The p38 MAPK–MK2 Axis Regulates E2F1 and FOXM1 Expression after Epirubicin Treatment

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Abstract

E2F1 is responsible for the regulation of FOXM1 expression, which plays a key role in epirubicin resistance. Here, we examined the role and regulation of E2F1 in response to epirubicin in cancer cells. We first showed that E2F1 plays a key role in promoting FOXM1 expression, cell survival, and epirubicin resistance as its depletion by siRNA attenuated FOXM1 induction and cell viability in response to epirubicin. We also found that the p38–MAPK activity mirrors the expression patterns of E2F1 and FOXM1 in both epirubicin-sensitive and -resistant MCF-7 breast cancer cells, suggesting that p38 has a role in regulating E2F1 expression and epirubicin resistance. Consistently, studies using pharmacologic inhibitors, siRNA knockdown, and knockout mouse embryonic fibroblasts (MEF) revealed that p38 mediates the E2F1 induction by epirubicin and that the induction of E2F1 by p38 is, in turn, mediated through its downstream kinase MK2 [mitogen-activated protein kinase (MAPK)-activated protein kinase 2; MAPKAPK2]. In agreement, in vitro phosphorylation assays showed that MK2 can directly phosphorylate E2F1 at Ser-364. Transfection assays also showed that E2F1 phosphorylation at Ser-364 participates in its induction by epirubicin but also suggests that other phosphorylation events are also involved. In addition, the p38–MK2 axis can also limit c-jun-NH2-kine (JNK) induction by epirubicin and, notably, JNK represses FOXM1 expression. Collectively, these findings underscore the importance of p38–MK2 signaling in the control of E2F1 and FOXM1 expression as well as epirubicin sensitivity. Mol Cancer Res; 10(9); 1189–202. ©2012 AACR.

Introduction

Breast cancer is the most common cancer among women worldwide, with the lifetime risk of women being diagnosed with breast cancer at about 1 in 8. Treatment of breast cancer usually involves initial surgical removal of the tumor, in conjunction with adjuvant treatment, including radiotherapy, endocrine therapy, cytotoxic chemotherapy, and molecular targeted therapies, to help reduce the risk of cancer recurrence and promote survival (1). Cytotoxic chemotherapeutic drugs, such as taxanes and anthracyclines, are key treatments of breast cancer and other solid tumors. In addition to adjuvant treatments, these drugs are often used as neo-adjuvant treatments to reduce tumor size before surgery and to reduce the chance of metastasis (1).

Anthracyclines are some of the most common cytotoxic chemotherapeutic drugs used in the adjuvant setting, and the preferred option for treatment of estrogen receptor (ER)–negative breast cancers (2). They are derived from the bacterium Streptomyces peucetius and include, among others, doxorubicin and epirubicin. Besides their essential role in the treatment of many cancers, anthracyclines can also induce adverse side effects such as cardiomyopathy and congestive heart failure (3). Their mechanisms of action include intercalating DNA strands, inducing free oxygen radicals, and inhibiting topoisomerase II (4). By intercalating DNA strands, anthracyclines can inhibit important intracellular biologic mechanisms such as DNA replication, DNA repair, and protein synthesis. Topoisomerase II is an enzyme that introduces temporary double-stranded breaks (DSB) to resolve topological problems that occur during DNA replication and transcription (5). When inhibited by anthracyclines, topoisomerase II is unable to reseal these DNA breaks, leading to the accumulation of permanent DSBs, which are toxic lesions that can ultimately lead to cell death predominantly by apoptosis (6). Resistance to chemotherapeutic drugs is one of the major causes for the failure of anticancer treatments. Treatment with several anti-cancer drugs, including anthracyclines, can often trigger cross-resistance to...
other unrelated chemotherapeutic drugs, resulting in a much greater problem known as acquired multidrug resistance (7). Several mechanisms that may affect resistance to anthracyclines have been identified and they include altered pharmacokinetics and drug metabolism, increased drug efflux, decreased drug uptake, and increased drug-induced DNA damage repair (8). Nevertheless, a better understanding of the cellular and molecular mechanisms underlying anthracycline action and resistance, as well as the cellular factors involved, is essential for devising novel strategies for overcoming anthracycline resistance and for the development of more effective, more potent but safer cancer therapeutic strategies.

Forkhead box (FOX) proteins are members of an evolutionarily conserved family of transcription factors with key roles in the regulation of a variety of cellular and physiologic processes including development, metabolism, differentiation, proliferation, apoptosis, migration, invasion, and longevity (9). The forkhead box M1 (FOXM1) transcription factor is associated with cell proliferation and survival (9). It is ubiquitously expressed in all embryonic tissues and in adult proliferating cells and has an important role in the regulation of a variety of processes, including G1–S and G2 –M cell-cycle progression, chromosomal integrity, genomic stability, and DNA damage repair (10, 11). Loss of FOXM1 has catastrophic effects, and Foxm1-deficient mice have been shown to be embryonically lethal, due to failure to enter mitosis (12). Consistently, it has been shown that FOXM1 is barely detectable in quiescent cells, but its expression levels increase dramatically when stimulated to re-enter cell cycle (13).

Phosphorylation is one of the posttranslational modifications that modulates FOXM1 expression, cellular localization, and activity (9). Several regulatory kinases have been shown to activate FOXM1 via phosphorylation throughout the different stages of the cell cycle, which consequently leads to its nuclear translocation. During G1 to S-phase, FOXM1 is associated mainly with cyclin E–Cdk2 complexes, whereas in G2 phase, it primarily binds to the cyclin B–Cdk1 complex (14). In late S-phase, FOXM1 can also be activated by Raf–MEK–MAPK (mitogen-activated protein kinase) protein kinase signaling, before entry into G2–M phase (15). Moreover, cyclin A–Cdk complexes are crucial for activation of FOXM1 during G2 cell-cycle phase, by blocking the auto-inhibitory interaction between the NRD and TAD domains of FOXM1 (16). FOXM1 stability and transcriptional activity have been shown to increase following phosphorylation by checkpoint kinase 2 (Chk2), as a survival signal in response to DNA damage (17).

Overexpression of FOXM1 is associated with development and progression of various types of human cancers, including liver (18), prostate (19), lung (20), colorectal (21), and breast (22). Studies in mice have also shown that overexpression of FOXM1 plays a significant role in the process of tumor initiation, development, and progression, whereas reduction of FOXM1 expression results in a massive decrease in tumor growth (19, 22). In addition, the role of FOXM1 in DNA damage repair has been studied in Foxm1-deficient mouse embryonic fibroblasts (MEF), where cells show an increase in the number of DNA breaks (23). These findings strongly suggest a critical role of FOXM1 in maintenance of genome stability and DNA damage repair. Consistently, FOXM1 has been found to have a principal role in determining anthracycline sensitivity. In the drug-responsive cancer cells, the repression of FOXM1 expression by anthracycline has been shown to be mediated through p53 induction, which in turn represses E2F activity by activating the retinoblastoma pRB proteins and downregulating E2F1 expression. Besides p53, the DNA damage-sensing kinase Ataxia-telangiectasia mutated (ATM) also contributes toward modulating epirubicin sensitivity by regulating FOXM1 expression (24). Interestingly, p53 expression is lost and ATM is overexpressed in the epirubicin-resistant MCF-7-EPI breast cancer cells, further suggesting that the antagonistic signals from ATM and p53 modulate the cytotoxic effects of epirubicin through modulating E2F1 and FOXM1 expression. The regulation of FOXM1 expression by epirubicin is mediated primarily at the transcriptional level through E2F1 on the FOXM1 promoter (24). The discordance observed previously between the kinetics of E2F1 protein and transcript expression in response to epirubicin also led us to speculate that E2F1 expression is also modulated by nontranscriptional p53-independent mechanisms (24). The nontranscriptional mechanisms underlying E2F1 and FOXM1 activation by epirubicin remain largely unknown. In this study, we explored the upstream signaling events that regulate E2F1 expression and found that the p38–MK2 signaling axis plays a central part in controlling E2F1 and FOXM1 expression, cell survival, and epirubicin response.

Materials and Methods

Cell culture and reagents

U2OS osteosarcoma and MCF-7 breast carcinoma cell lines originated from the American Type Culture Collection. Epirubicin-resistant MCF-7 (MCF-7-EPI)5 breast cancer cells, were nonclonal MCF-7 cells subjected to increasing amounts of epirubicin until cells acquire resistance to 10 μmol/L of epirubicin, originally described (24). p38α+/−, p38β−/−, p38δ−/− MEFs were kindly provided by Angel Nebreda (Institució Catalana de Recerca i Estudis Avançats, Barcelona, Spain) and JNK1/2−/−, p53−/−, MEFs were previously been described (25). CK2−/−, MK2−/−, MK3−/−, and MK2/3−/− MEFs were kind gifts from Matthias Gaestel (Institute of Biochemistry, Hannover Medical School, Hannover, Germany; ref. 26). All cell lines were in culture for less than 6 months and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma) containing 10% fetal calf serum, 2 mmol/L glutamine, and 100 U/mL penicillin/streptomycin in a humidified incubator at 37°C with an atmosphere of 10% CO2. The inhibitors SB203580 and SP600125 were acquired from Calbiochem (Merck), whereas SB202190 was from Sigma. Epirubicin was obtained as Epirubicin Hydrochloride (Hospira UK Limited) from Imperial College Healthcare NHS Trust Pharmacy (London, UK) and was used at 10 μmol/L unless mentioned otherwise.

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Western blot analysis and antibodies

Cells were lysed and SDS-PAGE was carried out as described (27). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology): anti-FOXO1 (C-20), anti-E2F1 (C-20 and KH95), anti-β-tubulin (H-235), and anti-MAPK-1 (V-15). Antibodies against phospho-p38 (Ser15; #9284), phospho-Chk2 (Thr68; #2197), phospho-MK2 (Thr33; Thr46; #2332), phospho-JNK (Ser276; #9251), phospho-c-jun (Ser73; #2197), phospho-p53 (Ser15; #9284), phospho-Chk2 (Ser345; #2544), cleaved caspase-3 (#9664), and PARP (#9542) were acquired from Cell Signaling Technology (Merck Millipore). E2F1 phospho-Ser-364 antibody (ab5391) was from Abcam. Secondary polyclonal goat anti-mouse or goat anti-rabbit antibodies conjugated to peroxidase were purchased from DAKO Cytomation. 

siRNA transfection

The following ON-TARGETplus SMARTpool siRNAs were purchased from Dharmacon: L-003505-00 (MAPK9/JNK2), L-003512-00 (MAPK14), L-003972-00 (p38α), L-003512-00 (MAPK14), L-003505-00 (MAPK9/JNK2), L-003512-00 (MAPK14), and L-003972-00 (p38α).

Cell-cycle analysis

Floating and adherent cells were harvested, collected by centrifugation, washed with PBS, fixed in cold 70% ethanol, and kept overnight at 4 °C. Cell pellets were washed twice with PBS and resuspended in PBS containing 25 μg/mL propidium iodide, RNase A, and Triton X-100 in PBS for 30 minutes at room temperature in the dark immediately before fluorescence-activated cell-sorting (FACS) analysis in a FACS Canto flow cytometer (Becton Dickinson). The cell-cycle profile was analyzed using the Cell Diva software (Becton Dickinson).

siRNA transfection

The following ON-TARGETplus SMARTpool siRNAs were purchased from Dharmacon: L-003572-00 (MAPK11/p38β), L-003514-00 (MAPK8/JNK1), L-003505-00 (MAPK9/JNK2), L-003512-00 (MAPK14/p38α), L-005014-00 (MAPKAPK3), and D-001210-01-05 (nonspecific). siRNA oligonucleotides corresponding to MAPKAP kinase-2 sense strand 5'-GGGCGCCCTGCGGGGCTCCCGTGGACGAGGAC-AGG-3' and antisense strand 5'-GCGGATCGTCATCATCTCCGCG-3' were synthesized by Sigma. siRNAs were transfected into cells using Oligofectamine (Invitrogen) according to the manufacturer’s protocol.

Plasmids

The luciferase reporter plasmid used was the FOXM1 promoter Apal construct, which has been described previously (11). The Renilla reporter (pRL-TK; Promega) was used as a transfection efficiency control in luciferase assays. The expression plasmids pCMV-HA-E2F1 and pCMV-E2F1 E132 have been previously described (28) and are kind gifts from Xin Lu (Ludwig Institute for Cancer Research, Oxford, UK). The expression plasmids pDNA3-MK2-WT and pDNA3-MK2-K76R have been previously described (29, 30). The site-directed S364A, S364D, S31A, and S31D mutations were introduced into the pCMV-HA-E2F1 plasmid by the QuickChange Mutagenesis system (Stratagene) using the following primers: E2F1-S364A-F: 5'-GAACCGCCCTTGTGTCGCCGATGGGCGCCCTGCGGGC-3' and E2F1-S364A-R: 5'-GGCGTCTCGTCCAGGGG-3'.

Luciferase assay and transfections

Four thousand cells were seeded in each well of a 96-well plate, left to attach overnight, and transfected with 20 ng of the human FOXM1 (Apal) promoter reporter and 5 ng of Renilla reporter plasmid, alone or in combination with 20 ng of an E2F1 expression vector [either pCMV-E2F1-(132E), pCMV-HA-E2F1, or the S364A, S364D, S31A or S31D pCMV-HA-E2F1 mutants], using a 3:1 ratio of Fugene-6 reagent (Roche). Thirty-six hours posttransfection, the activity of the reporter genes was determined with the steadylight plus Reporter Gene Assay System (Perkin-Elmer). Luminescence was read using a PHERAstar Plus microplate reader (BMG Labtech). Following this, the Renilla substrate coelenterazine was added and Renilla luciferase activity measured.

In vitro kinase assay

Active recombinant MK2 protein (1 μg) was incubated with purified GST–E2F1 wild-type or mutant GST–E2F1 S364A proteins (2 μg) for 30 minutes in kinase buffer (10 mmol/L HEPES at pH 7.5, 75 mmol/L KCl, 5 mmol/L MgCl2, 0.5 mmol/L EDTA, 2 mmol/L dithiothreitol, 100 μmol/L ATP) in the presence of 5 μCi [γ-32P]ATP. Reactions were stopped by the addition of 4× SDS loading buffer, and the protein mixtures were resolved by denaturing gel electrophoresis. Gels were subjected to autoradiography to monitor 32P incorporation. Equal levels of proteins were confirmed by Coomassie blue staining.

Results

Correlation of p38–MAPK induction with E2F1 and FOXM1 expression in response to epirubicin

The primary mechanism of action of epirubicin involves DNA intercalation and topoisomerase II inhibition, causing double-strand breaks, as well as the production of oxygen free radicals, which damage DNA. Among the pathways that are activated in response to epirubicin are the ATM-Chk2 and the p38 and c-jun-NH2 kinase (JNK) stress–activated MAPK signaling cascades. Thus, as a first step toward identifying the cellular molecules that integrate the epirubicin signals with E2F1 and FOXM1, we investigated by Western blot analysis, the expression patterns of kinases known to be activated by chemotherapeutic drugs in both
sensitive and resistant (MCF-7-EPIR) MCF-7 breast cancer cells following epirubicin treatment (Fig. 1A). FOXM1 expression was induced transiently by epirubicin in the MCF-7 cells and this was accompanied by the accumulation of E2F1, supporting the notion that E2F1 regulates FOXM1 expression. Both E2F1 and FOXM1 were expressed constitutively at high levels in MCF-7-EPIR cells. The results also showed that p-JNK, p-ERK, and p-Chk1 were induced by epirubicin in MCF-7 but not in MCF-7-EPIR, suggesting they are unlikely to be directly involved in the regulation of E2F1 and FOXM1 expression at least in the resistant cells. The results also showed that the phosphorylated active forms of p38 (p-p38; T180/T182) and to some extent p-Chk2 (T68), but not p-ERK (T202/T204), p-JNK (T183/T185), and p-Chk1 (S345), displayed similar expression patterns as E2F1 and FOXM1 in the epirubicin-sensitive MCF-7 cells. In the resistant MCF-7-EPIR, p38 and Chk2 appeared to be constitutively phosphorylated, whereas E2F1 and FOXM1

![Figure 1](image-url)
Epirubicin Regulates E2F1 and FOXM1 via the p38–MK2 Axis

expressed at high levels. These findings suggest that p38 and possibly Chk2 may modulate E2F1 and FOXM1 expression in both cell lines (Fig. 1A). We have shown previously that E2F activity plays a pivotal part in the regulation of FOXM1 expression and that both p53 and ATM, which co-regulate E2F1 expression, are deregulated in the drug-resistant MCF-7-EPIR cells (24). As a consequence, E2F1 is deregulated and expressed at constitutively high levels in the MCF-7-EPIR cells (see also Supplementary Figs. S3 and S4), which will not be a suitable cell model for studying the regulation of E2F1 and FOXM1 expression. To investigate further the potential regulation of E2F1 and FOXM1 expression by p38 and Chk2, we studied the expression levels of p-p38, p-JNK, p-Chk2, E2F1, and FOXM1 in the epirubicin-resistant and p53 and pRB wild-type U2OS cells upon epirubicin and H2O2 treatment, as the kinetics of activation of these kinases differs upon treatment with different DNA damaging and stress-inducing stimuli (Fig. 1B). The results showed that H2O2 induced an early and transient increase in E2F1 levels whereas epirubicin induced a delayed and sustained increase. Consistent with results from breast cancer cell lines, E2F1 levels accumulated with similar kinetics as FOXM1, p-JNK, and p-p38 in response to both epirubicin and H2O2 treatments. The results also showed that although p-Chk2 accumulated with similar kinetics as p-p38, p-JNK, and p-Chk2, E2F1 and FOXM1 in U2OS cells treated with H2O2, it was induced at a faster kinetics than p-p38, p-JNK, and E2F1 and FOXM1 in cells stimulated with epirubicin. As the Chk2 kinase has previously been described to mediate Ser-364 phosphorylation of E2F1 at Ser-364 (S364) was also monitored following H2O2 and epirubicin treatment. Surprisingly, the kinetics of S364 phosphorylation mirrored that of p38–MAPK and JNK activation, instead of Chk2 induction. These data suggest that p38 and possibly JNK, and not Chk2, is a major regulator of E2F1 expression and Ser-364 phosphorylation in response to epirubicin and H2O2. We next examined the effect of epirubicin in U2OS cells depleted of E2F1 using siRNA and the results revealed that knockdown of E2F1 impaired FOXM1 upregulation and sensitized U2OS cells to epirubicin-induced apoptosis, as revealed by the increased levels of cleaved PARP and caspase-3 (Fig. 1C). Together these data indicate that E2F1 is at least partially responsible for FOXM1 upregulation upon epirubicin treatment and its expression plays a key role in epirubicin response.

Epirubicin induces E2F1 accumulation and phosphorylation via p38

To examine the role of p38 signaling on E2F1 accumulation in response to epirubicin, U2OS cells were left untreated or treated with the specific p38 inhibitor SB203580 for 1 hour before exposure to epirubicin. Pretreatment with the p38 inhibitor prevented epirubicin-induced accumulation of E2F1 and its phosphorylation on the Ser-364 residue (Fig. 2A). Consistently, chemical inhibition of p38 substantially increased epirubicin-induced apoptosis. To further assess the requirement of p38 activity for E2F1 induction in response to epirubicin, we examined the effect of increasing concentrations of p38 inhibitor on E2F1 levels in U2OS cells treated with epirubicin for 6 hours, a time when E2F1 levels peak. The result showed a dose-dependent decrease in E2F1 and p-E2F1 levels, which was accompanied by a corresponding decrease in FOXM1 protein levels (Fig. 2B; Supplementary Fig. S1). To rule out potential off-target effects of the chemical p38 inhibitor, U2OS cells were transfected with p38si and p38B siRNA pools or nontargeting control siRNA for 48 hours and then treated with epirubicin for 0, 3, 6, and 9 hours. Silencing the expression of p38α and β isoforms caused a reduction in epirubicin-induced E2F1 accumulation and S364 phosphorylation, which was accompanied by a decrease in FOXM1 upregulation and enhanced apoptosis independently of p53 pathway (Fig. 2C). Consistently, quantification of the percentage of cells with sub-G1 DNA content by FACS also indicated that p38 inhibition sensitized U2OS cells to epirubicin-induced cell death (Fig. 6A).

The p38 substrate MK2 phosphorylates E2F1 on Ser-364 in response to epirubicin

Our results thus far suggested that p38 mediates E2F1 Ser-364 phosphorylation, which could have a role in the regulation of E2F1 expression. In consequence, we next sought to identify the kinase responsible for Ser-364 phosphorylation as well as the functional outcome of this posttranslational modification. The Chk2 kinase has previously been described to mediate Ser-364 phosphorylation on E2F1 (31), but its activation by epirubicin was not impaired by SB203580 treatment (Fig. 2A), suggesting that p38 does not mediate Ser-364 phosphorylation through Chk2. In addition, it is unlikely that p38 phosphorylates Ser-364 directly as the site does not conform to the consensus sequence motif for phosphorylation by p38 because it lacks a proline residue at position +1. However, the Ser-364 motif closely resembles that of the p38 substrate MAPKAPK-kinase 2 (MK2), whose activation was inhibited by p38 inhibitor or p38 siRNA (Fig. 2A and C). Collectively, these observations led us to hypothesize that MK2, functioning downstream of p38, mediates the E2F1 accumulation and Ser-364 phosphorylation in response to epirubicin. To test whether E2F1 is a direct substrate of MK2, we conducted an in vitro kinase assay using a full-length GST–E2F1 fusion protein and an active recombinant MK2 (Fig. 3A). As controls, we also carried out parallel in vitro kinase assays using a GST–E2F1 fusion protein in which the Ser-364 residue was mutated to Ala as well as the glutathione S-transferase (GST) protein as substrates. In the presence of γ32P-ATP, MK2 was able to phosphorylate GST–E2F1, but not GST alone, whereas mutation of Ser-364 to alanine resulted in substantially reduced incorporation of γ32P-ATP into the GST–E2F1 S364A protein (Fig. 3A). Although in silico analysis of the E2F1 amino acid sequence also revealed 2 additional putative phosphorylation sites (i.e., RXXS/ST) for MK2 at Ser-131 and Ser-235, these in vitro kinase assay results suggest that the Ser-364 residue is a major MK2 phosphorylation site on E2F1. To determine whether MK2 is responsible for the phosphorylation of E2F1 at Ser-364 in vivo in response to
epirubicin, we analyzed the effect of knocking down MK2 by siRNA in U2OS cells using the phospho-E2F1 (S364) antibody. Indeed, Ser-364 phosphorylation upon epirubicin treatment was considerably blunted in cells depleted of MK2 (Fig. 3B). In addition, E2F1 accumulation was reduced and apoptosis enhanced in the MK2-depleted cells, indicating that MK2 contributes to E2F1 phosphorylation on Ser-364 in response to epirubicin and suggesting that this phosphorylation event may contribute to E2F1 stabilization. Nevertheless, as MK2 depletion did not completely abrogate Ser-364 phosphorylation of E2F1, we reasoned that the highly homologous MK3 (75% amino acid identity) could perhaps partially compensate for the loss of MK2. However, combined RNA interference against MK2 and MK3 did not reduce further the levels of phosphorylated E2F1, suggesting that MK3 is not the kinase that cooperates with MK2 to mediate Ser-364 phosphorylation of E2F1 (Fig. 3C). It is notable that p-p38 expression was increased in the MK2- and MK3-depleted cells, and this could be due to a negative feedback signaling mechanism. However, E2F1 expression levels were decreased rather than increased in the MK2- and MK3-depleted cells, further indicating that MK2 but not p38 directly mediates E2F1 expression. Chk2 has been shown to be able to phosphorylate the Ser-364 residue in response to genotoxic stress and recently, siRNA-mediated knockdown of Chk2 has also been shown to prevent the accumulation of E2F1 protein in response to DNA damage (31). We reasoned that MK2 and Chk2 kinases might be functioning in parallel to mediate Ser-364 phosphorylation. As a result, we analyzed the effect of Chk2 depletion by siRNA and compared it with that of MK2 depletion in epirubicin-treated U2OS cells (Fig. 3D; Supplementary Fig. S2). The results showed that MK2 but not Chk2 depletion significantly reduced the epirubicin-induced Ser-364 phosphorylation. This argues in favor of MK2 being the predominant mediator of Ser-364 phosphorylation. Consistent with this, the results also revealed that only MK2, but not Chk2, knockdown concomitantly reduced E2F1 and FOXM1 induction and enhanced epirubicin-induced apoptosis (Fig. 3D; Supplementary Fig. S2). Together, such results suggest that Chk2 may contribute toward Ser-364 phosphorylation, but it is MK2 that predominantly phosphorylates E2F1 on Ser-364 and mediates its accumulation in response to epirubicin. These results also indicate that MK2 controls E2F1 and FOXM1 accumulation as well as cell survival and mediates E2F1 phosphorylation on the Ser-364 residue in response to epirubicin. Consistent with this, we also found that depletion of E2F1 expression or p38 activity can sensitize the MCF-7 and U2OS but not MCF-7-EPI® cells to epirubicin (Supplementary Figs. S3–S5). This is likely to be due to the fact that both p53 and ATM, which

![Figure 2](http://molecr.mcr.aacrjournals.org/content/mcr/10/9/1194/F2.large.jpg)

**Figure 2.** p38 MAPK mediates E2F1 and FOXM1 induction in response to epirubicin. A, pretreatment with the p38 inhibitor SB203580 prevents epirubicin-induced E2F1 accumulation and its phosphorylation at Ser-364. U2OS cells were either pretreated with 20 μmol/L SB203580 or untreated and 1 hour later exposed to 1 μmol/L epirubicin. At the times indicated at the top of each lane, cells were harvested and proteins extracted from lysates. Equal amounts of protein were used for Western blot analysis with the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control. B, the p38 inhibitor SB203580 reduces the expression of epirubicin-induced E2F1 in a dose-dependent manner, accompanied by a corresponding decrease in FOXM1 protein levels. Total cell lysates derived from U2OS cells pretreated with increasing concentrations of SB203580 (0, 5, 10, 20 μmol/L) followed by 6-hour exposure to 1 μmol/L epirubicin were subjected to Western blot analysis. An untreated sample (C-) was loaded as a negative control. Anti-β-tubulin served as a loading control. C, siRNA-mediated depletion of p38 impairs E2F1 Ser-364 phosphorylation and FOXM1 upregulation induced by epirubicin. U2OS cells were transfected with nontargeting siRNA (NS) or a mixture of siRNAs specific to p38α and p38β. Forty-eight hours later, cells were treated with 1 μmol/L epirubicin and harvested at the indicated time points. Whole-cell extracts were prepared and equal amounts of protein were subjected to Western blot analysis using the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control.
have a predominant role in the regulation of E2F activity, are
deregulated in the MCF-7-EPIR cells, leading to E2F1 and
FOXM1 to be expressed at constitutively high levels and not
sensitive to p38 regulation (ref. 24; see also Supplementary
Figs. S3–S5).

Ser-364 phosphorylation contributes to E2F1 induction/
activity
To directly examine the functional consequence of E2F1
Ser-364 phosphorylation, we generated phosphorylation
mutants for E2F1-Ser-364, with S364 mutated to alanine
(pCMV-HA-E2F1-S364A) and to glutamic acid (pCMV-
HA-E2F1-364E) as well as mutants for E2F1-S31 (pCMV-
HA-E2F1-S31A and pCMV-HA-E2F1-1-S31D), another
phosphorylation site targeted by DNA damage response and
shown to modulate E2F1 protein stability. These phosphor-
ylation mutants and the wild-type E2F1 (pCMV-HA-E2F1-
WT) were then co-transfected into U2OS cells with a
FOXM1 promoter–luciferase reporter construct and their
expression levels and transactivational activity studied before

![Figure 3](https://example.com/figure3.png)

**Figure 3.** The p38 substrate MK2 phosphorylates E2F1 on Ser-364 in response to epirubicin. A, the p38 substrate MK2 can phosphorylate E2F1 on Ser-364 in vitro. Recombinant active human MK2 (1 μg) was incubated with 2 μg of bacterially produced GST, GST-E2F1, or GST-E2F1 S364A as a substrate in the presence of γ^{32}P-ATP. Reaction products were resolved by SDS-PAGE and visualized by autoradiography (top). The corresponding Coomassie blue stained gel is shown in the bottom. B, MK2 contributes to E2F1 phosphorylation on Ser-364 in response to epirubicin. U2OS cells were transfected with nontargeting siRNA (NS) or siRNA against MK2, and 48 hours later treated with 1 μmol/L epirubicin and harvested at the indicated times. Whole-cell lysates were prepared, and equal amounts of protein were used for Western blot analysis using the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control. C, combined depletion of MK2 and MK3 does not completely abrogate epirubicin-induced E2F1 phosphorylation at Ser-364. U2OS cells were transfected with nontargeting siRNA (NS) or a mixture of siRNAs against MK2 and MK3. Forty-eight hours later cells were treated with 1 μmol/L epirubicin and harvested at the indicated times. Whole-cell lysates were prepared, and equal amounts of protein were used for Western blot analysis using the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control. D, MK2 or Chk2 depletion by siRNA both reduces E2F1 phosphorylation at Ser-364 but only MK2 ablation compromises E2F1 or FoxM1 upregulation in response to epirubicin. U2OS cells were transfected with nontargeting siRNA (NS), Chk2 siRNA, or MK2 siRNA. Forty-eight hours later, cells were treated with 1 μmol/L epirubicin and harvested at the indicated times. Whole-cell lysates were prepared, and equal amounts of protein were used for Western blot analysis with the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control.
Figure 4. FOXM1 phosphorylation is responsible for the induction of E2F1 protein expression in response to epirubicin. A, neither Ser-364 nor Ser-31 is solely responsible for the induction of E2F1 protein expression in response to epirubicin. U2OS cells were transfected with the empty pCMV plasmid, pCMV-E2F1-132E, wild-type (WT) pCMV-HA-E2F1 plasmid, or an pCMV-HA-E2F1 mutant plasmid bearing one of the following point mutations: S31A, S31D, S364A or S364D. Thirty-six hours later, transfected cells were either untreated or exposed to epirubicin for 6 hours and harvested. Whole-cell extracts were prepared, and equal amounts of protein were subjected to Western blot analysis using the antibodies indicated. B, E2F1 phosphorylation mutants have depleted transactivation activity. Four thousand cells were seeded in each well of a 96-well plate, allowed to attach overnight, and transfected with 20 ng of the indicated expression plasmids together with 5 ng Renilla reporter and 20 ng of the luciferase reporter gene driven by the E2F1 promoter. Twenty hours later, 6 replicates of each condition were left untreated, whereas 6 others were treated with 1 μmol/L epirubicin for 3 hours before measurement of luciferase activity. Graphs represent mean (± SD) luciferase activity corrected for Renilla luciferase activity. Values were normalized to that of the empty vector in untreated cells. C, S364 phosphorylation contributes to E2F1 activation. Four thousand cells were seeded in 96-well plates, allowed to attach overnight, and transfected with either empty vector, pCMV-E2F1-132E or increasing concentrations (0, 10, 20, or 30 ng) of wild-type HA-E2F1 or the E2F1-S364A point mutant together with 5 ng Renilla reporter and 20 ng of the luciferase reporter gene driven by the E2F1 promoter. Six replicates of each combination were measured. Graphs represent mean (± SD) luciferase activity in corrected for Renilla luciferase activity. Values were normalized to that of empty vector (–). D, MK2 and p38 contribute to FOXM1 transactivation by E2F1. U2OS cells were transfected with 20 ng of empty vector, pCMV-E2F1-132E, or wild-type HA-E2F1 together with 5 ng Renilla reporter and 20 ng of the luciferase reporter gene driven by the FOXM1 promoter (24) in the absence or presence of 20 ng pcDNA3-MK2-WT, 20 ng pcDNA3-MK2-K76R) or 20 μmol/L SB203580. Six replicates of each combination were measured. Graphs represent mean (± SD) luciferase activity in corrected for Renilla luciferase activity.
and after 3-hour epirubicin treatment (Fig. 4A). The DNA-binding defective mutant pCMV-E2F1-(132E) was also included in the study as a negative control. The Western blot analysis result showed that the ectopically expressed wild-type E2F1 as well as all the phosphorylation mutants increased in protein expression levels following epirubicin treatment. Given that these E2F1 expression constructs do not contain noncoding E2F1 sequences, the findings support the idea that E2F1 increases in protein stability in response to epirubicin. However, these data also suggest that neither Ser-31 nor Ser-364 is entirely responsible for the induction of E2F1 protein expression in response to epirubicin, as mutation to either of the S364 or Ser-31 phosphorylation sites failed to abrogate the increase in expression levels following epirubicin treatment (Fig. 4A). The luciferase reporter assays revealed that all the Ser-364 and Ser-31 phosphorylation mutants were able to transactivate FOXM1 promoter. However, the phosphorylation mutants were less effective in transactivating the FOXM1 promoter compared with the wild-type E2F1 but were more potent in relation to the E2F1-(132E) construct which displayed basal activity (Fig. 4B). The amino acids, glutamic acid (E) and aspartic acid (D), carry negative charges, which resemble the effects of amino acid phosphorylation. However, the phosphorylation mimics (E/D) do not always reproduce the structural and aggregation properties of phosphorylation in vitro (32). When the phospho-mimic mutants do not reproduce the effects of phosphorylation, they may function

Figure 5. p38 induces E2F1 expression via MK2 upon epirubicin treatment. A, SB203580 treatment induces JNK activation and downregulation of E2F1 and FOXM1 expression in response to doxorubicin. MCF-7 cells were either pretreated with 20 μmol/L SB203580 or untreated and 1 hour later exposed to 1 μmol/L epirubicin. At the times indicated at the top of each lane, cells were harvested and proteins extracted. Equal amounts of protein were used for Western blot analysis with the indicated antibodies. Anti-p38 was immunoblotted as a loading control. B, knockdown of p38 enhances the intensity and duration of JNK activation. U2OS cells were transfected with nontargeting siRNA (NS) or siRNAs specific to p38α and p38β. Forty-eight hours later, cells were treated with 1 μmol/L epirubicin and harvested at the indicated time points. Whole cell lysates were prepared, and equal amounts of protein were subjected to Western blot analysis with the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control. C, MK2 mediates the p38-dependent repression of JNK activity. U2OS cells were transfected with nontargeting siRNA (NS), Chk2 siRNA, or MK2 siRNA. Forty-eight hours later, cells were treated with 1 μmol/L epirubicin and harvested at the indicated times. Whole cell lysates were prepared, and equal amounts of protein were used for Western blot analysis with the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control. D, p38-dependent repression of JNK activity is mediated by MK2 and MK3. Wild-type or MK2−/− MK3−/− MEFs were treated with 1 μmol/L epirubicin for 0, 4, or 8 hours. Whole-cell extracts were prepared, and equal amounts of protein were subjected to Western blot analysis using the indicated antibodies. Anti-β-tubulin was immunoblotted as a loading control.
as phospho-defective mutants, as they cannot be phosphorylated. These data also indicate that the DNA-binding domain of E2F1 is required for the full stimulatory effect on the FOXM1 promoter. The effect of Ser-364 phosphorylation in E2F1 transactivation was also analyzed using the luciferase reporter assay driven by E2F1 promoter and it showed that Ser-364 is necessary for activation of E2F1 (Fig. 4C). Together, such data suggest that both Ser-31 and Ser-364 phosphorylation sites are involved in epirubicin response, but their phosphorylation is not indispensable for the induction of E2F1 protein stability following epirubicin treatment. We have shown previously that epirubicin activates FOXM1 transcription through an E2F site on its promoter (24). To examine the effect of MK2 or p38 inhibition on the transcriptional activity of E2F1 on the FOXM1 promoter, we investigated the ability of a dominant-negative MK2 (MK2-K76R) and p38 inhibitor SB203580 to repress the transcriptional activity of E2F1 on the FOXM1 promoter in a cotransfection study (Fig. 4D). The promoter–reporter assays showed that the

Figure 6. JNK inhibition attenuates whereas p38 inhibitor enhances epirubicin–induced apoptosis. A, JNK inhibition attenuates epirubicin–induced apoptosis, whereas p38 inhibition sensitizes U2OS cells to epirubicin–induced apoptosis. U2OS cells were either untreated or pretreated with either 25 μmol/L SP600125 or 15 μmol/L SB202190 during 1 hour before adding epirubicin to the media (final concentration, 1 μmol/L). Samples were collected by trypsinization 0, 6, or 24 hours after epirubicin addition, stained with propidium iodide, and analyzed by flow cytometry to determine the cell-cycle distribution (top). The chart below shows the quantitation of the percentage of cells with sub-G0 DNA content. B, opposing effects of p38 and JNK inhibition on epirubicin–induced FOXM1 upregulation and apoptotic cell death. U2OS cells were either pretreated with 20 μmol/L SB203580 or 25 μmol/L SP600125 or untreated and 1 hour later exposed to 1 μmol/L epirubicin. At the times indicated at the top of each lane, cells were harvested and proteins extracted from lysates. Equal amounts of protein were used for Western blot analysis with the indicated antibodies. Anti–β-tubulin was immunoblotted as a loading control. C, absence of JNK in MEFs augments the induction of FOXM1 expression by epirubicin and suppresses epirubicin–induced apoptosis. Wild-type, JNK1−/−/JNK2−/− MEFs were treated with 1 μmol/L epirubicin for 0, 4, or 8 hours. Whole-cell extracts were prepared, and equal amounts of protein were subjected to Western blot analysis using the indicated antibodies. Anti–β-tubulin was immunoblotted as a loading control. D, JNK depletion augments E2F1 and FOXM1 levels induced by epirubicin treatment and confers resistance to epirubicin–induced apoptosis. U2OS cells were transfected with nontargeting siRNA or siRNAs specific to either JNK1 or JNK2. Forty-eight hours later, cells were treated with 1 μmol/L epirubicin and harvested at the indicated time points. Whole-cell extracts were prepared, and equal amounts of protein were subjected to Western blot analysis using the indicated antibodies. Anti–β-tubulin was immunoblotted as a loading control.
transcriptional activity of E2F1 on the FOXM1 promoter can be inhibited by treatment with the p38 inhibitor SB203580 and by cotransfection with the dominant-negative MK2 (pcDNA3-MK2-K76R) but not the wild-type MK2 (pcDNA3-MK2-WT) expression vector. These results suggest that MK2 and p38 are required for the transcriptional activity of E2F1 on the FOXM1 promoter.

**p38 and MK2 suppress JNK activation by epirubicin**

To confirm further our findings, we next examined whether inhibition of the p38 pathway using SB203580 can also block the induction of E2F1 and FOXM1 by epirubicin and enhance its cytotoxic effect in the epirubicin-sensitive breast cancer MCF-7 cells (Fig. 5A). The results showed that pretreatment with SB203580 blunted the accumulation of P-E2F1 (S364), E2F1, and FOXM1 in response to epirubicin and enhanced the cytotoxic effects of epirubicin (also see Fig. 6A). Inhibition of p38 by SB203580 also augmented the induction of JNK activity by epirubicin, suggesting that p38 represses the induction of JNK activity by epirubicin (Fig. 5A; Supplementary Fig. S6). Intriguingly, the results also revealed that the downregulation of FOXM1 expression in the SB203580-treated cells preceded the decrease in E2F1, indicating that p38 can also regulate FOXM1 expression in an E2F1-independent manner. Notably, the downregulation of FOXM1 coincided with the induction of p-JNK and occurred after the inhibition of MK2 activity, as revealed by p-MK2 (T334). This led us to hypothesize that p38 might also enhance FOXM1 expression through activating MK2 and repressing JNK activity. To test this conjecture, we first analyzed the JNK1/2 activity in U2OS cells depleted of p38, MK2, or MK3 following epirubicin treatment by Western blot analysis using an anti-phospho-JNK antibody (Fig. 5B and C). Compared with the nontargeting siRNA-transfected cells, U2OS cells transfected with p38Δα and p38β siRNA (Fig. 5B), MK2 siRNA, or MK3 siRNA (Fig. 5C) displayed higher levels of phospho-JNK upon epirubicin treatment, suggesting that p38 can repress JNK activity through MK2/3. In agreement, JNK hyperactivation was also observed in MK2/3−/− and not wild-type MEFs (Fig. 5D). Importantly, MK2/3 knockout also resulted in impaired E2F1 and FOXM1 accumulation upon epirubicin treatment and sensitization to epirubicin-induced apoptosis, as indicated by the cleaved PARP and caspase-3. These observations are consistent with the idea that p38 induces FOXM1 expression through repressing JNK activity as well as activating MK2 and E2F1.

In agreement, JNK hyperactivation was also observed in MCF-7 cells pretreated with the p38 inhibitor SB2023580. This suggests that part of the effects we observe on inhibition of p38-MK2 expression or activity can be due to enhanced JNK activity. To elucidate the role of JNK, we used a reversible ATP-competitive JNK inhibitor, SP600125 (also known as JNK inhibitor II) to determine the effect of inhibiting JNK activity on epirubicin-induced E2F1 and FOXM1 accumulation and cell survival (Fig. 6A). The p38 inhibitor SB2023580 was also included in this experiment to study the effects of p38 inhibition. Compared with cells treated with epirubicin alone, JNK inhibition enhanced FOXM1 upregulation and reduced apoptotic cell death whereas p38 inhibition impaired FOXM1 induction and increased the amount of apoptosis, as indicated by PARP cleavage. The phosphorylation of p53 at Ser-15 was unaffected by either inhibitors and neither was the accumulation of total p53 protein, indicating that the modulation of FOXM1 expression and survival by p38 and JNK is not mediated through p53 (Fig. 6B). To analyze further the role of JNK, we compared the effect of epirubicin treatment in MEFs isolated from Jnk1/2−/− and wild-type mice (Fig. 6C). The results showed that the absence of JNK in MEFs augmented the induction of FOXM1 expression by epirubicin and suppressed the epirubicin-induced apoptosis. To confirm this further, we silenced Jnk1 and Jnk2 expression by transient siRNA transfection in U2OS cells (Fig. 6D). JNK depletion augmented E2F1 and FOXM1 levels induced by epirubicin treatment and substantially reduced caspase-3 cleavage in a p53-independent manner, as p53 levels were not induced. Quantification of the percentage of cells with sub-G1 DNA content by FACS also indicated that JNK depletion confers resistance to epirubicin-induced apoptosis (Fig. 6D). We next asked whether the effects of MK2 on FOXM1 were entirely due to repression of JNK activity. We expressed exogenously MK2 and a dominant-negative kinase dead form of MK2, MK2 (K76R), which mimics the effect of inhibiting MK2 activity (Supplementary Fig. S7) in Jnk1/2−/− deficient MEFs. While expression of wild-type MK2 had little effect on E2F1 or FOXM1 expression upon epirubicin treatment compared with empty vector-transfected cells, the kinase dead mutant caused a considerable reduction of FOXM1 induction but not E2F1 accumulation.

**Discussion**

The E2F family of transcription factors is known to determine the timely expression of genes crucial for entry into and progression through S-phase of the cell cycle (33). However, it has also become increasingly evident that cell-cycle control is only one facet of the many functions of E2F proteins, which also regulate gene expression essential for a wide range of other biologic processes, including DNA replication, mitosis, DNA damage repair, differentiation, and autophagy (34, 35). Moreover, at least one family member, E2F1, also modulates cell death by apoptosis (36). In this study, we have established that E2F1 protects the drug-resistant cancer cells from epirubicin-induced cell death, as E2F1-depleted U2OS cells were more sensitive to epirubicin treatment. In addition, E2F1 is transiently induced by epirubicin before subsiding to basal levels in the drug-sensitive breast carcinoma MCF-7 cells, whereas it is constitutively expressed at elevated levels in the resistant cells, further supporting a pro-survival and drug-resistant role for E2F1 in epirubicin treatment. These findings are consistent with previous findings that E2F1 is a transcriptional activator of FOXM1 (24), which plays a key role in cell survival, DNA damage response, and anthracycline.

www.aacrjournals.org Mol Cancer Res; 10(9) September 2012 1199

Published OnlineFirst July 16, 2012; DOI: 10.1158/1541-7786.MCR-11-0559

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resistance (24, 37). In agreement, a number of reports have described a prosurvival and DNA repair function for E2F1 in the context of UV- (38) or γ-irradiation–induced DNA damage (39). Moreover, E2F1 has been shown to be able to promote the recruitment of DNA repair factors to sites of DNA DSBs (40). However, these findings are at odds with other reports proposing that anthracyclines and other similar genotoxic drugs induce E2F1 expression to accelerate apoptosis. Nevertheless, together these results suggest that E2F1 may have a role in cell death and can function as a suppressor as well as activator in a context-dependent manner.

The present study focused primarily on identifying the upstream regulators of E2F1 and FOXM1 during epirubicin treatment. We showed that inhibition of p38 activity using the chemical inhibitor SB203580 or its expression by siRNAs or in knockout MEFs impairs E2F1 accumulation and Ser-364 phosphorylation and enhances apoptosis in epirubicin-treated cells. This indicates that p38 acts upstream of E2F1 and promotes E2F1 and FOXM1 expression and therefore cell survival in response to epirubicin. Our data revealed that the effect of p38 on E2F1 is at least partially mediated through the p38 substrate MK2. Consistently, MK2 inhibition or deletion by siRNA can attenuate the accumulation of E2F1 and FOXM1 as well as cell survival in response to epirubicin treatment. We also observed that MK2 can phosphorylate E2F1 directly in vitro primaril on Ser-364, and notably, MK2 is responsible for Ser-364 phosphorylation in vivo upon epirubicin treatment. Phosphorylation of E2F1 by Chk2 at Ser-364 has previously been shown to increase its protein stability in response to DNA damage (31). In agreement, our data also showed that MK3 and Chk2 are capable of phosphorylating Ser-364 and promoting E2F1 accumulation in response to epirubicin. However, our data suggest that MK2 is the kinase primarily responsible for E2F1 phosphorylation at Ser-364 in response to epirubicin, as only deletion of MK2, and not Chk2 or MK3, can effectively abrogate the E2F1 Ser-364 phosphorylation accumulation.

Previous work has also proposed a more direct relationship between p38 and E2F1, in which p38 phosphorylates E2F1 in vitro on Ser-403 and Thr-433. In keratinocytes, p38 activity is a major contributor to E2F1 phosphorylation, and these 2 phosphorylation sites have been shown to be critical for the modulation of E2F1 stability, subcellular localization, and thus correct execution of differentiation programs (41). In contrast, another study has also shown that E2F1 phosphorylation on Ser-403 and Thr-433 is induced by the anthracycline doxorubicin but has ruled out p38 as the kinase responsible, on the basis that p38 inhibitors failed to affect Ser-403 phosphorylation (42). A more recent report linked p38 to E2F1 regulation in response to etoposide via p38-mediated the retinoblastoma protein (pRB) phosphorylation on Ser-567. This phosphorylation event triggers the degradation of pRB by Mdm2, leading to the release of E2F activity (43). We cannot exclude the contributions of these MK2-independent mechanisms toward E2F1 regulation, but the MK2 deletion studies show that MK2 is the major regulator of E2F1 in response to epirubicin, as MK2 deletion by siRNA or gene knockout is sufficient to effectively suppress E2F1 induction by epirubicin.

Consistent with previous findings that Ser-364 phosphorylation enhances E2F1 stability (31), our data indicate that Ser-364 phosphorylation contributes toward E2F1 induction as the non-phosphorylatable S364A mutant has lower transactivation activity compared with wild-type E2F1. Nevertheless, the fact that epirubicin can still enhance E2F1 expression levels also reveals that other E2F1 phosphorylation sites as well as alternative mechanisms may also participate in the regulation of E2F1 expression. In agreement with the idea that Ser-364 phosphorylation cannot be solely responsible for E2F1 stabilization by epirubicin is the observation that the mouse E2F1 expression could also be induced by epirubicin in MEFs, despite the Ser-364 phosphorylation site not conserved in mice. Because 2 other residues in E2F1 also bear the consensus MK2 phosphorylation motif, it is possible that increased stability of E2F1 results from other MK2-dependent phosphorylation events. Alternatively, MK2 could also affect E2F1 turnover indirectly. For instance, MK2 has been reported to phosphorylate and activate Mdm2 on S166 (44), and Mdm2 can inhibit E2F1 degradation by displacing the E2F1 E3 ligase Skp2 (45). Collectively, these results suggest that the epirubicin-induced accumulation of E2F1 is mediated via p38 and its downstream kinase MK2.

In addition, our data also indicate that the p38-MK2 MAPK pathway can negatively regulate JNK activity. Accordingly, deletion of either p38 or MK2 activity results in JNK hyperactivation upon epirubicin treatment. Moreover, JNK pathway was hyperactivated in epirubicin-treated MEFs, suggesting that MK2 and MK3 cooperate to inhibit JNK signaling. Consistent with our findings, a number of studies have previously reported that JNK activity is overactivated upon pharmacologic p38 inhibition in several cell types and in p38α-deficient mouse cells when subjected to treatment with a diversity of stimuli, including lipopolysaccharide (LPS), interleukin (IL)-1, and sorbitol. Mechanistically, this JNK hyperactivation can be due to a reduction in DUSP1 (MKP1) phosphatase level or an increase in upstream MAP2K activity, such as that of MKK4/7 and MKK6/3. In our model, there are no appreciable changes in DSUP1 expression levels upon p38/MK2 inhibition but indeed increased MKK3/6 phosphorylation could be observed, suggesting the involvement of a negative feedback signaling loop to enhance JNK and p38 activity via MAP2Ks (Supplementary Fig. S8).

Our results indicate that JNK inhibition results in enhanced FOXM1 expression and reduced apoptosis in response to epirubicin. Conversely, JNK hyperactivation as a consequence of p38 inhibition greatly enhances susceptibility to apoptosis. A previous study has described that JNK1 phosphorylates E2F1 in vitro and that cotransfection of JNK1 reduces the DNA-binding activity of E2F1 (46). Notably, the fact that E2F1 expression can be induced by E2F1 activity renders E2F1 activity, becoming synonymous with its expression. We observed that JNK activity has a negative impact on FOXM1 levels, but its role on E2F1...
phosphorylation and expression is less well understood. Because p-JNK and p-p38 expressed in similar kinetics in response to epirubicin treatment, it is possible that JNK also contributes to E2F1 phosphorylation and expression, which warrants further investigation.

In summary, our data suggest that the p38–MK2 axis can regulate FOXM1 expression and, therefore, epirubicin resistance by 2 mechanisms. First, the p38-activated MK2 can phosphorylate and stabilize E2F1, which, in turn, regulates the expression of FOXM1 involved in cell survival and DNA repair. Second, MK2 can inactivate JNK to relieve its repression on FOXM1 expression. Collectively, these findings underscore the importance of p38–MK2 signaling in the control of E2F1 and FOXM1 expression as well as drug sensitivity in response to epirubicin. Our study also identifies the E2F1 Ser-364 as a MK2 phosphorylation acceptor site in response to epirubicin. These findings could have important implications for therapeutic interventions as well as monitoring chemotherapy treatment and predicting sensitivity.

References


Molecular Cancer Research

The p38 MAPK–MK2 Axis Regulates E2F1 and FOXM1 Expression after Epirubicin Treatment


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Access the most recent version of this article at:
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