoplasm. The results shown in Fig. 4*C* show that the majority of FOXO3a in control and SP600125-treated cells was located in the cytoplasm, and that following treatment with paclitaxel, LY294002, or Triciribine, FOXO3a was predominantly located in the nucleus. Consistent with the immunocytochemical staining results, treatment of MCF-7 cells with SP600125 blocked the paclitaxel-induced nuclear translocation of FOXO3a. However, treatment of cells with SP600125 failed to inhibit the nuclear translocation of FOXO3a induced by inhibition of Akt activity through LY294002 or Triciribine. These data represent the first evidence in mammalian cells for a role for JNK1/2 in regulating FOXO3a nuclear translocation. Furthermore, these data suggest that the main determinant of FOXO3a subcellular localization is Akt activity, and that the effect of JNK1/2 on FOXO3a subcellular distribution occurs predominantly via modulation of PI3K/Akt signaling rather than by acting directly on FOXO3a.

Conditional activation of JNK1/2 inhibits Akt and FOXO3a phosphorylation. To confirm that FOXO3a is a downstream target of JNK1/2, we used RM3 cells, a clone of Rat-1 cells stably expressing the conditional protein kinase ΔMEKK3:ER* (27). ΔMEKK3:ER* consists of the isolated kinase domain of MEKK3 fused in frame to the hormone-binding domain of the human estrogen receptor. Treatment of RM3 cells with 4-hydroxytamoxifen causes dissociation of hsp90 and rapid activation of the kinase domain. Treatment with 4-hydroxytamoxifen increased the levels of phospho-JNK1/2 in RM3 cells but not in the parental Rat1 cells

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(Fig. 5*A*). JNK activation coincided with decreased levels of phospho-Akt and phospho-FOXO3a. JNK activation coincided with decreased levels of phospho-Akt and phospho-FOXO3a. However, tamoxifen treatment of RM3 cells also activated p38 and ERK1/2. To confirm that the reduced levels of phospho-Akt and phospho-FOXO3a observed in 4-hydroxytamoxifen–stimulated RM3 cells were the result of JNK1/2 activation and not a consequence of combined activation of JNK1/2, p38, and ERK1/2, the experiments were repeated in the presence of SP600125, SB203580, or PD98059. As shown in Fig. 5*B*, the JNK1/2 inhibitor

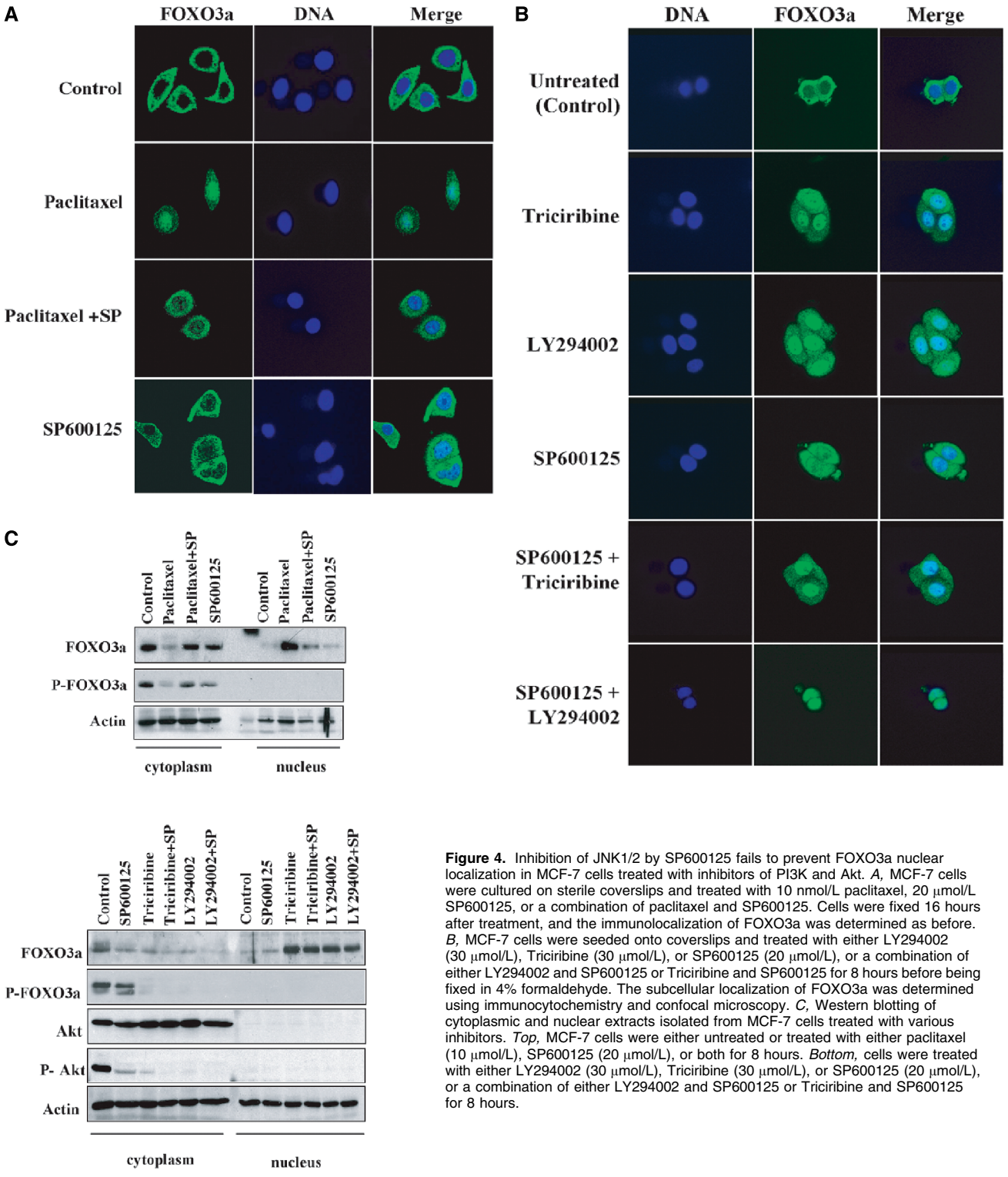

Figure 4. Inhibition of JNK1/2 by SP600125 fails to prevent FOXO3a nuclear localization in MCF-7 cells treated with inhibitors of PI3K and Akt. *A,* MCF-7 cells were cultured on sterile coverslips and treated with 10 nmol/L paclitaxel, 20 μmol/L SP600125, or a combination of paclitaxel and SP600125. Cells were fixed 16 hours after treatment, and the immunolocalization of FOXO3a was determined as before. *B,* MCF-7 cells were seeded onto coverslips and treated with either LY294002 (30 μmol/L), Triciribine (30 μmol/L), or SP600125 (20 μmol/L), or a combination of either LY294002 and SP600125 or Triciribine and SP600125 for 8 hours before being fixed in 4% formaldehyde. The subcellular localization of FOXO3a was determined using immunocytochemistry and confocal microscopy. *C,* Western blotting of cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with various inhibitors. *Top,* MCF-7 cells were either untreated or treated with either paclitaxel (10 μmol/L), SP600125 (20 μmol/L), or both for 8 hours. *Bottom,* cells were treated with either LY294002 (30 μmol/L), Triciribine (30 μmol/L), or SP600125 (20 μmol/L), or a combination of either LY294002 and SP600125 or Triciribine and SP600125 for 8 hours.

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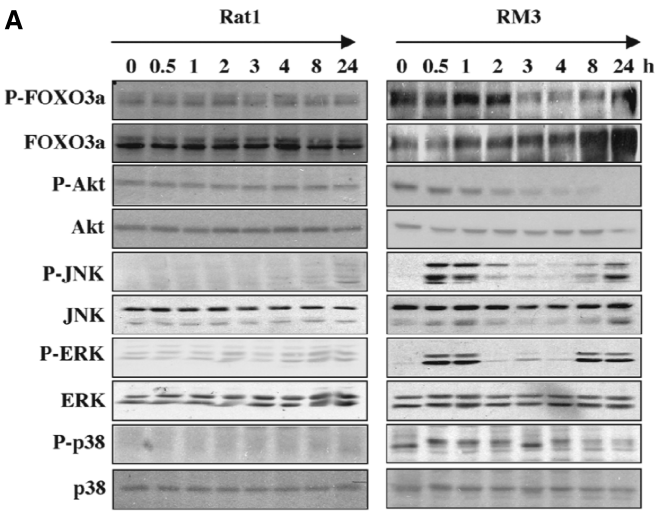
SP600125 completely abolished the decline in phospho-FOXO3a upon tamoxifen treatment of RM3 cells. In contrast, inhibition of MEK1 or p38 signaling had no effect on the phosphorylation status of FOXO3a, indicating that conditional activation of JNK1/2 is sufficient to antagonize Akt activity and attenuate FOXO3a phosphorylation.

MEFs nullizygous for the *JNK1* and *JNK2* loci fail to suppress phosphorylation of Akt and FOXO3 in response to paclitaxel. The ability of SP600125 to reduce phospho-Akt and -FOXO3a levels following paclitaxel treatment suggested crosstalk between the JNK1/2 and PI3K/Akt/FOXO pathways. To confirm that SP600125 exerts its effect by targeting JNK activity and not through nonspecific inhibition of other kinases, we treated wild-type MEFs as well as MEFs that lack both *Jnk1* and *Jnk2* alleles with paclitaxel. As shown in Fig. 6*A*, the levels of phospho-JNK1/2 increased markedly upon paclitaxel treatment in wild-type MEFs but not in JNK1/2-deficient cells. In parallel, the levels of phospho-c-Jun, a downstream JNK1/2 target, also increased in wild-type MEFS. Notably, the levels of phospho-Akt but not total Akt were constitutively elevated JNK1/2 null MEFs when compared with wild-type control cells. Furthermore, paclitaxel treatment resulted in a time-dependent reduction of phospho-Akt and phospho-FOXO3a levels in wild-type MEFs but not in the JNK1/2-deficient MEFs. These observations further show that JNK1/2 activation is required for paclitaxel-mediated Akt inhibition and FOXO3a activation. We next used confocal microscopy to monitor the subcellular localization of FOXO3a in response to paclitaxel treatment (Fig. 6*B*). In wild-type cells, endogenous FOXO3a was detectable in both the cytoplasmic and nuclear compartments of untreated cells but accumulated into the nuclei upon paclitaxel stimulation. Although FOXO3a also resided in the cytoplasm and nuclei of untreated JNK1/2 deficient cells, there was no evidence of nuclear translocation upon paclitaxel treatment. Taken together, the data shown here suggests that JNK1/2 double knockout cells lack the ability to repress Akt activity following drug treatment, thereby relieving Akt repression of FOXO3a, which is manifested in the failure of FOXO3a translocation to the nucleus following paclitaxel treatment (Fig. 6*B*).

Discussion

We have previously shown that in a paclitaxel-sensitive breast cancer cell line, paclitaxel treatment resulted in induction of FOXO3a expression, and FOXO3a induced transcription of the proapoptotic *Bim* gene, thereby resulting in apoptosis (6). In this report, we have shown that treatment of MCF-7 cells with paclitaxel also resulted in the accumulation of FOXO3a in the nucleus. This nuclear accumulation correlated well with the paclitaxel induced reduction of active Akt, suggesting that the inhibition of Akt by paclitaxel caused a reduction of phosphorylated FOXO3a levels, which lead to nuclear localization of FOXO3a. Furthermore, we observed that blocking the paclitaxel-induced activation of JNK1/2 but not ERK1/2 or p38 could prevent apoptosis. Indeed, treating MCF-7 cells with the JNK1/2 inhibitor SP600125 prevented the nuclear accumulation of FOXO3a, which is a prerequisite for transcriptional activation of proapoptotic genes that we have previously observed (6). Additional experiments also showed that the effect of JNK1/2 on FOXO3a nuclear localization in response to paclitaxel was dependent on PI3K-Akt signaling. The use of specific inhibitors of PI3K and Akt resulted in the nuclear translocation of FOXO3a, which could not be blocked by SP600125. The use of a cell line expressing the conditional kinase ΔMEKK3:ER*, an



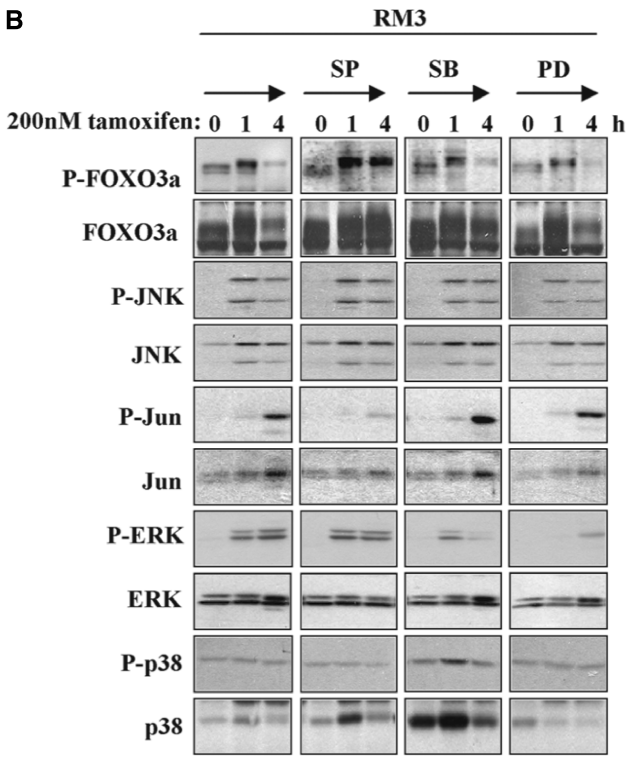


Figure 5. Ectopic activation of JNK1/2 inhibits Akt and FOXO3a phosphorylation. RM3 cells, which stably express ΔMEKK3:ER*, and the Rat-1 parental cell line were treated with 200 nmol/L 4-hydroxytamoxifen. Protein lysates were prepared at the times indicated, and protein expression was analyzed by Western blotting. *B,* RM3 cells were treated with 200 nmol/L tamoxifen alone or in the presence of either 20 μmol/L SP600125, 30 μmol/L PD98059, or 30 μmol/L SB203580 for the times indicated. Protein lysates were prepared at the times indicated, and protein expression was determined by Western blotting.

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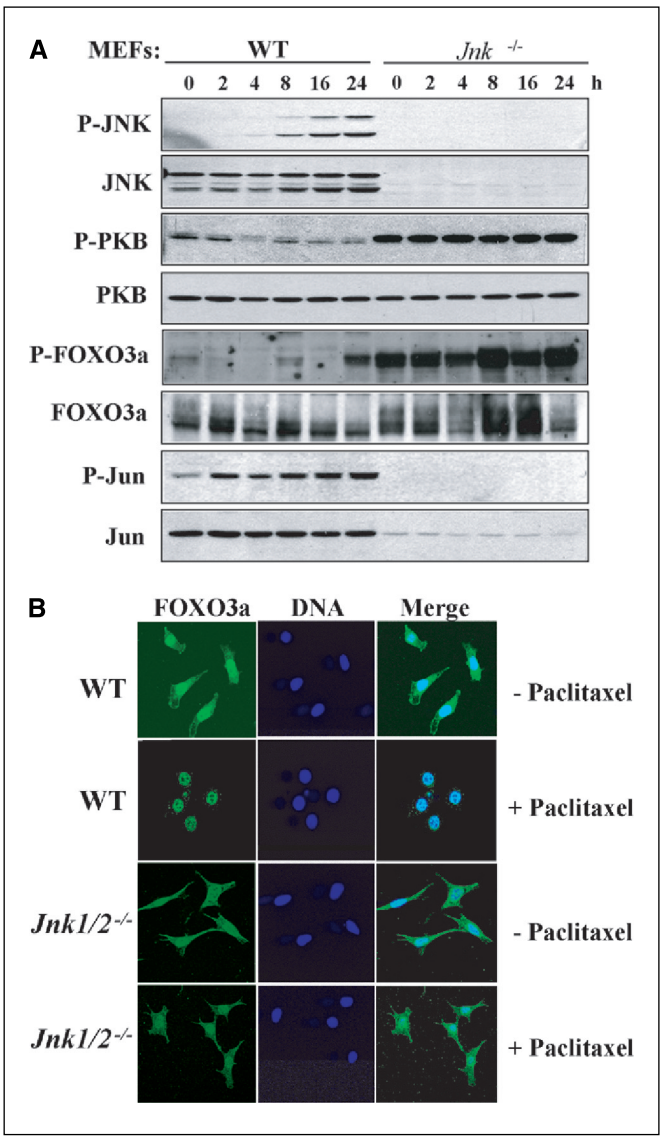

Figure 6. Paclitaxel treatment of MEFs nullizygous for the *Jnk1/Jnk2* loci fails to repress Akt activity or induce nuclear translocation of FOXO3a. MEFs derived from *JNK1/JNK2* knockout mice, as well as control cells were treated with 25 nmol/L paclitaxel. *A,* protein lysates were prepared at the times indicated, and protein expression levels were analyzed by Western blotting. *B,* MEFs derived from *JNK1/JNK2* knockout mice, as well as control cells were grown on sterile coverslips treated with 25 nmol/L paclitaxel for 16 hours and fixed in 4% formaldehyde. The subcellular localization of FOXO3a was determined using immunocytochemistry and confocal microscopy.

upstream activator of the JNK1/2 pathway, resulted in inhibition of Akt and FOXO3a phosphorylation. Furthermore, fibroblasts null for both the *Jnk1/Jnk2* loci contained less nuclear FOXO3a than wild-type controls and also exhibited higher levels of phosphorylated FOXO3a. Taken together, these results suggest that the main determinant of FOXO3a subcellular distribution is the PI3K-Akt signaling pathway, and this pathway may be subject to regulation by JNK1/2. This is the first report detailing the ability of JNK1/2 to regulate both Akt activity and thus the nuclear localization of FOXO3a in mammalian cells. Furthermore, the observation that inhibition of JNK1/2 protected a panel of breast cancer cell lines from paclitaxel-induced apoptosis combined with the fact that paclitaxel failed to repress both Akt and FOXO3a phosphorylation and FOXO3a nuclear translocation in JNK1/2 null MEFs suggests that this mechanism is widespread.

The data shown here clearly shows that treatment of MCF-7 cells with paclitaxel resulted in an initial transient stimulation of Akt followed by an immediate inhibition, which has been observed in other cell types (10). Indeed, the inhibition of Akt activity seems necessary for the induction of paclitaxel-induced apoptosis, because there are a number of reports that show that either enhanced activation of endogenous Akt or ectopic expression of a constitutively active Akt protects cells against the cytotoxic effects of paclitaxel (10, 11, 30, 31). Indeed, we have also observed that MCF-7 cells expressing a dominant-negative Akt expression construct undergo apoptosis more readily than control cells.[5] One explanation for this is that the inhibition of Akt would directly affect the phosphorylation status of FOXO3a, thereby resulting in its nuclear translocation and subsequent activation of target genes, which is in agreement with our previous observations (6).

Following the treatment of MCF-7 cells with paclitaxel, we observed an induction of JNK activity as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun. The initial increase in phospho-JNK1/2 paralleled that of Akt, but unlike phospho-Akt, this increase was prolonged. Our observations that JNK1/2 activity was elevated and prolonged in both MCF-7 and in MEFs coupled with the observation that inhibition by SP600125 suggests that the activation of JNK1/2 is required for paclitaxel-induced apoptosis. The activation of JNK1/2 and the inactivation of Akt following paclitaxel treatment suggested that there maybe crosstalk between these two signal transduction pathways. Indeed, our results indicated that activation of JNK in a 4-hydroxytamoxifen–inducible MEKK3-expressing cell line resulted in the reduction of Akt activity, as did our observation that MEF cells derived from JNK null mice contained higher levels of active Akt. Although the mechanism of this postulated inhibition of Akt by JNK1/2 is unclear, there is evidence that JNK is able to block insulin signaling via IRS phosphorylation (32).

With respect to the data shown here in which inhibition of JNK1/2 signaling prevented the nuclear localization of FOXO3a and protected cells from paclitaxel induced apoptosis, there are examples of JNK signaling affecting FOXO family members. Recent data has shown that JNK signaling can extend life span in *Drosophila* using a mechanism that represses insulin signaling and requires *Drosophila* FOXO (33). Furthermore, in *Caenorhabditis elegans*, it has been shown that the homologue of mammalian FOXO family members, DAF-16, is responsible for longevity and is regulated by insulin (34). It has also been shown that DAF-16 is directly phosphorylated by *C. elegans* JNK following heat shock, thereby increasing stress resistance and longevity via a mechanism that involves nuclear translocation of DAF-16 (35). In MEFs, it has been shown that the small GTPase RAL stimulates JNK activity, and that JNK1/2 phosphorylate FOXO4 directly at Thr^{447} and Thr^{451}, thereby enhancing FOXO4 activity (36). Although we have no direct evidence that JNK1/2 phosphorylate FOXO3a, we have observed a slower migrating form of FOXO3a in Western blots that is absent in the presence of SP600125. Whether this is a direct or indirect phosphorylation induced by JNK or a secondary modification caused indirectly by JNK1/2 is as yet unknown and will form the basis of further investigation.

[5] A. Sunters and E.W.-F. Lam, unpublished observations.

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The nuclear localization of FOXO3a is a prerequisite for transcriptional transactivation and, accordingly, the majority of FOXO3a was located in the cytoplasm in exponentially growing MCF-7 cells. However, this was not the case in either JNK1/2 null or control fibroblasts. In both these cases, the cells remained healthy, despite the apparent high levels of FOXO3a found in the nucleus of the mouse fibroblasts. This observation would suggest that there is a threshold for the amount of FOXO3a present in the nucleus that is required to induce cell cycle arrest and apoptosis (34, 37, 38). An attractive explanation would be that the apoptosis induced by paclitaxel is in part mediated by increased nuclear translocation of FOXO3a to a level at which exceeds this threshold, thereby allowing transcription of FOXO target genes. However, it is likely that there are other factors, which potentiate the activity of FOXOs once they are within the nucleus, such as direct phosphorylation by JNK1/2 (36) and other kinases. Thus, FOXO3a may be regulated by JNK on two levels, one whereby stress-induced JNK1/2 activity results in Akt inhibition and nuclear translocation as seen here, coupled with direct phosphorylation activity, which modulates the transcriptional activity as previously reported (36). This would also help to explain the continued survival of the control and JNK1/2 null MEFs, which had high levels of nuclear FOXO3a, which may not be fully active.

In summary, we have shown through the use of conditional protein kinases, selective protein kinase inhibitors, and JNK1/2 double null fibroblasts that JNK1/2 signaling plays a key role in determining the extent of apoptosis in response to paclitaxel. This may be in large part due to the JNK-dependent dephosphorylation, nuclear accumulation, and activation of FOXO3a. This suggests that patients with high levels of functional FOXO3a and JNK1/2 may respond more favorably to paclitaxel regimens. It is also possible that coadministration of agents that activate JNK may synergize with paclitaxel to promote tumor cell death, and that JNK activation may be a therapeutically desirable end point. This study is the first demonstration that JNK1/2 signaling regulates FOXO3a function and, in turn, the sensitivity of breast cancer cell lines to paclitaxel. This indicates that FOXO3a and JNK1/2 may be important targets in breast cancer and in the prediction of drug sensitivity.

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Paclitaxel-Induced Nuclear Translocation of FOXO3a in Breast Cancer Cells Is Mediated by c-Jun NH_2-Terminal Kinase and Akt

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