C-jun Inhibits Mammary Apoptosis In Vivo

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INTRODUCTION

The c-jun oncprotein is an essential component of an activator protein transcription complex. On hetero-dimerization with other Jun/Fos members it forms an active activator protein-1 complex (AP-1), which regulates the expression of various target genes harboring AP-1–binding DNA elements within their promoters. AP-1 controls the expression of genes that regulate several crucial cellular functions such as cell cycle progression, migration, proliferation, and apoptosis. c-jun is activated upon phosphorylation by c-jun N-terminal kinase (JNK), which responds to diverse biological stress signals (Kyriakis, 1999).

c-jun functions as both a positive and negative regulator of apoptosis (Shaulian and Karin, 2002). Nerve growth factor (NGF) withdrawal induced neuronal apoptosis is reduced by c-jun inhibition, and mice expressing the c-jun phosphorylation site mutant (c-jun A65/73) are resistant to kainate-induced apoptosis (Ham et al., 1995; Behrens et al., 1999). In contrast, c-jun inhibits TNF-α–induced apoptosis via p53/p21 in transformed fibroblasts (Eferl et al., 2003), and p53 is induced in c-jun−/− liver tumors. p53 however is not activated in c-jun−/− hepatocytes (Eferl et al., 2003), suggesting acute c-jun deficiency induces apoptosis eliminating p53-overexpressing cells.

Reactive oxygen species (ROS) are thought to play an important role in cell death: apoptosis, programmed necrosis, cellular proliferation, premature senescence, genomic instability, and cellular invasiveness. On one hand, ROS induced by oncogenic Ras or ErbB2 has been shown to induce antiproliferative signals that block cellular proliferation and growth (Dolado et al., 2007). ROS can cooperate with oncogenic signals and in some circumstances ROS production is associated with activation of growth receptors including transforming growth factor-β and platelet-derived growth factor (PDGF; Sattler et al., 1999; Balaban et al., 2005). The carcinogenic effect of ROS has been linked to increased cellular proliferation rate seen in many cancer cell lines (Fernandez-Pol et al., 1982; Church et al., 1992). Mitochondria are the major source of cellular ROS, derived from leakage of electrons from carriers in the electron transport chain (Scherz-Shouval et al., 2007) produced during the turnover of complex I and complex II. Molecular oxygen is converted to superoxide (O2−), which in turn develops into ROS. Nonmitochondrial ROS are derived from other compartments including peroxisomes, plasma membrane, the endoplasmic reticulum, Golgi complex, lysosomes, and the nucleus. ROS may be generated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which includes a catalytic subunit of the NADPH oxidase (NOX) family (Lambeth, 2004). ROS formation and efflux may be altered by the mitochondrial inner transmembrane potential (Δψm), pH gradient and the outer mitochondrial membrane potential, O2− production is enhanced by high Δψm ROS leave the mitochondria either via diffusion or via active carriers such as the inner membrane anion channel (IMAC) or the voltage-dependent anion channel (VDAC; Boveris and Chance, 1973).
Superoxide dismutases are the antioxidant enzymes that catalyze the dismutation of O$_2$ to H$_2$O$_2$. There are two forms of intracellular superoxide dismutases (SODs) in mammalian cells: nuclear and cytoplasmic CuZnSOD (SOD-1) and mitochondrial matrix localized MnSOD (SOD-2).

In addition to passive or active diffusion from mitochondria, ROS undergo enzymatic elimination via MnSOD (Melov, 2000) or CuZnSOD (Okado-Matsumoto and Fridovich, 2001). Further detoxification is undertaken by glutathione peroxidase (GPx; Nohl and Jordan, 1980) which uses reduced glutathione (GSH) and/or catalase in which converts H$_2$O$_2$ to H$_2$O (Radi et al., 1991).

The c-jun gene plays a central role in the diverse functions of the AP-1 complex. Although the number of studies is small, analysis of acute loss of target genes have revealed distinct results compared with germ line deletion studies (Sage et al., 2003). The current studies were conducted first, therefore, to determine the signaling pathways regulated by endogenous c-jun in vivo. To this end, c-jun$^{+/+}$ mice were deployed, and excision was conducted using cre recombinase. Laser capture microdissection (LCM) was conducted of cells deleted of c-jun, and genome-wide expression analysis was conducted to determine signaling pathways regulated by c-jun in vivo. Second, JNK and the c-jun genes have been linked to both the inhibition and induction of cellular apoptosis (Xia et al., 1995; Kuan et al., 1999; Xia and Karin, 2004). The current studies were therefore conducted to examine the effect of acute c-jun excision on cellular survival.

**MATERIALS AND METHODS**

### Transgenic Mice and Expression Plasmids

Transgenic animals carrying floxed c-jun alleles, c-jun$^{flo}$, and mouse mammary tumor virus (MMTV)-Cre were previously described (Zena et al., 2003). The mammary gland mRNA was obtained from virgin female mice at 2 mo of age. All experimental procedures conducted on these mice were approved by the ethics committee of Thomas Jefferson University. Quantitation of mammary epithelial cell number was conducted as described (Atabai et al., 2005). Apoptosis in situ was determined using the TdT in situ apoptosis kit (DAB peroxidase (GPx; Nohl and Jordan, 1980) which uses reduced glutathione (GSH) and/or catalase in which converts H$_2$O$_2$ to H$_2$O (Radi et al., 1991).

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### Regenerants and Antibodies

The following reagents and antibodies were used: H$_2$DCFDA (Invitrogen, Carlsbad, CA); anti-Cre (EMD Biosciences, San Diego, CA); anti-c-jun (H-79), anti-survivin, and anti-ribo-guanine nucleotide disassociation inhibitor (GDI; Santa Cruz Biotechnology, Santa Cruz, CA); VDAC-1 (Abcam, Cambridge, MA); and DAPI (4′,6′-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO). AmpliDex Red H$_2$O$_2$ detection kit was from Invitrogen. Reagents and their final concentration used in experiments were N-acetyl-l-cysteine (NAC; 5 mM), diphenyleneiodonium chloride (DPI; 10 lM), ammonium pyrrolidinedithiocarbamate (PDTC; 10 lM), rotenone (1 mg/l M), and H$_2$O$_2$ were purchased from Sigma-Aldrich. Measurements of H$_2$O$_2$ production were conducted using AmpliDex Red Hydrogen Peroxide/Peroxidase Assay Kit from Invitrogen (Mishin et al., 2010). Catalase activity was determined using the Catalase Assay Kit from Caymen Chemicals (Ann Arbor, MI; Sinha, 1972), and the In Gel MetalloSOD activity was done as previously described (Beatir and Fridovich, 1971).

### Cell Culture, Viral Cell Transduction, and Reporter Gene Assays

Cells were maintained under standard tissue culture conditions in DMEM supplemented with 10% FBS and 100 mg/ml each of penicillin-streptomycin at 37°C with 5% CO$_2$. Adenovirus propagation was previously described (Wang et al., 2003). Infection was done at a multiplicity of infection (MOI) of 50, cells were cultured overnight, and media were changed before experiment.

### Assays for Assessment of Cellular Apoptosis

FACS-based cell cycle assays were performed using propidium iodide staining in citrate buffer. Assays of cellular apoptosis were performed using annexin V staining on living cells using the annexin V kit from BD Biosciences (San Jose, CA) followed by the FACS sorting of fluorescent apoptotic cells.

### Laser Capture Microdissection

Mammary glands from the following bistransgenic mice genotypes: wild-type (cre$^{+/-}$; resulting genotype: c-jun$^{+/+}$), heterozygotes (cre$^{+/-}$; resulting genotype: c-jun$^{+/+}$), or knockouts (cre$^{+/-}$; resulting genotype: c-jun$^{-/-}$) were frozen in optimal temperature compound on dry ice. The frozen tissue blocks were trimmed and cryosectioned at ~20°C in a cryostat machine onto cold, RNase-free sterile glass slides. Each slide was mounted in the LCM machine, and ~200–1000 mammary epithelial cells were picked by firing lasers on to the resin-bearing caps. RNA was isolated using microRNAasy RNA extraction kits (Qiagen, Valencia, CA), followed by DNase treatment and concentrated in a small volume using DNA-free kit (Zymo Research, Orange, CA). RNA quantity and quality was measured in a NanoDrop 1000 spectrophotometer. This RNA was then subjected to hybridization to Affymetrix RNA chips (Santa Clara, CA) for assessing the relative expression of genes affected by c-jun excision.

### Gene Expression Arrays and Pathways Analysis

RNA samples extracted from laser capture microdissected mammary epithelial cells and wild-type c-jun$^{+/-}$ and knockout c-jun$^{-/-}$ mammary glands were processed for Affymetrix 430 2.0 arrays to determine gene expression. RNA quality was determined by gel electrophoresis. Probe synthesis and hybridization were performed as previously described (Li et al., 2008). Analysis of the arrays was performed using the R statistics package (www.r-project.org) and the limma library of the Bioconductor software package (www.biocductor.org). Arrays were normalized using robust multiarray analysis, and a p of 0.05 was applied as statistical criterion for differ-

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Table 1. List of oligonucleotide primers used in PCR, RT-PCR, real-time qRT-PCR analysis, and chromatin immunoprecipitation assays

RESULTS

Endogenous c-jun Inhibits Signaling Pathways Promoting Apoptosis In Vivo

*c-jun* has been shown to either inhibit or induce cellular apoptosis in different cell types (Seaman, 1976; Udou et al., 2004; Vleugel et al., 2006; Hettinger et al., 2007; Podar et al., 2007). We conducted studies to determine the role of endogenous *c-jun* in mammary epithelial cell survival. Because *c-jun* somatic deletion is embryonically lethal, we interscrossed floxed *c-jun* (*c-jun*^fl/fl^) transgenic mice with MMTV-Cre mice and conducted LCM of mammary epithelial cells from mouse mammary glands (*Materials and Methods; Figure 1A*). mRNA derived from mammary epithelial cells of six separate transgenic mice was subjected to microarray analysis and subsequently to pathways analysis using ASSESS (*Figure 1A*). Gene expression pathways and the induction of apoptosis associated with ROS production were induced upon *c-jun* deletion (*Figure 1A*). To examine whether *c-jun* deletion–regulated expression of specific pro- or antiapoptotic genes, we conducted RT-PCR–based expression analysis of 87 genes (using low-density apoptosis arrays) previously shown to be involved in apoptosis. Thirty-nine proapoptotic genes were induced, and 16 anti-apoptotic genes were repressed upon deletion of *c-jun* (*Figure 1B*). The relative change in gene expression of the antia apoptotic and proapoptotic genes are shown in a log scale (*Figure 1C*). Deletion of *c-jun* induced expression of apoptotic genes and reduced expression of antiapoptotic genes (*Figure 1C*). The inhibitor of apoptosis protein (IAP) BIRC5 (survivin) was repressed substantially upon deletion of *c-jun* (*Figure 1C*). RT-PCR–based analysis of *c-jun* gene expression within laser capture–microdissected mammary epithelial cells derived from Cre recombinase expressing bitransgenic mammary glands also showed reduction in *c-jun* expression (*Figures 1D*).

To determine whether the excision of *c-jun* within the mammary epithelial cells had affected mammary gland development and mammary ductal morphology, we performed mammary gland whole-mount analyses. Mammary glands squashes prepared by staining the entire #4 mammary glands from *cre*^f/f^/*c-jun*^−/−^ (resulting genotype: *c-jun*^−/−^ or KO) and *cre*^f/f^/*c-jun*^+/−^ (resulting genotype: *c-jun*^+/−^) to littersmate controls *cre*^+/−^/*c-jun*^wt/wt^ (resulting genotype: *c-jun*^+/−^ or WT) were photographed and carefully compared (Supplemental Figure 1A). The morphological structure and architecture was unchanged between genotypes. Mammary gland ductal branching complexity number was also largely unaltered (Supplemental Figure 1A). Studies of the cellular components of the mammary gland of the mice were next conducted. The relative...
number of cells per field and the area covered by cells lining mammary glands were carefully assessed and characterized by pixels per field and demonstrated ~50% reduction in the number of epithelial cells observed per field and the same for their area. (Supplemental Figure 1B).

Endogenous c-jun Inhibits ROS Production via Mitochondrial Complex I and NOX1

To determine the role of endogenous c-jun in ROS production, c-jun<sup>f/f</sup> MEFs (Figure 2A) were transduced with an adenoviral expression vectors encoding cre (Ad-Cre) or a control vector (Ad-Null). The excision of c-jun was documented by RT-PCR analyses for c-jun mRNA normalized to the internal control Rpl-19 and deletion of the floxed site by PCR (Figure 2AIII). ROS production was visualized by 2′,7′-dichlorofluorescein diacetate (DCFDA) and quantitated by FACS sorting (Figure 2, B and C), which demonstrated an approximate doubling of DCFDA positivity (Figure 2B).

Confocal microscopy combining staining of mitochondria with mitotracker dye evidenced a colocalization of DCFDA...
with mitochondria (yellow; Figure 1C). Additional nonmitochondrial DCFDA was also seen. Reintroduction of c-jun by retroviral transduction into c-jun/W110002/W1100002 cells reversed the increase in DCFDA positivity by FACS (data not shown). To examine further the source of endogenous ROS repressed by c-jun, ROS inhibitors were used. NAC and PDTC thought to act as antioxidants. PDTC inhibits initiator-I inhibitors activity (Hayakawa et al., 2003). Rotenone, an inhibitor of the mitochondrial electron transport chain, and DPI are inhibitors of the NOX enzymes that are the major mediators of nonmitochondrial ROS (Kamata and Hirata, 1999). Addition of DPI, PDTC, or rotenone reduced DCFDA fluorescence (Figure 2D) suggesting both NOX- and mitochondrial-dependent sources of ROS in c-jun/W1100002/W1100002 cells.

**Endogenous c-jun Maintains Mitochondrial Membrane Potential**

Apoptosis assessed by annexin V staining was increased fourfold in c-jun/W1100002/W1100002 cells (Figure 3A). EM of the mitochondria demonstrated mitochondrial blebbing (Figure 3B) characteristic of apoptotic cell death. The size of mitochondria assessed by mitotracker staining was unchanged in c-jun/W1100002/W1100002 cells (Figure 3C). The mitochondrial membrane potential (Dym) regulates ROS formation and efflux. O$_2^{-}$ production increased the number of cells with medium and low Dym. Quantitation of JC-1 staining was conducted to measure Dym and demonstrated a 3–5-fold increase in staining in c-jun/W1100002/W1100002 cells (Figure 3D).

To determine the role of c-Jun and c-Jun–regulated ROS in cellular growth and apoptosis, cellular proliferation assays were conducted. c-jun/W1100002/W1100002 MEFs have a severe proliferative defect that can be passed only a few times before entering premature senescence. To avoid any independent effects of serial passage and senescence, the studies were conducted acutely on early-passage MEFs. c-jun/W1100002/W1100002 cells were transduced with an adenovirus expressing cre via an IRES to the GFP protein (Supplemental Figure 3A). Immunohistochemical staining confirmed cells transduced with Cre lacked c-Jun protein. GFP-positive cells were selected and analyzed. c-jun/W1100002/W1100002 MEFs demonstrated a reduced proliferation rate assessed by cellular counting (Supplemental Figure 3A) or by MTT assays (Supplemental Figure 3B). c-jun/W1100002/W1100002 MEFs displayed enhanced sensitivity to H$_2$O$_2$-induced apoptosis (Supplemental Figure 3C).

**c-jun–dependent ROS Production Governs MnSOD, Catalase, and GPX Expression**

We examined further the mechanisms governing endogenous c-jun–mediated inhibition of ROS production. Increased ROS production in the c-jun/W1100002/W1100002 cells may be a consequence of either reduced ROS elimination or increased ROS production. O$_2^{-}$ is converted to H$_2$O$_2$ by matrix manganese superoxide dismutase (MnSOD; Temkin and Karin, 2007), whereas catalase and GPX either separately or synergistically convert H$_2$O$_2$ to H$_2$O. To determine the mechanism by which c-jun inhibited ROS production, we examined the role of c-jun in regulating MnSOD and catalase abundance. We examined the abundance of MnSOD by quantitative real-time RT-PCR upon acute excision of c-jun using Ad-Cre. As endogenous c-jun mRNA levels decreased (Figure 4AI), MnSOD mRNA transcript abundance increased (Figure 4AII). O$_2^{-}$ released to the mitochondrial intramembrane space is dismutated by CuZnSOD (Okado-Matsumoto and Fridovich, 2001). ROS are further detoxified by GPX (Nohl and Jordan, 1980), which uses GSH in catalysis. The relative abundance of MnSOD, CuZnSOD, catalase, and GPX
were assessed upon acute deletion of c-jun to determine the role of endogenous c-jun in regulating the key components involved in eliminating ROS (Figure 4A, III–V). Quantitative RT-PCR transcript analysis was conducted, and mean data of multiplicate studies demonstrated that c-jun excision induced MnSOD 2.5-fold. More modest changes occurred with catalase and GPX (1.5- to 2-fold). CuZnSOD mRNA expression was unchanged (Figure 4A, III–V). The finding that MnSOD mRNA increased and CuZnSOD was unchanged upon excision of c-jun in fibroblasts was confirmed by microarray analysis of the c-jun−/− mammary gland in which MnSOD was increased.
(1.6-fold, mean of n = 3), and CuZnSOD mRNA abundance was unchanged. We had previously shown that ROS induces MnSOD expression (Tanaka et al., 2002). We therefore examined

Figure 3. c-jun determines mitochondrial membrane potential and apoptosis. (A) Annexin V staining for assessment of apoptotic cells after c-jun excision (data are mean ± SEM for n > 5). (B) Electron micrographs of the floxed c-jun cells with (No virus or Ad-Null) or without c-jun expression (Ad-Cre). (C) Mitochondrial membrane potential measurements using JC-1 mitochondrial probe after c-jun excision (data are mean ± SEM). (D) Quantitation of JC-1 staining and measurement of Dym, indicating a 3-5-fold increase in staining in c-jun−/− cells.
whether inhibition of ROS production in \textit{c-jun}^{-/-} cells was responsible for the increase in MnSOD. The addition of DPI (Figure 4B), rotenone, or PDTC (not shown) reversed the hyperactivation of MnSOD. Thus, increased ROS from NOX1 and mitochondrial sites produced in \textit{c-jun}^{-/-} cells enhances MnSOD expression. Consistent with an important role for MnSOD in converting O$_2$ to H$_2$O$_2$, H$_2$O$_2$ production (Figure 4C) was reduced by DPI. Consistent with the induction of expression in \textit{c-jun}^{-/-} cells, MnSOD enzyme activity was also increased in \textit{c-jun}^{-/-} cells (Figure 4D). The induction of MnSOD activity in \textit{c-jun}^{-/-} cells was reduced by DPI (Figure 4D). To determine whether the increase in H$_2$O$_2$ abundance in \textit{c-jun}^{-/-} cells was due to reduced catalase activity, catalase activity assays were conducted. Catalase activity was reduced by 60\% upon deletion of \textit{c-jun} (Figure 4D). The reduction in catalase activity was partially reversed by DPI, suggesting NOX-dependent ROS are a source of reduced catalase activity in \textit{c-jun}^{-/-} cells.
Endogenous c-jun Induces Survivin (BIRC5) in Part via ROS

Microarray and RT-PCR analysis of mRNA from LCM of the mammary epithelial cell targeted MMTV-Cre/c-jun\textsuperscript{fl/fl} bi-transgenics demonstrated a reduction in the abundance of several antiapoptotic genes (Figure 1, B and C). The abundance of BIRC5 (survivin), in particular, was down-regulated. To determine whether BIRC5 was also a direct target of endogenous c-jun, acute excision of c-jun was conducted and BIRC5 abundance was determined (Figure 5A). c-jun excision was associated with a decrease in BIRC5 (survivin) mRNA (by RT-PCR) and protein levels. Survivin abundance in c-jun\textsuperscript{−/−} cells was reduced in part by the addition of ROS inhibitors (Figure 5B). DPI and PDTC treatment increased survivin abundance in c-jun\textsuperscript{−/−} cells 3–5-fold (Figure 5B, lanes 5 vs. 7 and 8). Reintroduction of c-jun restored c-jun and survivin levels and reversed aberrant ROS production (Figure 5C).

Collectively these studies demonstrate acute c-jun excision increased proapoptosis and inhibited antiapoptotic gene expression in vivo. The mammary epithelium in vivo c-jun excision or c-jun\textsuperscript{−/−} cultured cells induced expression of genes that catalase ROS, mitochondrial membrane potential and both mitochondrial and NOX-based ROS, which induced MnSOD, and catalase expression. The inhibition of ROS production by endogenous c-jun reduced cellular apoptosis and promoted contact-independent growth. Because c-jun functions as a pivotal downstream regulator of diverse biological processes governing cellular proliferation and growth, these studies provide a direct link between c-jun and mitochondrial function.

DISCUSSION

The current studies demonstrate an essential role for endogenous c-jun in maintaining mammary epithelial cellular survival in vivo. Double transgenic mice (c-jun\textsuperscript{−/−},MMTV-Cre) and conditional knockout MEFs were generated to identify the key signaling pathways regulated by endogenous c-jun in vivo. Genome-wide expression studies conducted of mRNA derived from laser capture–microdissected mammary epithelium from these double transgenic mice identified the key pathways regulated by endogenous c-jun. In the mammary epithelium and fibroblasts the key pathways regulated by endogenous c-jun in vivo. RT-PCR analysis of 87 genes confirmed that 39 apoptotic genes were induced and 16 antiapoptotic genes were repressed upon deletion of c-jun.

Herein, acute c-jun excision increased apoptosis. The induction of cellular senescence, which occurs upon several cellular divisions, is associated with the induction of p53/p21\textsuperscript{CIP1} (MacLaren et al., 2004). To distinguish the effects of c-jun excision from the effects of cellular senescence the molecular events, accompanying acute somatic excision of...
c-Jun were characterized. The apoptosis induced upon acute excision of c-Jun herein was associated with increased annexin V staining, characteristic of phosphatidyl-serine exposure on the cellular membrane seen in apoptosis and features of caspase 3 cleavage and activation (Supplemental Data) indicative of the intrinsic mitochondrial pathways of apoptosis. Mitochondrial morphology reflected the characteristic feature of mitochondrial cristae swelling and inner mitochondrial membrane swelling.

The current studies define a central role for endogenous c-Jun gene in maintaining ROS homeostasis. The decreased membrane potential of c-Jun−/− cells would be anticipated to increase ROS production and enhance efflux (Tirosh et al., 2003). Acute c-Jun excision enhanced ROS production and expression of the enzymes that catalase and detoxify ROS (MnSOD and GPX). The induction of gene expression occurred within 16–18 h. The induction of the cellular ROS-catalyzing enzymes was accompanied by a decrease in mitochondrial membrane potential. ROS are known to induce expression of MnSOD (Tanaka et al., 2002). The enhanced production of MnSOD in c-Jun−/− cells was abolished by addition of DPI, indicating that ROS production from mitochondrial complex I and NOX are inhibited by endogenous c-Jun. Thus, endogenous c-Jun inhibits MnSOD production via NOX1 and mitochondrial complex 1.

Apoptosis is regulated via extrinsic pathways that are triggered by ligand binding, whereas the intrinsic apoptotic pathways involves mitochondria, which release cytochrome c that binds and activates Apaf1 (apoptotic protease activation factor 1). Apaf1 induces assembly of the apoptosome, leading to activation of caspase 9 members of the IAP family including survivin and others (ML-IAP, XIAP, CIAP1, CIAP2, NIAP, and Apollon). These factors block apoptosis downstream of cytochrome c release by interfering with caspase 9 activity and processing and inhibiting the terminal effectors caspase-3 and -7. Increased ROS production has been linked to cellular apoptosis through sustained JNK activation and an additional pathway (Kamata et al., 2005; Chang et al., 2006) that is inhibited by a prosurvival NFκB (nuclear factor κB) pathway (Temkin and Karin, 2007). The current studies extend these prior observations by demonstrating that the endogenous c-Jun functions to inhibit ROS production (Supplemental Figure 3). The IAP BIRC5 (survivin) was reduced upon deletion of c-Jun. Survivin is a 16.5-kDa protein, with a single baculovirus IAP repeat (BIR). Mitochondrial survivin orchestrates a novel pathway of apoptosis inhibition, which contributes to tumorigenesis (Dohi et al., 2004). On deletion of c-Jun, ROS levels rise, and survivin and other apoptotic gene levels fall, inducing apoptosis (Supplemental Figure 3). c-Jun induced survivin abundance in a ROS-dependent manner. ROS generated independently of c-Jun and other factors also regulate survivin expression. The survivin gene is regulated by a variety of factors including chronic hypoxia, growth factors, HIF1α (hypoxia-inducible factor), and paracrine factors. The independent role of these factors in ROS signaling to the survivin gene remains to be determined.

REFERENCES


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