THE p53 RESPONSE IS DIFFERENTIALLY REGULATED BY NUMEROUS MECHANISMS INITIATED BY STRESS

by

DAVID E. WHITE

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

THE p53 RESPONSE IS DIFFERENTIALLY REGULATED BY NUMEROUS MECHANISMS INITIATED BY STRESS

by

David E. White

Advisor: Professor Jill Bargonetti

When the living environment is favorable, the p53 response is stringently suppressed by the cell. Activation of p53 by genotoxic stress leads to the initiation of growth suppressive pathways. These pathways are mediated, in part, from the protein products of genes upregulated by p53. We found that Mdm2 protein co-localized with p53 on the p53 binding sites of target genes prior to activation. Once p53 is activated, Mdm2 breifly dissociated from p53. At later timepoints, however, Mdm2 was found to reassociate with p53 on chromatin. The reformation of this inhibitory complex correlated with decreased transcription from these target genes. We also found that the induction of growth arrest or apoptotic pathways by p53 did not correlate with the expression of p53 target genes. The level of p53 protein and p53 target gene expression required to shift a cell from a growth arrest to an apoptotic cell fate differ according to the damaging agent used. In examining the p53-dependent and independent apoptotic pathways induced by mitomycin C (MC) and its derivative 10-decarbamoyl mitomycin C (D-MC), we found that MC and D-MC induced cell death in cells expressing wild type p53 provoked slightly different apoptotic pathways. MC treatment promoted the stabilization of p73, greater levels of p53 phosphorylation at serine 15, but the p53 induced by this drug exhibited less transcriptional activity than the p53 protein induced by D-MC. In a p53 null environment, however, only D-MC was capable of inducing apoptosis. The

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apoptotic pathway utilized by D-MC occurred independently of p53 apoptotic target gene activation and was found to be dependent on both caspase and serine protease activity.

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This manuscript is dedicated to my parents, Phillip and Linda White. Every accomplishment I acheive is a tribute to their unending support and guidance. On the days when they're not directly providing advice, consolment, and often times, monetary help, they indirectly play a part in the advancement of my pursuits through the ideals, values, and wisdom they have imparted me. I would not have made it this far without them. I hope that I may use this degree to honor their selflessness, and that any success I have in the future be their's as well.

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Abreviations

Actinomycin D, Act D; activating transcription factor, ATF; 4-(2 aminoethyl) benzenesulfonyl fluoride, AEBSF; antibody, Ab; apoptosis inducing factor, AIF; ataxia-telangiectasia-mutated, ATM; camptothecin, CPT; chromatin immumoprecipitation, ChIP; cycloheximide, CHX; 10-decarbamoyl mitomycin C, D-MC; 2,7-diaminomitosene, 2,7-DAM; dithiolthreitol, DTT; doxycycline, DOX; electrophoretic mobility shift assay, EMSA; etoposide, ETOP; extracellular signal-regulated kinase, ERK; fetal bovine serum, FBS; fluorescence activated cell sorting, FACS; growth arrest and DNA damage clone 45, gadd45; glyceraldehyde 3-phosphatase dehydrogenase, gapdh; histone acetyltransferase, HAT; histone deacetylase complex, HDAC; ionizing radiation, IR; mitomycin C, MC; N-acetyl-Leu-Leu-Norleu-al, LLnL; mouse double minute 2, mdm2; mouse embryonic fibroblast, MEF; methylmethane sulfonate, MMS; myeloid leukemia 1, ML-1; nuclear export signal, NES; nuclear localization signal, NLS; p53 inducible gene 3, pig-3; p300/CBP associating factor, P/CAF; phenylmethylsulfonyl fluoride, PMSF; phosphate buffered saline, PBS; phosphatidyl inositol 3-kinase, PI3K; polyacrylimide gel electrophoresis, PAGE; poly (ADP-ribose) polymerase 1, PARP-1; polymerase chain reaction, PCR; propidium iodide, PI; purine, Pu; pyrimidine, Py; responsive element, RE; reverse transcriptase, RT; rottlerin, ROTT; super consensus sequence, SCS; TATA binding protein, TBP; TBP associating factor, TAF; tetracycline, TET; 12-O-tetradecanoylphorbol 13-acetate, TPA; transactivation domain, TA; trichostatin A, TSA; ultraviolent radiation, UV; Zeocin, ZEO.

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Chapter 1. Introduction

One of our cell's strongest defenses against unregulated growth and the perpetuation of abhorrent genetic mutations is the activation of the tumor suppressor protein p53. Over the last fifteen years, knowledge of p53's role in the cell has expanded from a potential oncogene (Lane, 1987) to a critical regulator of anti-cancer pathways. p53 has been shown to be mutated in over 60% of cancers (Hollstein et al., 1991). The loss of p53 in mice (Donehower et al 1992) or humans with Li Fraumeni syndrome (Ko and Prives, 1996) is accompanied by the early onset of multiple tumors and increased mortality (Jones et al., 1996; Law et al., 1991). In response to stress, p53 protein becomes stabilized. These increased levels of p53 act as a potent transcription factor that transactivate a number of target genes whose protein products mediate either cell cycle arrest or apoptosis. Growth arrest prevents cellular transformation by blocking unregulated cell division in response to oncogenic stimulation or by creating a pause the cell cycle to allow for DNA repair in response to damage. Apoptosis sacrifices the cell if the amount of DNA damage existing in the cell is insurmountable (Ko and Prives, 1996).

1.1 Domains of p53 and their role in p53 transcriptional activity

The p53 protein contains five domains which individually contribute to the ability of p53 to effectively activate its target genes. The transcriptional activation domain is located within the first 63 amino acids in the N-terminus (Figure 1.1). This region contains numerous amino acids that are targeted for phosphorylation by kinases activated by stress. Phosphorylation on these N-terminal residues dissociates p53 from its

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inhibitory complex with mouse double minute 2 (Mdm2), as well as promotes the association of p53 with members of the transcriptional machinery like TATA binding protein (TBP) and numerous TBP associating factors (TAFs)(Buschmann et al., 2001; Chen et al., 1993), as well as the histone transacetylase proteins p300 (Avantaggiati et al., 1997) and p300/CBP associating factor (P/CAF)(Jin et al., 2002). Deletion of this region has been shown to inactivate the transcriptional activity of p53 (Wang et al., 1994; Zhu et al., 2000). Initial studies demonstrated that single or double point mutations in the transactivation domain were not sufficient to inactivate p53 function (Chen et al., 1996), however, later studies found that numerous mutations within or deletions of this region were capable of inhibiting p53's ability to promote transcription and induce apoptosis and growth arrest (Venot et al., 1999; Zhu et al., 2000). The proline-rich domain resides within residues 64 – 99 in the N-terminus of p53 (Figure 1.1). Deletion studies have shown that the proline-rich domain is necessary for the ability of p53 to mediate apoptosis, but not cell cycle arrest (Zhu et al., 1999). The proline rich domain serves as an additional anchorage site for p300, and deletion of this domain not only inhibits the ability of p53 to associate with p300, but also decreases p53 stability by making p53 more susceptible to downregulation by Mdm2 (Berger et al., 2001; Dornan et al., 2003; Liu et al., 2003). The central DNA binding domain located between residues 100 and 300 (Figure 1.1) is essential for the ability of p53 to bind site-specifically to DNA (Bargonetti et al., 1993). Over 60% of cancers contain mutations in this region of p53 (Harris and Hollstein, 1993; Hollstein et al., 1991). In fact, p53 with mutations in this domain exhibit oncogenic properties (Michalovitz et al., 1991). The tetramerization domain is located between amino acids 316-356 of p53 (Figure 1.1). p53 binds to DNA

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as a tetramer through interactions between p53 monomers at this region (Friedman et al., 1993). Deletion of this domain abrogates the ability of p53 to mediate transcription (Wang et al., 1994). The tetramerization domain also contains a nuclear localization signal between residues 316 and 325, which has been shown to be important for the retention of p53 in the nucleus (Shaulsky et al., 1991). Additionally, a nuclear export signal (NES) has been discovered in this region. Tetramerization of p53 masks the NES which further promotes its nuclear retention (Stommel et al., 1999). The C-terminal 37 amino acids constitute the basic domain (Figure 1.1). Although the deletion of this domain has been shown to activate p53 transcriptional activity *in vitro* (Hupp et al., 1992), mutants lacking this region exhibit reduced transcriptional activity of some genes and fail to induce apoptosis *in vivo* (Chen et al., 1996; Wang et al., 1996).

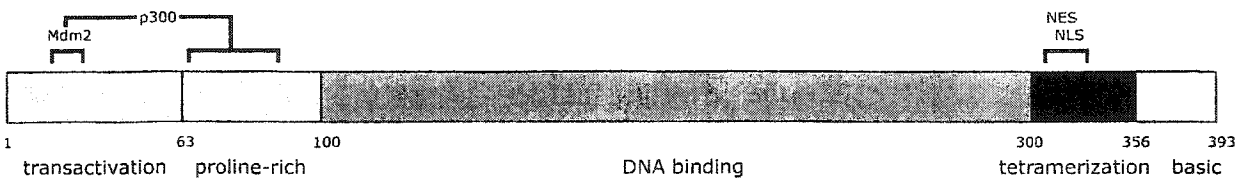

Figure 1.1 p53 protein has five functional domains.

1.2 p53 responsive elements and their role in the regulation of target genes by p53

p53 mediates growth arrest and apoptosis by binding site specifically to enhancer elements on its target genes. The p53 consensus sequence consists of two copies of 5'-PuPuPuC(A/T)(T/A)GPyPyPy -3'(el-Deiry et al., 1992). The region encompassing the p53 binding site has been shown to be nucleosome free and poised for transcriptional activation on numerous p53 target genes (Graunke et al., 1999; Xiao et al., 1998). p53

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binding sites are not localized to one specific part of a gene, and have been shown to reside in promoters, upstream of promoters, or within introns, regardless of the function of the gene. It is not known whether the location of the p53 binding sites affects how p53 regulates transcription from these loci. Intronic p53 binding sites have been placed upstream of minimal promoters in numerous studies to show p53 responsiveness (Kastan et al., 1992; Selvakumaran et al., 1994; Thornborrow et al., 2002). To date, only one study has shown that p53 can mediate transcription of genes independently of its binding site. p53 has been shown to promote transcription from the *gadd45* promoter in association with the Wilm's tumor suppressor protein 1 (WT-1) without the p53 binding site located in the 3rd intron (Zhan et al., 1998).

Although the location of the p53 responsive element has not been shown to affect how p53 regulates target genes, differences exist in the ability of p53 to interact with the p53 responsive elements from apoptotic target genes (Qian et al., 2002). Chromatin immunoprecipitation (ChIP) studies have revealed that p53 has a lower binding affinity for p53-responsive elements on apoptotic genes (Kaeser and Iggo, 2002; Szak et al., 2001). Additionally, ChIP has been used to demonstrate that the transcriptional machinery assembled with p53 on apoptotic target genes is different than the transcriptional complex found with p53 on growth arrest-promoting genes (Espinosa et al., 2003). These differences suggest that there may be distinct pathways that converge on p53 which contribute to p53's ability to determine cell fate.

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1.3 p53-dependent growth arrest

One way in which p53 responds to stress is by blocking cell growth. In order for cellular division to take place effectively, there must be favorable conditions within the cell. Low oxygen levels (hypoxia), low ribonucleotide levels, DNA damage, and abnormal proliferative signals (oncogenic stimulation) have been shown to activate p53. Once activated, p53 transactivates a number of downstream target genes that halt cellular division until the conditions improve and once again favor growth (Kastan et al., 1991). p53 has been shown to promote growth arrest at the G1/S checkpoint by transcriptionally activating the cyclin dependent kinase (CDK) inhibitor *waf1/cip1* (El-Deiry et al 1993). The protein product of this gene, p21, binds to and inhibits the cyclin/CDK complexes that hyperphosphorylate the tumor suppressor retinoblastoma (Rb) protein which is complexed to E2F. In the absence of p21, the hyper-phosphorylation of Rb releases the transcription factor E2F, which then promotes the cell cycle progression from G1 into S phase by activating a number of genes promote DNA replication (Medrano et al., 1995). p53 promotes a G2/M cell cycle arrest by transcriptionally activating two other target genes, *gadd45* (Kastan et al., 1992) and *14-3-3σ* (Hermeking et al., 1997). Although it is not clear how Gadd45 mediates G2 arrest, it has been shown that overexpression of Gadd45 protein induces a G2 arrest (Wang et al., 1999). Additionally, *gadd45* null mice exhibit defects in G2 arrest, DNA repair, and genomic stability (much like *p53* null mice)(Sheikh et al., 2000). The cytoplasmic protein 14-3-3σ, on the other hand, mediates G2 arrest by inhibiting the cyclin dependent kinase phosphatase Cdc25C (Hermeking et al., 1997). Cdc25C promotes progression into mitosis by removing inhibitory phosphate groups from cycinE/Cdk2 (Figure 1.2) (Gu et al., 1992).

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The tumor suppressor protein p14arf also plays a role in regulating the cell cycle through p53. p14arf has been shown to be induced by numerous oncogenes, including Ras and E1A (de Stanchina et al., 1998; Ries et al., 2000). In response to oncogenic stimulation, p14arf transcript and protein levels increase in the cell. p14arf stabilizes p53 protein by binding to p53's N-terminus and competitively inhibiting the association of Mdm2 with p53. This disruption of the Mdm2-p53 complex promotes growth arrest through the p53-dependent activation of *waf1* (Figure 1.2) (Kamijo et al., 1998).

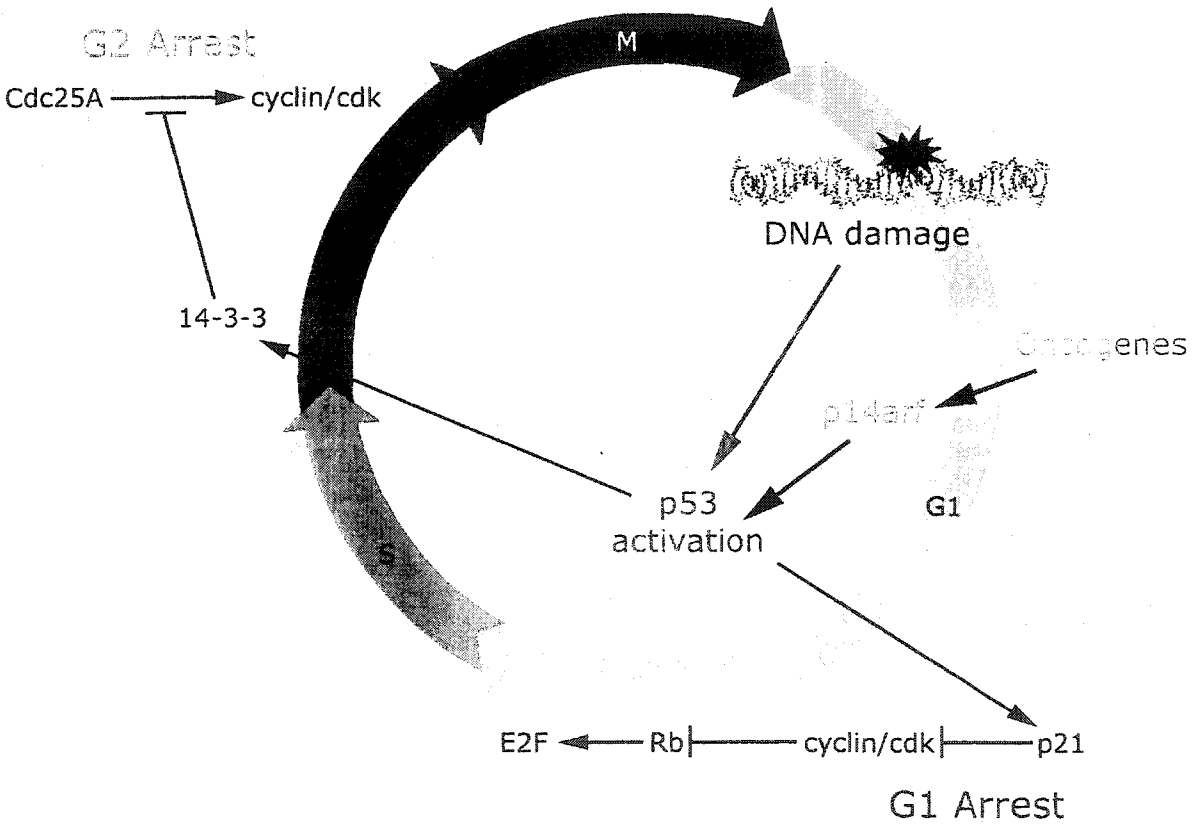

Figure 1.2 p53 promotes cell cycle arrest at both G1 and G2/M in response to DNA damage or oncogenic stimulation.

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1.4 p53-dependent apoptosis

There are two principle pathways that can drive p53-dependent apoptosis: the extrinsic and intrinsic pathways (Kiechle and Zhang, 2002). The extrinsic pathway originates from the plasma membrane, while the intrinsic pathway begins at the mitochondria. Both pathways utilize members of a family of cysteine proteases, called caspases (Mashima et al., 1995). Two classes of caspases are activated during apoptosis, initiator caspases and effector caspases. Caspases exist in the cell in an inactive pro-caspase form. Activation occurs when the N-terminal pro domain is cleaved off. Initiator caspases lie directly upstream of the effector caspases. In addition, adaptor proteins reside upstream of the initiator caspases. The extrinsic and intrinsic pathways have different initiator caspases. The extrinsic pathway utilizes caspase 8, while the intrinsic pathway utilizes caspase 9. These pathways both signal to the predominant effector caspase 3, which digests most of the principle cellular components during apoptosis (Burns and El-Deiry, 1999).

p53 promotes apoptosis through the extrinsic pathway by transcriptionally activating genes coding for death receptors from the TNF-receptor superfamily, like *fas/apo1* and *killer/dr5* (Muller et al., 1998; Wu et al., 1997). The induction of these apoptotic target genes enhances the sensitivity of the cell towards the p53-independent expression of ligands for these receptors following stress. Following ligand binding, the Fas receptor associates with the adaptor protein Fas associating death domain (FADD). This complex then recruits receptor interacting protein (RIP) and the initiator procaspase-

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8, which in turn promotes apoptosis through the activation of caspase-8 (Figure 1.3) (Juo et al., 1998).

The activation of apoptosis via the intrinsic pathway involves the interplay between pro-survival and pro-apoptotic members of the Bcl-2 family of proteins. Members of the Bcl-2 family share numerous regions of homology referred to as Bcl-2 homology (BH) domains. Bcl-2, Bcl-XL, Bax, and Bak are referred to as multidomain members of the Bcl-2 family, because they share numerous BH domains in common. Other Bcl-2 family members only contain one BH domain, the BH3 domain, which is the region of interaction between Bcl-2 family members. These BH3-only proteins promote apoptosis by binding to and inhibiting the pro-survival activity of Bcl-2 and Bcl-XL. In response to stress, Bax protein localizes to the mitochondrial membrane where it inserts its hydrophobic C terminus. The insertion of Bax into the mitochondrial membrane and the binding of Noxa or Puma to Bcl-2 alter the membrane potential and promote the release of cytochrome C into the cytoplasm (Figure 1.3) (Nakano and Vousden, 2001; Oda et al., 2000; Wu and Deng, 2002). As a member of the electron transport chain, cytochrome C plays an important role in ATP production, but once released from the mitochondria, cytochrome C contributes to caspase activation. The pro-survival Bcl-2 family members, Bcl-2 and Bcl-XL prevent apoptosis by preserving the integrity of mitochondrial membrane, which prevents the release of cytochrome C (Wu and Deng, 2002). Overexpression of Bcl-2 has been shown to inhibit p53-dependent apoptosis by preventing the translocation of Bax to the mitochondria (Murphy et al., 2000).

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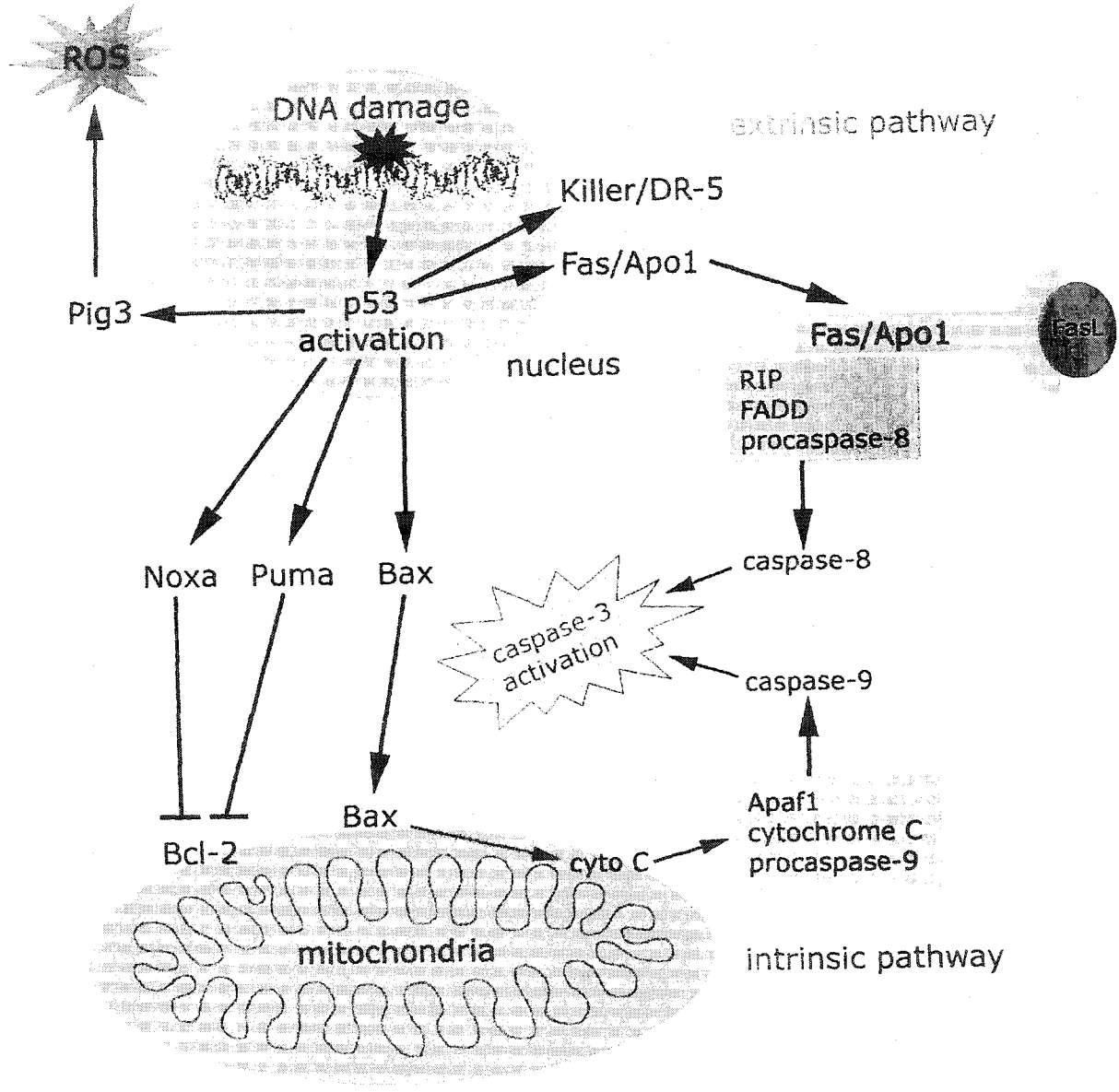

Figure 1.3 p53 mediates apoptosis in response to DNA damage via extrinsic and intrinsic pathways.

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p53 promotes apoptosis through the intrinsic apoptotic pathway by disrupting the delicate ratio of pro-survival proteins to pro-apoptotic proteins by transcriptionally activating a subset of apoptotic target genes, including *bax*, *noxa*, and *puma* (Figure 1.3). p53 has been shown to transcriptionally activate *bax* in association with the transcription factor Sp1 (Thornborrow and Manfredi, 2001), while *noxa* and *puma* transcription have only been shown to require p53 for their upregulation (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). The induction of these pro-apoptotic target genes by p53 offsets the inherent protection administered by the pro-survival factors, Bcl-2 and Bcl-XL. Increased levels of Bax, Noxa, or Puma promote the release of cytochrome C from the mitochondria (Nakano and Vousden, 2001; Nomura et al., 1999; Oda et al., 2000). Cytoplasmic cytochrome C forms a complex with the adaptor protein Apaf1 and the initiator caspase-9, called an apoptosome (Figure 1.3). The formation of the apoptosome activates caspase-9 within this complex (Perkins et al., 2000; Zhuang and Cohen, 1998).

The initiator caspases from both the extrinsic and intrinsic pathways converge on the principle effector caspase-3. Caspase-3, and to a lesser extent caspase-9 and 7, cleave numerous proteins, like poly(ADP)ribose polymerase-1 (PARP-1) and caspase activated DNase (CAD), that prevent or promote the methodical digestion of cellular components. There exists a significant amount of redundancy in these apoptotic pathways, since some cell lines, like MCF-7, do not express caspase-3, yet have been shown to efficiently undergo apoptosis in response to stress (Johnson et al., 2000).

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Numerous downstream target genes of p53 also contribute to the onset of apoptosis by lowering the cell's apoptotic threshold. p53 inducible gene-3 (*pig-3*) codes for a cytoplasmic protein that may act as a flavoprotein to promote the induction of reactive oxygen species (ROS) (Figure 1.3), which propentiates apoptotic pathways (Flatt et al., 2000). p53 has also been shown to transcriptionally activate a number of genes for proteases that play a role in the apoptotic mechanism. The genes for the serine protease HtrA2 (Jin et al., 2003a) and the effector caspase-6(MacLachlan and El-Deiry, 2002) are both downstream targets of p53.

1.5 Mdm2 and the regulation of p53 protein in the absence of stress

Under normal conditions, the levels of p53 protein are kept low through interactions with the oncogene Mdm2. Mdm2 binds to a region on the amino terminus of p53 (Bottger et al., 1997). When bound to p53, Mdm2 acts as E3 ubiquitin ligase ubiquitinating p53 at multiple lysine residues on its carboxyl terminus (Honda et al., 1997). In association with p300, Mdm2 promotes the polyubiquitination and subsequent degradation of p53 (Figure 1.4) (Jin et al., 2004). In addition, Mdm2 silences the regions adjacent to p53 binding sites by ubiquitinating adjacent histone proteins (Minsky and Oren, 2004). Mdm2 also inhibits p53 function by interfering with the ability of p53 to interact with members of the transcriptional machinery (Figure 1.4) (Thut et al., 1997). Following genotoxic stress, p53 protein is phosphorylated on numerous residues that disrupt its association with Mdm2 (Figure 1.4) (Shieh et al., 1997). The dissociation of p53 from Mdm2 activates p53's transcriptional activity and increases its half-life (Kubbutat et al., 1997). *Mdm2* is a target gene of p53 (Barak et al., 1993). If p53 protein

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levels increase in the absence of stress, the resulting *mdm2* induction serves to destabilize p53 in a negative feedback loop. Just as p53 is critical for maintaining genomic stability and blocking abnormal cellular proliferation, Mdm2 is necessary for regulating p53's growth suppressive activity. In fact, deletion of the *mdm2* gene is embryonic lethal (Montes de Oca Luna et al., 1995). Deletion of the *p53* gene rescues *mdm2* null mice, which are then able to come to term. However, these *mdm2/p53* double knockout mice exhibit a phenotype similar to *p53* null mice and develop tumors at an early age (Jones et al., 1996).

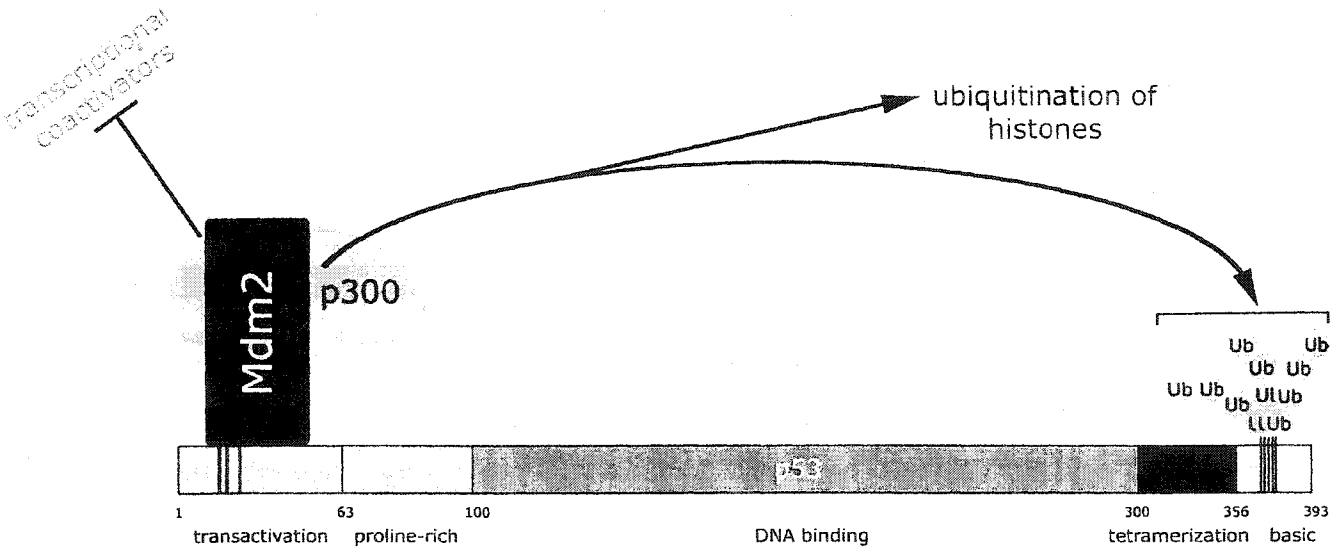

Figure 1.4 In the absence of stress, Mdm2 inhibits the ability of p53 to mediate transcription by binding to the transactivation domain and ubiquitinating p53 and nearby histone proteins.

Mdm2 protein levels remain stable in the nucleus through its phosphorylation by the protein kinase AKT (Mayo and Donner, 2001; Ogawara et al., 2002). AKT kinase phosphorylates Mdm2 at serines 166 and 168 (Mayo and Donner, 2001). These

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phosphorylation events not only increase the nuclear retention of Mdm2, but also stimulate its E3 ubiquitin ligase ability through its association with p300. Although Mdm2 has been shown to mono-ubiquitinate proteins on its own, p300 association enables Mdm2 to polyubiquitinate proteins like p53, targeting them for degradation in proteasomes (Grossman et al., 2003). Basal *mdm2* expression is maintained by two promoters, a p53-independent P1 promoter upstream of the 1st exon, and a p53-dependent P2 promoter located in the first intron (Juven et al., 1993). Although the regulation of this intronic p53-responsive promoter has been examined in numerous studies, it is not currently known if anything other than basal transcription occurs from the P1 promoter.

1.6 Effects of post-translational modification on p53 function following stress

The ability of p53 to activate downstream pathways in response to cytotoxic stimuli, like DNA damage, may rely on its post-translational modification. Numerous stress kinases converge on p53 following damage and phosphorylate it on serine and threonine residues in its amino and carboxyl termini (Lakin and Jackson, 1999). The PI3K family members, DNA-PK, ATM and ATR, have been shown to phosphorylate p53 at serine 15 (Canman et al., 1998; Lakin et al., 1999; Lees-Miller et al., 1992). Phosphorylation of p53 at serine 15 promotes its further phosphorylation at threonine 18 and serine 20, which reside within the Mdm2 binding site on p53 (Dumaz et al., 1999; Saito et al., 2002). Phosphorylation of p53 at these sites is believed to inhibit Mdm2 binding and promote p53's transcriptional activity (Figure 1.5) (Ashcroft et al., 1999; Craig et al., 1999; Unger et al., 1999a). Different types of genotoxic stress which activate these stress kinases have been shown to differentially affect the phosphorylation status of

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p53 (Lakin and Jackson, 1999). While some genotoxic agents, like UV and CPT, have been shown to induce phosphorylation of p53 on threonine 18 and serine 20 (Ashcroft et al., 2000; Sakaguchi et al., 2000), other agents, like actinomycin D (Act D), do not promote N-terminal phosphorylation events. Surprisingly, p53 induced by Act D transcriptionally activated *waf1* and *mdm2* and also promoted apoptosis (Ashcroft et al., 2000). These data suggest that phosphorylation of this region of p53 may not be necessary for p53 to mediate downstream pathways. A recent study demonstrated that p53 is phosphorylated at serine 46 by an unidentified kinase associated with p53DINP1 (Okamura et al., 2001). Although mutation of serine 46 does not affect the ability of p53 to bind to its responsive elements, phosphorylation at this site was critical for the ability of p53 to promote apoptosis (Bulavin et al., 1999).

The phosphorylation of p53 on its N-terminus in response to stress not only serves to relieve repression by Mdm2, but also promote p53 acetylation. Acetylation of p53 by the histone transacetylase p300 may increase p53's transcriptional activity directly by changing its conformation (Avantaggiati et al., 1997; Gu and Roeder, 1997), or by using p53 as a scaffold to remodel the chromatin on target genes during transcription (Figure 1.5) (Espinosa and Emerson, 2001). Recent studies using chromatin immunoprecipitations have detected acetylated p53 bound to its responsive element on p53 target genes that were determined to be transcriptionally active (Szak et al., 2001). p300 binds to p53 within the same region recognized by Mdm2 and acetylates numerous lysine residues on p53's C-terminus (Ito et al., 2001; Kobet et al., 2000). Phosphorylation of p53 at serine 15 has also been shown to promote association with

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p300 (Dumaz and Meek, 1999; Kar et al., 2002). Additionally, Zhu et al found that although p53 mutants containing point mutations on these critical residues in the N-terminus retained their ability to bind to chromatin, their ability to associate with p300 was diminished. These N-terminal mutant variants of p53 were less capable of transcriptionally activating *waf1/cip1*, which correlated with lower levels of acetylated histone proteins on this target gene (Zhu et al., 2001b). Other studies, however, suggest that the acetylation of p53 may require more than just N-terminal phosphorylation. Acetylation of p53 has been shown to occur on p53 in response to Act D in the absence of N-terminal phosphorylation (Ito et al., 2001).

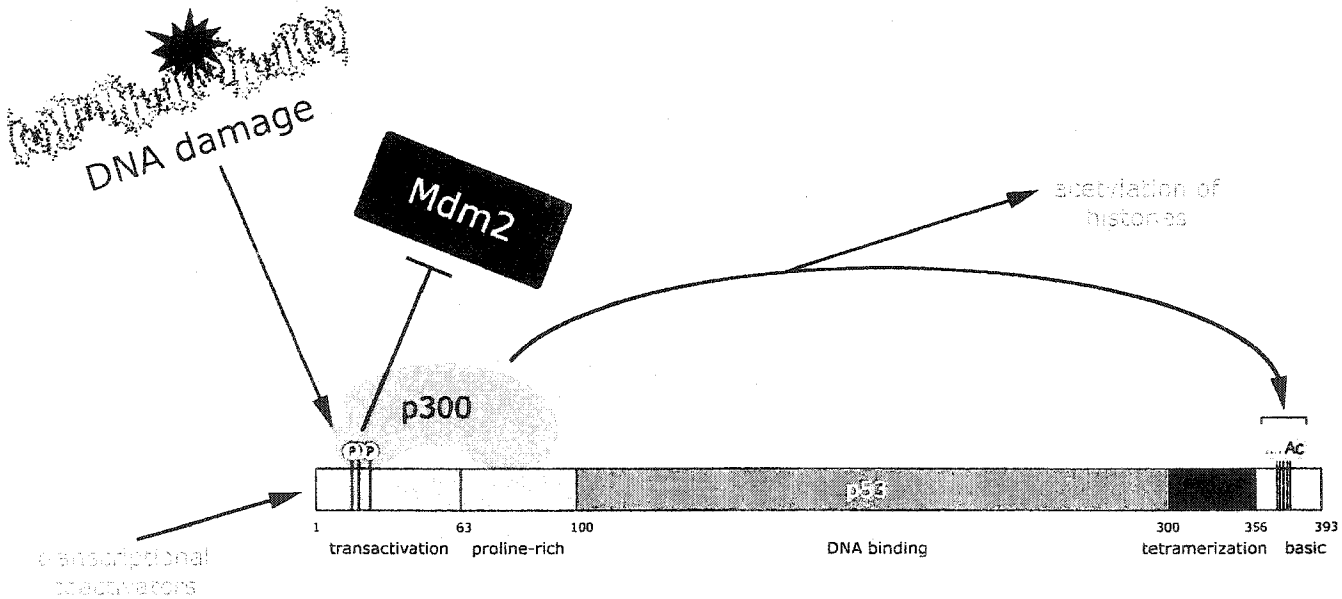

Figure 1.5 In the presence of stress, p300 binds to the phosphorylated N-terminus of p53 and acetylates on C-terminal lysine residues on p53.

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1.7 p53 family members

p53 has two homologues, p63 and p73, that are considered members of the p53 family of proteins. p63 and p73 share homology with p53 in three principle regions, the transactivation domain, the central DNA binding domain, and the tetramerization domain (Figure 1.6). Like p53, both p63 and p73 are capable of binding to p53 responsive elements (Levrero et al., 1999). p63 and p73 exist in primarily two forms, containing or lacking the N-terminal transactivation domain. The expression of these two isoforms of p63 and p73 occurs via two promoters. The delta varient lacks the transactivation domain and originates from an the internal promoter (Dohn et al., 2001; Grob et al., 2001). This form of p63 and p73 can bind to the p53 responsive element, but because it lacks the transactivation domain it cannot promote transcription (Figure 1.6). Overexpression of Δ-p73 or p63 can inhibit the p53 response by competing with p53 for binding to its responsive elements, as well as by associating with and inactivating the p53 tetramer (Chan et al., 2004; Yang et al., 2002). The transactivation proficient (TA)-p73 or p63 varient arises from the activation of the P1 promoter, and exists in two splice variants, a full length alpha form (Figure 1.6) and a slightly shorter beta form. Full length p73 and p63 have been shown to play a role in the ability of p53 to promote apoptotic pathways in response to DNA damage (Flores et al., 2002). Interestingly, only p73 has been shown to be stabilized in response to stress. Numerous studies have shown that p73α can be induced, in a c-Abl dependent manner, by several types of DNA damage including cisplatin and IR and promote apoptosis. Furthermore, full length p73 is capable of mediating apoptosis in the presence or absence of p53 (Agami et al., 1999; Toh et al., 2004; Yuan et al., 1999; Zhu et al., 2001a).

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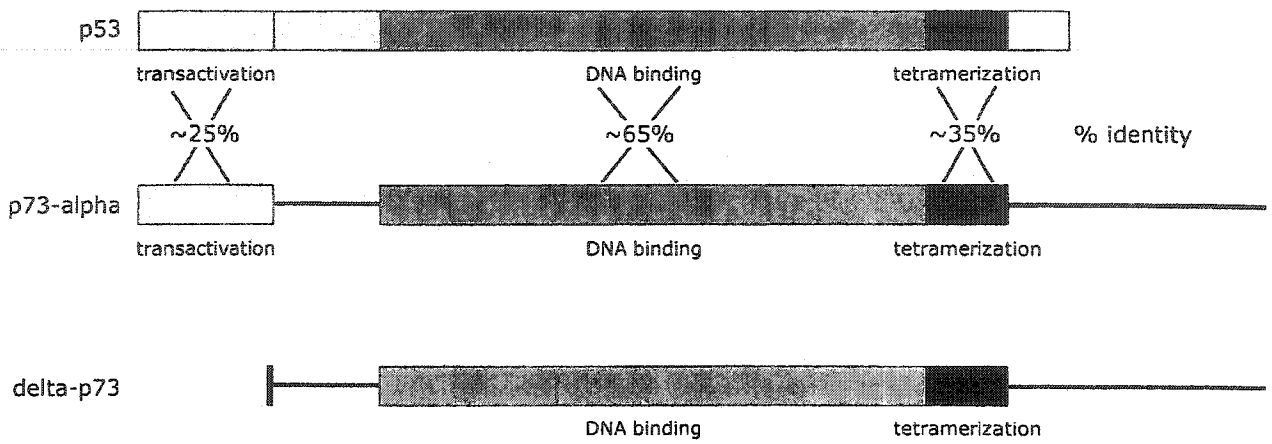

Figure 1.6 Domains shared by p53 and p73.

1.8 Regulation of p53 response pathways

The decision to promote either growth arrest or apoptosis may be based on the context of the cell in which p53 activation occurs. The cellular context is dependent upon the proteins that are present prior to and following the genotoxic event. It is these elements that permit or prevent the mediation of response pathways by p53 (Oren, 1994). The expression of determinant factors prior to p53 induction may establish conditions that promote either growth arrest or apoptotic responses. The expression of these factors may be tissue specific, allowing for cells derived from different tissues expressing similar levels of p53 to elicit different downstream pathways. Recent studies utilizing tetracycline regulatable systems have shown that overexpression of p53 protein in the Li Fraumeni cell line, MDAH041, resulted in growth arrest (Agarwal et al., 1995), while high levels of p53 in the colon cancer cell line DLD-1 induced apoptosis (Yu et al., 1999). In another study examining numerous cell lines from a similar origin, colorectal cancer, it was found that the overexpression of p53 induced apoptosis in one cell line and growth

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arrest in another. Furthermore, when these cell lines were fused together, the resulting hybrid underwent apoptosis in response to p53, suggesting that the apoptotic cellular context was dominant over the context of the cells prone to undergo p53-dependent growth arrest (Polyak et al., 1996).

Although p53 is believed to be central to the induction of either growth arrest or apoptosis, a number of cytoplasmic pathways composed of kinases are also stimulated or inhibited by stress. These stress responsive pathways play a role in dictating the cellular outcome not only by signaling to the p53 pathway, but also by activating a number of factors that either promote survival or mediate apoptosis. In order for p53 to promote apoptosis, the pathways that promote survival must be overcome (Aranda-Anzaldo and Dent, 2003; Holbrook et al., 1996). Activation of the extracellular signal-regulated kinase (ERK) and the phosphatidyl inositol 3-kinase (PI3K) pathways promotes survival in the cell. The ERK pathway promotes cellular survival by activating a number of transcription factors like Sp1 that induce DNA repair proteins like ERCC1, XRCC1 and XPC(Yacoub et al., 2003; Yacoub et al., 2001). Furthermore, ERK signaling promotes the expression of pro-survival genes like *bcl-xl*, *mcl-1*, and *bcl-2* (Boucher et al., 2000), as well as *mdm2* through Ras and Raf, which lie upstream of ERK (Figure 1.7) (Ries et al., 2000). Inhibition of the ERK pathway has been shown to occur in response to some damaging agents, like vinblastine. Additionally, use of ERK pathway inhibitors has been shown to sensitize cells to apoptosis-inducing agents (Brantley-Finley et al., 2003). The PI3K pathway promotes survival by inducing pro-survival genes like *mcl-1* and *c-flip1* through the activation of AKT (Nam et al., 2003; Osaki et al., 2004). AKT has also been

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shown to phosphorylate Mdm2 (Figure 1.7), which increases its ability to localize in the nucleus negatively regulate p53 (Mayo and Donner, 2001). The PI3K pathway has also been shown to be inhibited by stress (Zundel and Giaccia, 1998). Furthermore, inhibition of the PI3K survival pathways sensitizes cells to apoptosis inducing agents (Martelli et al., 2003). The activation of p53 can counteract these pro-survival pathways by activating target genes like *pten* that inhibit the PI3K pathway member AKT, and *bax*, *puma*, and *noxa* which overcome the pro-survival effects of *bcl-2* and *bcl-xl* expression in response to ERK signaling (Mayo and Donner, 2002; Wu and Deng, 2002).

Other kinase driven pathways, like the SAPK/JNK and the p38 mitogen activated kinase (MAPK) pathways, respond to stress by promoting apoptotic signals that contribute to the p53 response. Topoisomerase inhibitors, IR, UVC, and MMS have been shown to activate the SAPK/JNK pathway (Brantley-Finley et al., 2003). Activated JNK has been shown to phosphorylate numerous factors that promote apoptosis, including the tumor suppressor p53 (Fuchs et al., 1998), the pro-apoptotic proteins Bim and Bmf (Figure 1.7) (Lei and Davis, 2003), and the transcription factor c-Jun (Brantley-Finley et al., 2003). The phosphorylation of c-Jun by JNK leads to the formation of the potent transcription factor AP-1. Numerous members of the Jun family have been shown to form heterodimers with members of the Fos family, as well as with activating transcription factor (ATF), to form AP-1 (Ameyar et al., 2003). Since *mdm2* and *gadd45* both contain binding sites for AP-1, the formation of AP-1 through the activity of JNK may play a role in the regulation of p53 target genes (Graunke et al., 1999; Phelps et al., 2003). Furthermore, p53 has been shown to cooperatively transactivate the metastasis

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suppressor gene *kail* with c-Jun in response to ETOP treatment (Mashimo et al., 2000). The p38/MAPK pathway has also been shown to promote apoptosis or G2/M growth arrest, depending on the cell type and the type of stress exuded on the cell (Sheikh et al., 2000). In contrast to the JNK pathway, it has not been determined how p38 mediates these downstream pathways, but it may involve the stabilization of p53 through an association with GADD45a (Figure 1.7). Studies using *gadd45a* null MEFs have shown that GADD45a and p38 activity were required for p53 stabilization in response to UVB and oncogenic stimulation by H-Ras (Jin et al., 2003b). p38 and GADD45a have also been shown to associate *in vivo* (Bulavin et al., 2003). p38 may also promote p53 stabilization by targeting p53 for phosphorylation. Both JNK and p38 have been shown to phosphorylate p53 (Figure 1.7) (Bulavin et al., 1999; Hu et al., 1997).

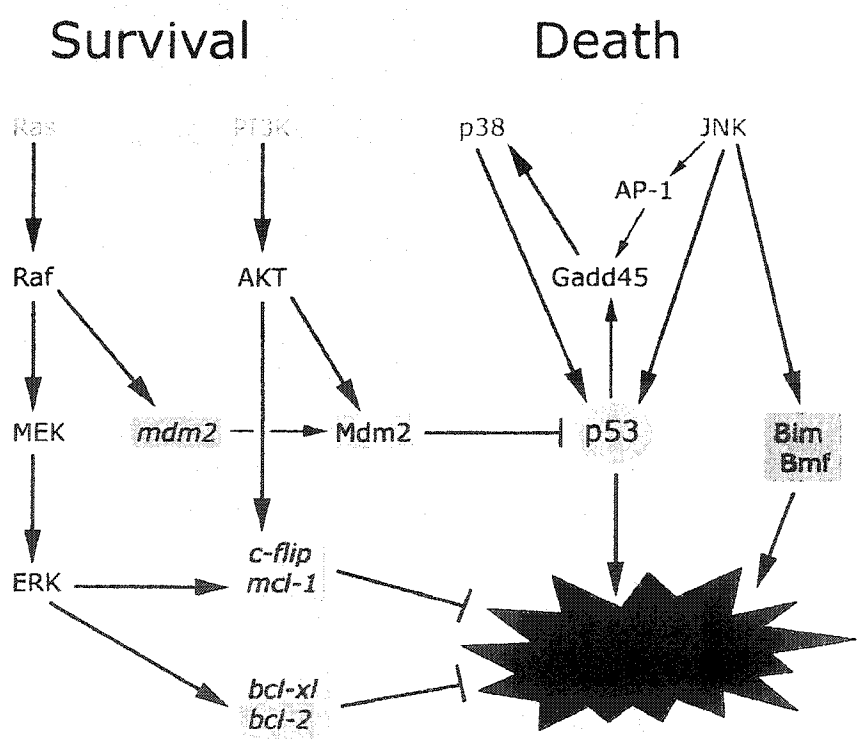

Figure 1.7 Kinase pathways can prevent or promote p53-dependent apoptosis.

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1.9 Dissertation Hypothesis

Numerous pathways converge on p53 and affect its ability to mediate its tumor suppressive function. It is these pathways emulating from the cell membrane towards the nucleus and from the site of damage in the nucleus itself that set up the conditions for the ensuing p53 response. This dissertation focuses on two separate aspects that dictate the ability of p53 to regulate downstream target genes: the activation of p53 on chromatin, and the ability of this activated p53 to direct cell fate. In addition, the p53-dependetn and independent apoptotic pathways utilized by 10-decarbamoyl mitomycin C is also examined.

Although Mdm2 has been shown in numerous studies to associate with p53 and inhibit p53's transcriptional activity, it has never been clearly shown that Mdm2 mediates this repression of p53 that is bound to DNA. We hypothesized that not only is Mdm2 bound to p53 prior to activation, but that following activation Mdm2 dissociates from p53 on chromatin permitting active transcription. Using chromatin immunoprecipitation we were able to show that Mdm2 co-localizes with p53 on p53 responsive elements prior to activation. Following activation, Mdm2 dissociates from p53 and this dissociation correlates with transcriptional activity. Surprisingly, we found that Mdm2 re-associates with p53 at later time-points, and this reformation of Mdm2's inhibitory complex with p53 was followed by decreases in transcription from the loci we examined.

Although many mechanisms have been discovered which affect the ability of p53 to promote growth arrest or apoptosis, it has still not been determined how p53 dictates

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cell fate in response to genotoxic stress. Using the myeloid leukemia cell line ML-1 that contains a well-documented p53 response, we determined whether p53 mediates growth arrest or apoptotic pathways by differentially regulating its downstream target genes. Surprisingly, we found no correlation between the types of target genes induced by similar levels of p53 protein and the cellular outcome that resulted from drug treatment. Furthermore, we found that different DNA damaging agents require different levels of p53 protein and target gene expression to shift the cell from growth arrest to apoptotic cell fates. Our data suggest that the ability of a DNA damaging agent to promote apoptosis through p53 relies on their ability to activate cooperative pathways that contribute to the p53 response.

The antibiotic mitomycin C (MC) has been used as a cancer treatment for numerous years. Studies examining how MC inhibits tumor growth have found that MC mediated DNA damage induces p53 protein and p53-dependent apoptosis (Abbas et al., 2002; Blagosklonny and El-Deiry, 1998). MC is much less effective at inducing cell death in cell lines that lack functional p53 (a defect found in most known cancers). Recent work in our laboratory by Abbas et al found that the analog of MC, decarbamoyl-mitomycin C (D-MC) induced apoptosis in cell lines regardless of their p53 status (Abbas et al., 2002). In order to further investigate the mechanism by which D-MC mediates apoptosis in cell lines expressing and lacking wild type p53, we examined the regulation of two proteins known to contribute to p53-dependent and independent cell death, p73 and the caspases. Since p73 has been shown to transactivate a number of p53 target genes and promote apoptosis in the absence of p53 (Lee and La Thangue, 1999; Zhu et al.,

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1998; Zhu et al., 2001a), we proposed that D-MC may be mediating p53-independent apoptosis by stabilizing and activating the p53 homologue p73. We found, however, that neither p73α protein nor p53 apoptotic target genes were induced by D-MC in cells that lacked p53. p73 has also been shown to contribute to p53's apoptotic potential (Flores et al., 2002). To determine a possible role of p73 in apoptosis induced by MC and D-MC, we examined the p73 protein levels in drug treated ML-1 cells. Surprisingly, we found that MC, but not D-MC, treatment stabilizes p73 protein in a p53-dependent manner. The p53 induced by MC, however, did not display a greater ability to transcriptionally activate downstream when compared to D-MC. These data suggest that although p73 was induced by MC treatment in ML-1 cells, it did not play a role in heightening the p53 response. When we examined the activity of the caspases in response to MC and D-MC in ML-1 cells, we found that MC and D-MC treatment differentially activate the downstream caspases. Additionally, we found that there were significantly lower quantities of caspase activity in response to D-MC in cells lacking p53. However, both caspases and serine proteases were required for the D-MC mediated p53-independent apoptotic pathway.

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Chapter 2. Materials and Methods

2.1 Reagents

Camptothecin, Etoposide, N-acetyl-Leu-Leu-Norleu-al (LLnL), Mitomycin C, Cycloheximide, Tetracycline and Doxycycline (DOX) were purchased from Sigma. Zeocin was purchased from Invitrogen. D-MC was synthesized from MC by Maria Tomasz and colleagues. RPMI 1640, McCoy's 5A, Fetal Bovine Serum (FBS), Gentomycin (G418) and the Penicillin-Streptomycin solution were purchased from Mediatech. Hygromycin B was purchased from Calbiochem. Powdered DMEM and Sodium Bicarbonate were purchased from Gibco/BRL and prepared on the premises. Rottlerin and 12-O-tetradecanoylphorbol 13-acetate (TPA) were purchased from Calbiochem.

2.2 Cell Culture

The myeloid leukemia cell line ML-1 and erythromyeloid leukemia cell line K562 were grown in RPMI 1640 supplemented with 10% FBS and 2500 units of Penicillin-Streptomycin. The ML-1 cells were a generous gift from Michael Kastan. The K562 cells were purchased from the American Type Culture Collection. The colon cancer cell line DLD-1 was grown in McCoy's 5A media supplemented with 10% FBS and 2500 units of Penicillin-Streptomycin. The p53-regulatable cell line, D-A2, was grown in McCoy's 5A media supplemented with 10% FBS, 2500 units of Penicillin-Streptomycin, 0.4 mg/ml G418, 20 ng/ml Doxycycline, and 0.25 mg/ml Hygromycin B. The DLD-1 and D-A2 cells were a generous gift from Burt Vogelstein. The MDAHO41 and TR9-7

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cells were a generous gift from Munna Agarwal. The TR9-7 cells were grown in DMEM supplemented with 10% FBS, 2500 units of Penicillin-Streptomycin, 0.4 mg/ml G418, 5 ug/ml Tetracycline, and 0.25 mg/ml Hygromycin B. p53 and p19arf mouse embryonic fibroblasts were a generous gift from Charles Sherr. All cells were incubated at 37°C with 5% CO2.

2.3 Protein Extract Preparation

Protein extracts were prepared using a variation of the Dignam protocol (Dignam et al., 1983). Cells were washed twice in 1x Phosphate Buffered Saline (PBS), pH 7.3 (136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4). The washed cells were spun down and the resulting cell pellets were resuspended in 5 packed cell volumes of Buffer A (10 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiolthreitol (DTT), 50 ug/ml Aprotinin, 0.1 uM Trichostatin A (TSA), 1% Phosphatase Inhibitor Cocktail 1 (Sigma), 0.96 mg/ml Leupeptin). The samples were then immediately spun down at 2000 rpm for 10 min at 4°C. The supernatant was discarded and the resulting pellet was resuspended with 2 packed cell volumes of Buffer A. Each sample was lysed with two strokes of a 20 gauge needle, and then incubated on ice for 10 min. Following the incubation, the cells were spun down at 12000 rpm for 10 min at 4°C. The resulting supernatant was the cytoplasmic extract. The remaining pellet was then resuspended in one packed cell volume of Buffer C (20 mM HEPES, pH 7.8, 25 % glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 uM EDTA, pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiolthreitol (DTT), 50 ug/ml Aprotinin, 0.1 uM Trichostatin A (TSA), 1% Phosphatase

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Inhibitor Cocktail 1 (Sigma), 0.96 mg/ml Leupeptin), and then lysed with 3 strokes of a 20 gauge needle. Each sample was rocked for 30 min at 4°C. Following this incubation, each sample was vortexed briefly and then spun down at 15000 rpm for 30 min at 4°C. The supernatant was saved as the nuclear extract.

2.4 Flow Cytometry

FACS analysis was carried out on a BD Biosciences scan. Cells were spun down at 1850 rpm for 7 min, washed twice with phosphate buffered saline (PBS), and resuspended in 20 ml of PBS containing 2% bovine serum albumin and 0.1% sodium azide. Ethanol (9 ml) was then added drop wise while vortexing. Propidium Iodide staining and RNase treatment were carried out at 37°C for 30 min 24 hr prior to flow cytometry.

2.5 Western Blot Analysis

Protein samples were electrophoresed on a 10% denaturing poly-acrylimide gel and then electrotransferred to nitrocellulose (Amersham). Blots were probed with either the monoclonal p53 specific antibodies (421, 240, and 1801) as indicated, or the monoclonal anti-poly (ADP-ribose) polymerase 1 (PARP-1) antibody (Pharmingen), or the monoclonal anti-procaspase-3, 7, 8 or 9 antibodies (Oncogene Research), or a phosphor-c-Jun antibody (Oncogene Research), or the phosphor-Chk2 antibody (Oncogene Research), or the monoclonal Mdm2 specific antibodies (4B2, a generous gift from Arnold Levine) and D-7 (Santa Cruz Biotechnology), or the monoclonal anti-p21 antibody AB-1(human) or AB-4 (murine) (Oncogene Research), or a polyclonal anti-

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actin antibody (Sigma). The immunoblotted proteins were detected by chemiluminescence.

2.6 Quantitative RT-PCR

RNA was isolated from cells using the QIAshredder columns and the RNeasy Mini Kit (Qiagen). For each sample, 5 ug of RNA were used for cDNA synthesis using the high capacity cDNA archive kit (Applied Biosystems). The TaqMan primer probes for *glyceraldehyde 3-phosphatase dehydrogenase*, *fas/apo1*, and *waf1/cip1* were obtained from Applied Biosystems Assays on Demand, while the *mdm2* (exons 5,6), *noxa*, *puma*, *bax*, *pig-3*, and *gadd45* specific TaqMan primer probes were also obtained from Applied Biosystems, but were designed by Celera Discovery Systems. The manufacture's conditions were followed for TaqMan PCR with the Applied Biosystems 5700 Sequence Detection System (Perkin Elmer Lifesciences). One cycle for the UNG Incubation at 50°C for 2 min and for priming the reaction at 94°C for 10 min was followed by 40 cycles of 94°C for 15 sec denaturation and 60°C for 1 min annealing. Fluorescence was detected during each annealing step and plotted automatically for each sample.

2.7 Electrophoretic Mobility Shift Assays

For this study, synthetic oligonucleotides were purchased from Operon. The super consensus site contained three adjacent p53 half sites. The sequence of this oligonucleotide was:

5'-TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC-3'

3'-GGCCCGTACAGGCCCGTACAGGCCCGTACAGCTCGA-5'

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Labeling of the oligonucleotides was performed with the large fragment of DNA polymerase I and [32P]dCTP. Electrophoretic mobility shift assay experiments (15 ul) were carried out in reaction mixtures with 150 pmol of 32P-oligonucleotide. 5 ug of nuclear extract was added and the reaction was incubated for 20 min at room temperature in a reaction buffer containing 20 mM HEPES, pH 7.8, 100 mM KCl, 1 mM EDTA, pH 8.0, 1 mM DTT, 1 ug of sheared salmon sperm DNA, and 10% glycerol. Reactions were carried out in the presence or absence of pAb421 as indicated. Samples were separated by 4% polyacrylimide gel electrophoresis (gels were pre-run at 100 V for 15 min at 4°C) at 200V for 3–3.5 hr. Gels were dried for 1 hr at 55 °C and autoradiography was performed.

2.8 Chromatin Immunoprecipitation

Prior to addition to cells, 37% Formaldehyde (Sigma) was boiled and then allowed to cool to room temperature. The boiled Formaldehyde was then diluted to an 11% solution in Solution 1 (0.1M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 8.0). This diluted Formaldehyde solution was then used for the next step where cells were incubated for 30 min at 37°C with a final concentration of 1% Formaldehyde. The crosslinking reaction was quenched by adding glycine to a final concentration of 0.125 M. The cells were then incubated at room temperature for 5 min. After collection, the cells were washed two times with ice cold 1x PBS, pH 7.3, and then spun down at 1850 rpm for 7 min at 4°C. The resulting cell pellet was resuspended in 5 ml of 100 mM Tris-Cl, pH 9.4 with 20 mM DTT and incubated for 15 min at 30°C. The cells were spun down at 2000 rpm for 5 min. The resulting pellet was resuspended in 2 ml of Solution 2 (0.25% Triton

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X-100, 0.5% IGE-PAL (Sigma), 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris-Cl, pH 8.0, 1 mM PMSF) and incubated on ice for 10 min. The samples were then spun down at 2000 rpm for 5 min at 4°C. The cell pellet was resuspended in 2 ml of Solution 3 (0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-Cl, pH 8.0, 1 mM PMSF), and incubated at room temperature for 10 min. The samples were spun down at 2000 rpm for 5 min at 4°C. The resulting pellet was resuspended in 1 ml of Solution 4 (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-Cl, pH 8.0, 1 mM PMSF) and then sonicated on ice 10 times for 10 sec. The sonicated extract was spun down at 14000 rpm for 10 min at 4°C. The supernatant was saved, and its volume measured. An appropriate volume of 10x RIPA buffer (1% Triton X-100, 1% Deoxycholate, 1.4 M NaCl, 1% SDS) was added to each sample to make the total concentration of RIPA buffer in solution 1x. From each sample, 0.2 ml was removed and designated the "input" fractions. Antibody (or in the case of the beads alone samples, no antibody) was then added to the rest of the sample and was set to rock overnight at 4°C. 2 ug of AB-6, D-7, and anti-Bcl-XL (Oncogene Research), 10 ug of purified 4B2, or a 1:200 dilution of anti-phosphoserine 15-p53 was used for these immunoprecipitations. The next day, 50 ul of Protein A/G Plus beads (Santa Cruz) were added to each sample. The samples were then rocked an additional two and a half hours at 4°C. The immunoprecipitated samples were then spun down at 3400 rpm for two and a half minutes at 4°C. The beads were washed once in Wash 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), once in Wash 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), once in Wash 3 (0.25 M LiCl, 1% IGE-PAL, 1% Deoxycholate, 1mM EDTA, 10 mM Tris-Cl, pH 8.1), and twice in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0). After each wash

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was added the samples were rocked for 10 min at 4°C, then spun down at 3400 rpm for 2 and a half minutes at 4°C. After the last TE wash, 0.1 ml of Elution buffer (1% SDS, 0.1 M NaHCO3) was added to immunoprecipitated and input samples. All samples were incubated at 65°C overnight to remove the formaldehyde crosslinks. The following day, the resulting DNA was isolated using the QIAquick PCR Purification Kit (Qiagen). Isolated DNA was stored at 4°C until analysis.

2.9 PCR and Qualitative Analysis of ChIP Samples

For each sample, 5 ul of the total 50 ul volume of DNA isolated from the chromatin immunoprecipitation or the input samples was used in each 25 ul PCR reaction (50% TaqMan Universal PCR Mix (Applied Biosystems), 0.3 pmoles/ul primers). The primer sequences used to analyze DNA isolated using ChIP are as follows:

mdm2: (forward primer) 5'-CGGGAGTTCAGGGTAAAGGT-3', (reverse primer) 5'-AGCAAGTCGGTGCTTACCTG-3'

waf1/cip1: (forward primer) 5'-GTGGCTCTGATTGGCTTTCTG-3', (reverse primer) 5'-CTGAAAACAGGCAGCCCAAG-3'

The number of cycles utilized to visualize the resulting amplicons differed according to the amount of product obtained, but the conditions for each PCR reaction remained the same (50°C for 2 min once, 94°C for 10 min once, 94°C and then 60°C for the number of cycles warranted). The entire PCR reaction was then electrophoresed on an 8% nondenaturing polyacrylimide gel, which was then stained with EtBr. The gel was de-stained in water until the PCR products were clearly visible.

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2.10 MTT Assay

Following treatment, at least 2.5 x 10^5 of ML-1 or K562 cells were spun down at 1850 rpm for 5 min at room temperature (RT). The supernatant was removed and the resulting cell pellet was resuspended in 1 ml of complete RPMI media containing 0.5 mg/ml MTT. Resuspended cells were plated in a 24 well plate and incubated from one to three hours at 37 C. The MTT treated cells were spun down at 1850 rpm for 5 min at RT, and the resulting cell pellet was thoroughly resuspended in 0.04N HCl diluted in Isopropanol. Samples were incubated for 5 min at RT, and then spun down at 14,000 rpm for 2 min. 250 ul of supernatant was transferred into a 96 well plate and the absorbance was read at 620 nm.

2.11 Annexin Staining

Following treatment, at least 5.0 x 10^5 of ML-1 or K562 cells were spun down at 1850 rpm for 5 min at room temperature (RT). The resulting pellet was resuspended in 500 ul of 1x Binding Buffer containing 1.25 ul of Annexin. Samples were incubated for 15 min at RT in the dark. The cells were spun down at 2000 rpm for 5 min at RT. The supernatant was removed and the pellet was resuspended in 500 ul of 1x Binding Buffer. 10 ul of Propidium Iodide was added to each sample, and the samples were kept on ice until analysis.

2.12 Guava Caspase Assay

Following treatment, at least 5.0 x 10^4 of ML-1 or K562 cells were spun down at 1850 rpm for 5 min at room temperature (RT). The resulting pellet was resuspended in 100 ul

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of 1x Apoptosis Wash Buffer (Guava Technologies). 5 ul of 20x SR-VAD-fmk reagent was added to each sample. Each sample was then briefly vortexed and incubated for 1 hr at 37 C in 5% CO2. The samples were resuspended once or twice during the incubation. One ml of 1x Apoptosis Wash Buffer was added to each sample. Each sample was vortexed and then spun down at 400g for 5 min. The supernatant was aspirated, and the resulting pellet was washed three times in 1 ml Wash Buffer (Guava Technologies). After the final wash, the pellet was resuspended in 100 ul of Wash Buffer. 5 ul of caspase 7-AAD was added to each sample. Each sample was then incubated for 10 min at RT, and then another 100-400 ul of Wash Buffer was added prior to analysis in the Guava Fluorescence Activated Cell Sorter (Guava Technologies).

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Chapter 3. Mdm2 associates with chromatin only in the presence of p53 and precedes decreases in transcription from p53 target genes

3.1 Introduction

In response to genotoxic stress, the tumor suppressor p53 conducts numerous pathways, such as cell cycle arrest, apoptosis, and DNA repair, which avert cellular transformation (Bargonetti and Manfredi, 2002; Velculescu and El-Deiry, 1996). Acting principally as a transcription factor, p53 binds to DNA as a tetramer, localizing to specific p53 responsive elements on genes whose protein products mediate growth suppressive activity indicative of the p53 pathway (el-Deiry et al., 1994; Farmer et al., 1992; Zhan et al., 1994a). In the absence of stress, p53 levels remain low due primarily to association of p53 with its negative regulator, Mdm2 (Giaccia and Kastan, 1998; Kastan et al., 1991). *Mdm2* is a p53 target gene (Juven et al., 1993) whose protein product regulates p53 activity by multiple mechanisms. Mdm2 protein binds to a region on the N-terminus of p53 and inhibits the transcriptional activity of p53 by blocking the ability of p53 to associate with transcriptional machinery (Barak et al., 1993; Oliner et al., 1993; Thut et al., 1997). In the presence of cytotoxic stimuli, like DNA damage, stress kinase pathways signal to and phosphorylate p53 on numerous sites (Bean and Stark, 2002). Phosphorylation of p53 on amino acids within the Mdm2 binding site (like serine 15, threonine 18, and serine 20) disrupt Mdm2-p53 complex formation (Sakaguchi et al., 2000; Shieh et al., 1997; Unger et al., 1999a). Additionally, Mdm2 acts as an E3 ubiquitin ligase targeting p53 for degradation in proteasomes (Honda et al., 1997; Ogawara et al., 2002). These phosphorylation events not only stabilize p53 protein in the

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cell, but also promote the transcriptional activity of p53 (Colman et al., 2000). A mathamatical model proposed by Samuel Levs Bar predicted that the p53-Mdm2 negative feedback loop is cyclical. This model was substantiated by the observed oscillations in the levels of Mdm2 and p53 proteins within the cell following the activation of p53 in response to DNA damage (Lev Bar-Or et al., 2000).

Recent studies have begun to delineate how p53 mediates transcription of its target genes by determining which cooperative factors co-localize with p53 at its responsive element. The regions encompassing the p53 binding sites on target genes, like *mdm2* and *gadd45*, have been found to be nucleosome free and poised for transactivation (Graunke et al., 1999; Xiao et al., 2000; Xiao et al., 1998). Studies using chromatin immunoprecipitation (ChIP) have found p53 bound to its binding sites prior to activation (Espinosa et al., 2003; Szak et al., 2001). Additionally, increasing levels of p53 were detected on these DNA binding sites with ChIP following activation of p53 by stress (Espinosa et al., 2003; Kaeser and Iggo, 2002; Szak et al., 2001). High levels of p53 expressed in the absence of stress have also been immunoprecipitated with DNA containing p53 binding sites from the *gadd45* gene (An et al., 2004). Chromatin immunoprecipitation has also been used to show that p53 promotes transcription by co-localizing with numerous components of the transcription initiation complex (Espinosa et al., 2003), the histone acetyltransferases (HAT) GCN5 and p300 (An et al., 2004), and the HAT complex scaffolding protein TRRAP (Ard et al., 2002). Recent work by Minsky and colleagues used chromatin immunoprecipitation to show the co-localization of Mdm2 with p53 on the *waf1* p53 responsive element in cells overexpressing Mdm2

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(Minsky and Oren, 2004). It is not known, however, whether p53 bound to the promoters of its target genes is associated with Mdm2 prior to damage, and if this association is truly relieved on all p53 target genes following damage. Using chromatin immunoprecipitation, we have examined the role of Mdm2 in the regulation of p53-dependent *mdm2* and *waf1/cip1* transcription. Interestingly, we found that Mdm2 only associated with chromatin in the presence of p53. Not surprisingly, oscillations in the associated Mdm2 at p53 responsive elements (Lev Bar-Or et al., 2000) occurred freeing p53 from Mdm2 during times of active transcription. A decrease in Mdm2 co-localization was observed early in the p53 response. Higher levels of Mdm2 protein returned to the p53 responsive elements at a later time-point despite the presence of phosphoserine-15 p53. The re-association of Mdm2 with p53 correlated with decreases in *mdm2* and *waf1* transcription. Taken together, these data suggest that the dissociation of Mdm2 from p53 is important in promoting p53-dependent transcription, while the reformation of the chromatin-associated Mdm2-p53 complex may act to shut off transcription after the initial p53 response has been set in motion.

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3.2 Results

Similar levels of p53 induced in the presence of CPT and ETOP differentially regulate *mdm2* and *waf1*.

The relationship between Mdm2 and p53 in the regulation of cell growth has been examined in numerous studies, but it is not known how these two antagonistic proteins interrelate on chromatin. In this study, we examine the localization of p53 and Mdm2 on the p53 responsive elements of two p53 downstream target genes, *mdm2* and *waf1/cip1*. In order to investigate the relationship between this association and gene activation in the presence and absence of DNA damage, we used the p53 inducible cell line, D-A2. Previous studies have shown that DNA damaging agents, like camptothecin (CPT) and etoposide (ETOP), differentially affect the ability of p53 to regulate its downstream target genes (Arriola et al., 1999; Ashcroft et al., 2000). Etoposide treatment has been shown to induce *mdm2* transcription in a p53-dependent manner (Arriola et al., 1999), while CPT has been shown to repress p53-mediated transcription of *mdm2* in the tetracycline-regulated wild type p53 expressing cell line, TR9-7 (Xiao et al., 2000). Here, we analyze the localization of p53 and Mdm2 on the promoters of *mdm2* and *waf1* gene during periods of transcriptional activation or repression. We first looked at the ability of high levels of p53 to up regulate *mdm2* and *waf1* in the presence of ETOP and CPT (Figures 3.1A & B). As expected, removal of doxycycline for 6 hrs resulted in an induction in both *mdm2* and *waf1* transcripts. Although our past results demonstrated the inhibition of only *mdm2* by CPT treatment (Xiao et al., 2000), D-A2 cells grown in the presence of 0.5 μM CPT for four hours, two hours after doxycycline withdrawal, repressed p53-

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dependent transactivation of both *mdm2* and *waf1* genes. In contrast, treatment with as high as 25 μM ETOP did not diminish the ability of p53 to upregulate *mdm2* and *waf1* transcripts (Figures 3.1A & B).

In order to determine whether the reduction in *mdm2* and *waf1* transcripts that occurred in the presence of CPT was due to decreases in the levels of p53 protein, p53 protein levels in drug treated D-A2 cells were analyzed by Western blot using the p53-specific antibody 1801 (Figure 3.1C). Previous work in our lab had shown that CPT treatment did not affect the level of p53 expressed by the tetracycline-regulated constructs in TR9-7 cells (Xiao et al., 2000). Consistent with this observation, no significant decrease in p53 protein was detected in the presence of drugs. To verify that the damage response pathways initiated by CPT and ETOP treatment were inducing p53 phosphorylation at serine 15, p53 protein in drug treated D-A2 cells was examined by Western blot (Figure 3.1D). p53 was barely detected in either the D-A2 cells (+DOX) or the parental DLD-1 cell line (Figure 3.1D, lanes 1 and 2). Higher exposures of D-A2 (+DOX) cell extract, however, did reveal some leakiness from the p53-expressing constructs (data not shown). Unexpectedly, low levels of phosphoserine-15 p53 were observed in untreated D-A2 cells (-DOX) (Figure 3.1D, lane 3), suggesting that the overexpression of p53 may be exerting stress on the cell and activating stress kinase pathways that signal to p53. Higher levels of phosphoserine 15-p53, however, were observed in the D-A2 cells (-DOX) treated with drugs (Figure 3.1D, lanes 4 though 7). These data, taken together, suggest that the differential regulation of *mdm2* and *waf1*

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transcription by p53 exposed to different drugs was not due to lower levels of induced p53 protein or differential phosphorylation of p53 at serine 15 by the various treatments.

Figure 3.1 p53 target genes are differentially regulated by similar levels of p53 protein. *Quantitative PCR was carried out on cDNA obtained from RNA isolated from D-A2 cells grown in the presence (+DOX) or absence (-DOX) of doxycycline for 6 hrs. Where indicated, samples were treated with camptothecin (CPT) or etoposide (ETOP) for 4 hrs following two hours of growth in the absence of doxycycline. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of mdm2 (A) or waf1/cip1 (B) transcripts was calculated over the D-A2 (+DOX) sample. The results shown are representative of two independent experiments. Nuclear extracts were isolated from DLD-1 cells (lane 1), D-A2 cells (+DOX, lane 2), D-A2 (-DOX, lane 3) or D-A2 (-DOX) treated with camptothecin (lane 4) or increasing dosages of etoposide (lanes 5 through 7). 100 ug of the resulting nuclear extract was electrophoresed on a 10% SDS-PAGE and subjected to immunoblotting using 1801 Ab (C, upper panel) or an anti-phosphoserine-15 p53 antibody (D). To show equal loading between lanes these samples were also immunoblotted for actin (D, lower panel).*

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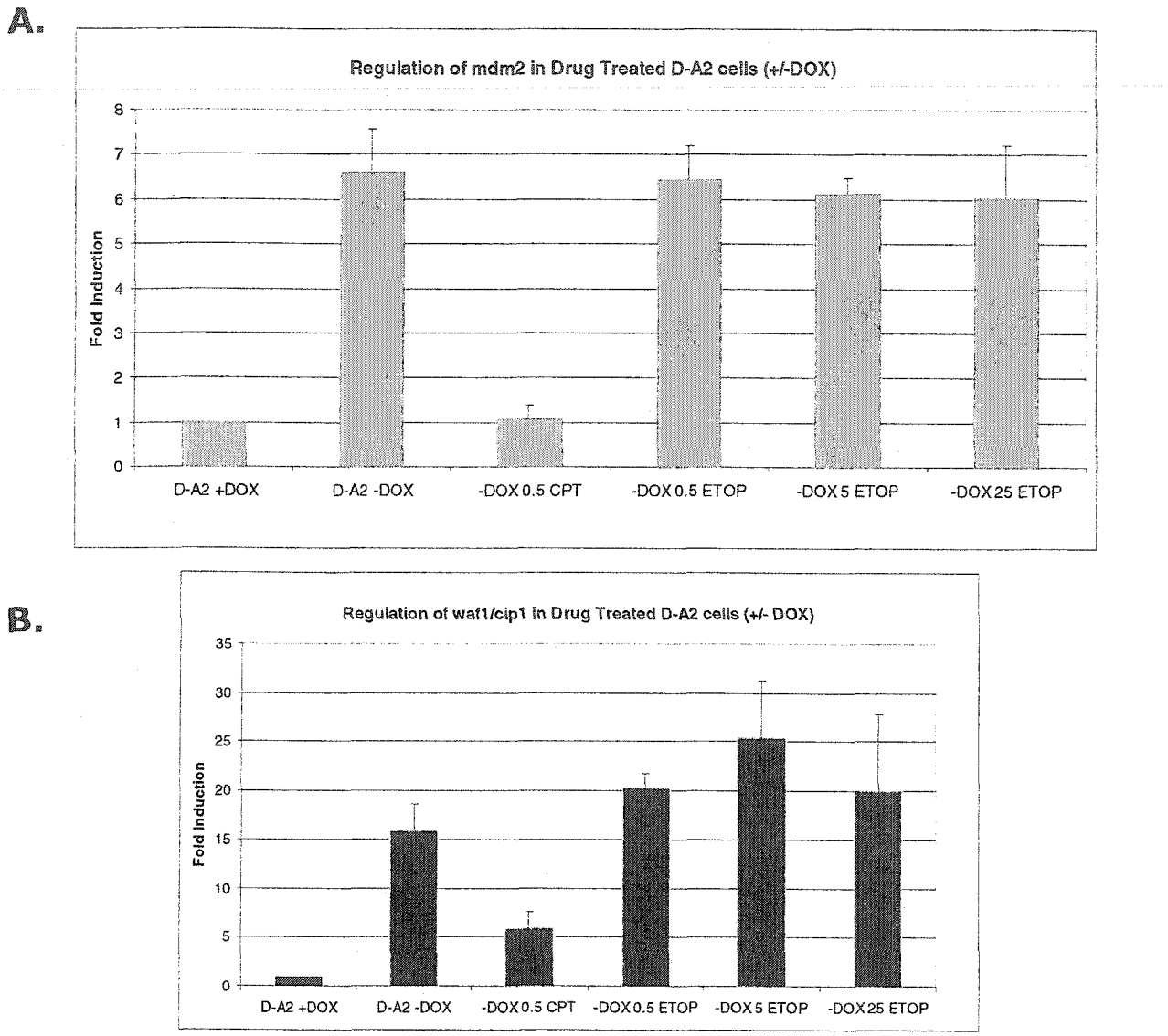

Figure 3.1 cont.

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The differential regulation of p53 target genes was not due to alterations in the ability of p53 protein to associate with its responsive element.

Although the expression of p53 protein was not affected by drug treatment, this did not address whether this p53 was associated with chromatin. Chromatin immunoprecipitation (ChIP) experiments were utilized to determine whether drug treatment promoted the differential regulation of *mdm2* and *waf1* genes by affecting the ability of p53 to localize to its responsive element. Chromatin-associated p53 has been demonstrated at the *waf1* and *mdm2* response elements in response to numerous DNA damaging agents (Kaeser and Iggo, 2002; Szak et al., 2001). Semi-quantitative PCR using primers specific for the region encompassing the *waf1* (5') or the *mdm2* p53 response elements was utilized to analyze immunoprecipitated DNA. As expected, we were unable to detect any DNA containing either p53 binding site from immunoprecipitations with p53-specific antibodies from the parental p53 null cell line, DLD-1 (Yu et al., 1999) (Figure 3.2A & B, lanes 2 and 3). Similar levels of DNA from both p53 target genes were immunoprecipitated from D-A2 cells (-DOX) in the presence or absence of drug (Figure 3.2C & D, lanes 4 through 6) using the p53-specific antibody, AB-6. Whereas DNA containing the *waf1* or *mdm2* p53 responsive elements from D-A2 (-DOX) was barely detectable in immunoprecipitations using phosphoserine-15 p53 specific antibodies, greater quantities of DNA containing these sites were immunoprecipitated from cells treated with CPT or ETOP (Figure 3.2C & D, compare lane 7 to lanes 8 and 9). No differences in the levels of chromatin-bound phosphoserine-15 p53 were observed between CPT and ETOP treatments. Taken together, these data suggest that the differential regulation of target genes by p53 was not due to any variation

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in the ability of p53 to bind to its responsive elements *in vivo*. The lowest panels from these D-A2 ChIPs represent one set of INPUT samples, indicating that all samples were normalized for chromatin prior to the immunoprecipitation. No detectable DNA from either gene was immunoprecipitated in the absence of antibody or from D-A2 cells (-DOX) with an antibody specific for the mitochondrial protein, Bcl-XL, substantiating the specificity of the D-A2 ChIPs (Figures 3.2A & B, lane 4, C& D, lanes 1 through 3). These data suggest that the repression of *mdm2* and *waf1* transcription by CPT was not due to the inability of p53 to bind to its responsive elements.

Figure 3.2 DNA damage drug treatment does not affect the ability of p53 to bind to its responsive elements on *mdm2* or *waf1/cip1*. *Chromatin immunoprecipitation was performed on formaldehyde crosslinked DNA from the DLD-1 parental cell line for mdm2 (A) and waf1/cip1 (B) from ChIP using antibodies specific for p53 (lane 2, upper panel) or phosphoserine-15 p53 (lane 3, upper panel). As a negative control, beads without antibody were also used (A and B, lane 1, upper panel). One fifth of the DLD-1 input DNA from each sample was also amplified using the same primers for mdm2 (A, lower panel) or waf1/cip1 (B, lower panel) promoters for 34 and 31 cycles, respectively. PCR was also carried out on DNA isolated from D-A2 cells grown in the absence of doxycycline for 6 hrs (lanes 1, 4, 7) alone, or in the presence of 0.5 uM camptothecin (lanes 2, 5, 8) or 5 uM etoposide (lanes 3, 6, 9) for 4 hrs after 2 hrs DOX removal. PCR was carried out on these samples using primers specific for the promoter region including the p53 responsive elements on mdm2 (C, upper panel) for 33 cycles or waf1/cip1 (D, upper panel) for 30 cycles. One fifth of the input DNA from each sample*

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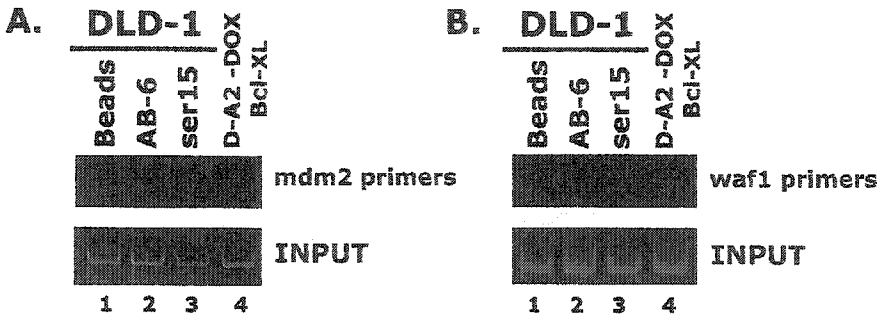
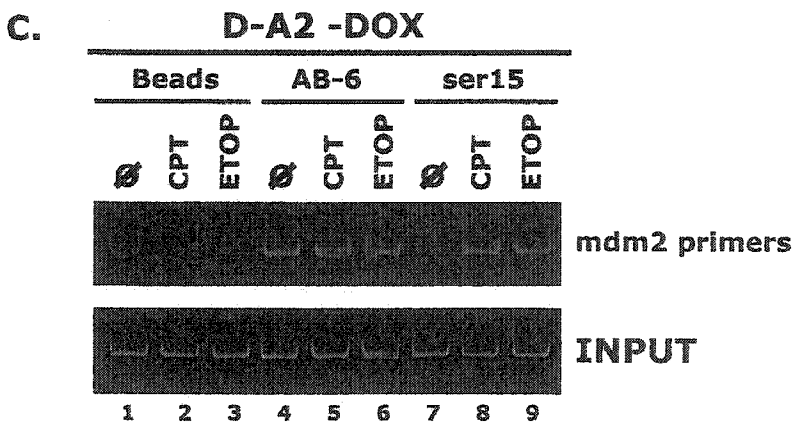
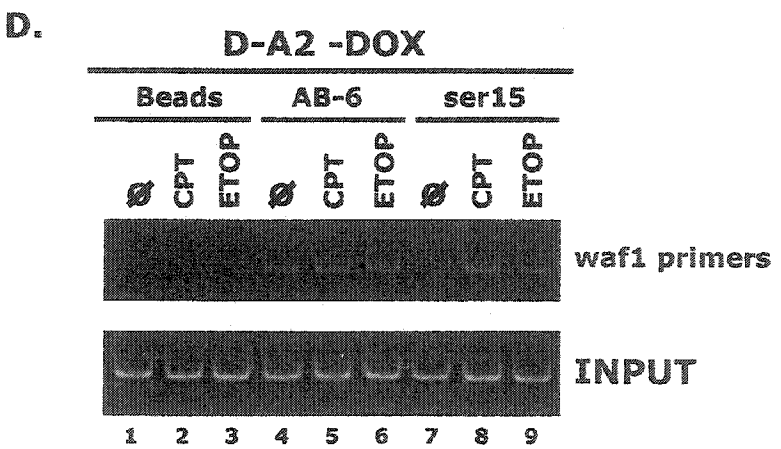
was also amplified using the same primers for mdm2 (C, lower panel) or waf1/cip1 (D, lower panel) promoters for 33 and 28 cycles, respectively. The p53 antibody AB-6 (lanes 4 through 6) and the anti-phosphoserine-15 p53 antibody (lanes 7 through 9) were utilized for the immunoprecipitations noted in the upper panels. As a negative control, beads without antibody were also used (lanes 1 through 3, upper panels).







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The localization of Mdm2 on p53 responsive elements requires the presence of p53.

Although no differences in the ability of p53 to associate with chromatin in response to drug were observed using ChIP, it was clear that the transcriptional activity of p53 was diminished in cells treated with CPT. Mdm2 has been shown to inhibit the transcriptional activity of p53 through an association at the N terminus of p53 (Barak et al., 1993; Oliner et al., 1993; Thut et al., 1997). To address whether the lower levels of p53 target gene expression detected in response to CPT were due to the association of Mdm2 with p53 on chromatin, chromatin immunoprecipitations were carried out on drug treated D-A2 cells using Mdm2-specific antibodies. No detectable DNA was immunoprecipitated from DLD-1 cells using these antibodies (Figures 3.3A & B), however, DNA encompassing the p53 responsive elements on the *mdm2* and *waf1* genes was immunoprecipitated from D-A2 cells (-DOX) and D-A2 cells (-DOX) treated with ETOP (Figures 3.3C & D). Interestingly, MDM2-specific antibodies immunoprecipitated comparatively lower levels of DNA from both *waf1* and *mdm2* genes in cells treated with CPT (Figures 3.3C & D, lanes 2 and 5). These data, taken together, imply that Mdm2 protein is unable to interact with chromatin in the absence of p53, but it can co-localize with p53 at its responsive element. Additionally, the presence of a DNA damage signal and phosphorylation of p53 at serine 15 only slightly reduces the quantity of Mdm2-bound with p53 on chromatin. The only conditions under which we saw no Mdm2 associated with p53 was when *mdm2* was repressed by CPT treatment (Figure 3.1A & B). To determine whether the lower levels of Mdm2 bound to chromatin exhibited in CPT-treated samples was due to decreased Mdm2 protein levels, we analyzed nuclear extracts from drug treated D-A2 cells by Western Blot. High levels of

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Mdm2 protein were detected in D-A2 cells (-DOX) using the 4B2 antibody (Figure 3.3E, lane 3). This 4B2-reactive Mdm2 species was downregulated in response to CPT, but not ETOP treatment (Figure 3.3E, lanes 4 and 5). These data correlated with the transcriptional data obtained from these samples (Figure 4.1A). No basal expression of 4B2 reactive Mdm2 was detected in DLD-1 cells or in D-A2 cells prior to doxycycline removal (Figure 3.3E, lanes 1 and 2). The levels of p21 protein also corresponded to the transcriptional data observed in these samples (Figure 3.1B). These data, taken together, suggest that the decreased levels of chromatin-bound Mdm2 protein were due to decreased Mdm2 protein resulting from the repression of *mdm2* transcription by CPT treatment.

Figure 3.3 The localization of Mdm2 on p53 responsive elements requires the presence of p53. *Chromatin immunoprecipitation was performed on formaldehyde crosslinked DNA from the DLD-1 parental cell line for mdm2 (A) and waf1/cip1 (B) from ChIP using antibodies specific for Mdm2 (lanes 2 and 3, upper panel). As a negative control, beads without antibody were also used (A and B, lane 1, upper panel). One fifth of the DLD-1 input DNA from each sample was also amplified using the same primers for mdm2 (A, lower panel) or waf1/cip1 (B, lower panel) promoters for 34 and 31 cycles, respectively. PCR was also carried out on DNA isolated from D-A2 cells grown in the absence of doxycycline for 6 hrs (lanes 1, 4, 7) alone, or in the presence of 0.5 uM camptothecin (lanes 2, 5, 8) or 5 uM etoposide (lanes 3, 6, 9) for 4 hrs after 2 hrs DOX removal. PCR was carried out on these samples using primers specific for the promoter region including the p53 responsive elements on mdm2 (C, upper panel) for 33 cycles or*

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waf1/cip1 (D, upper panel) for 31 cycles. One fifth of the D-A2 input DNA from each sample was also amplified using the same primers for mdm2 (C, lower panel) or waf1/cip1 (D, lower panel) promoters for 33 and 31 cycles, respectively. The Mdm2 antibodies D-7 (lanes 1 through 3) and 4B2 (lanes 4 through 6) were utilized for the immunoprecipitations noted in the upper panels. Nuclear extracts were isolated from DLD-1 cells (lane 1), D-A2 cells (+DOX, lane 2), D-A2 (-DOX, lane 3) or D-A2 (-DOX) treated with camptothecin (lane 4) or 0.5 uM etoposide (lanes 5). 100 ug of the resulting nuclear extract was electrophoresed on a 10% SDS-PAGE and subjected to immunoblotting using the Mdm2 specific antibody 4B2 (E, upper panel) or anti-p21 (E, middle panel). To show equal loading between lanes these samples were also immunoblotted for actin (E, lower panel).

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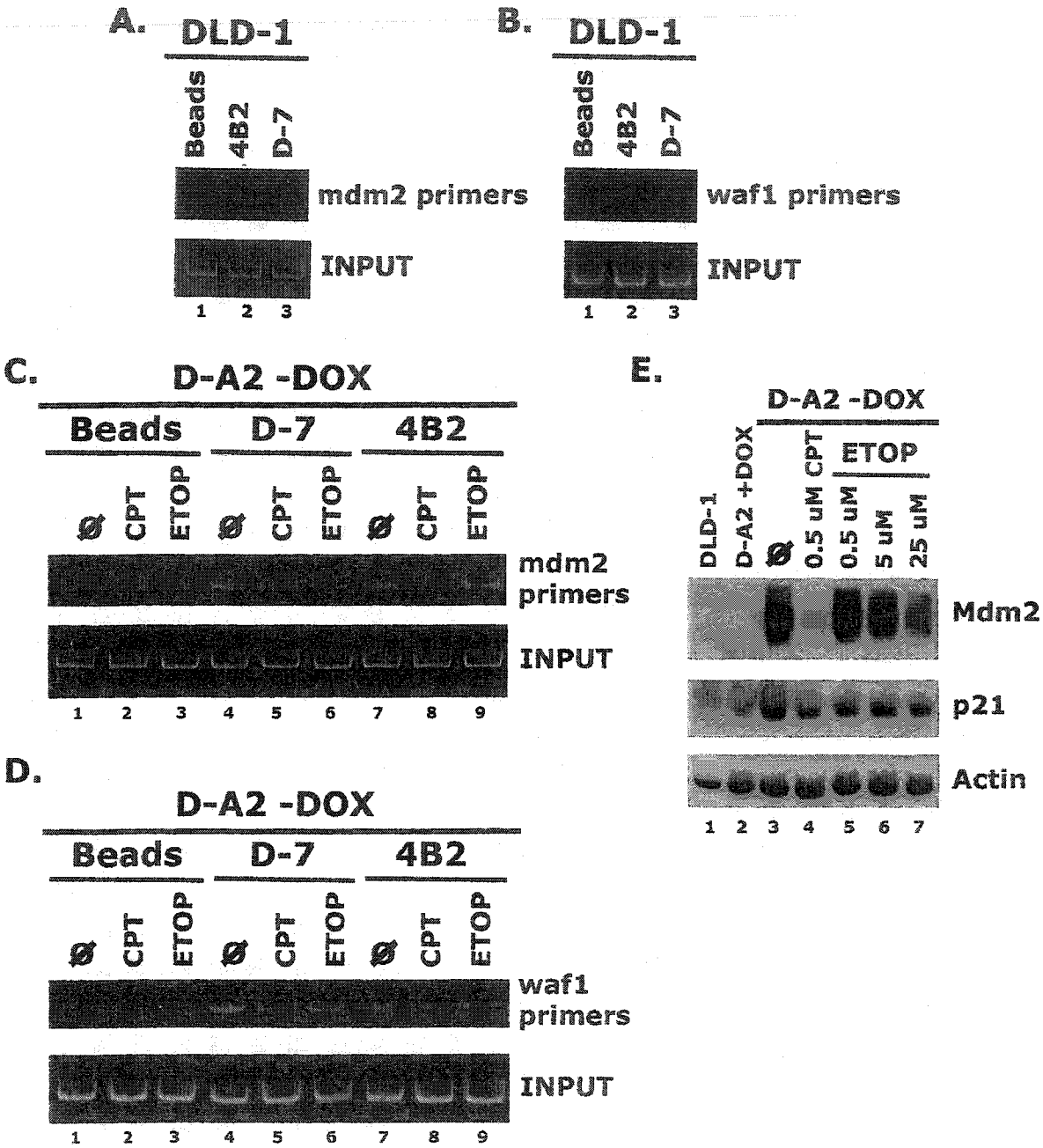

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Mdm2 dissociates from chromatin during p53-activated transcription, and then re-associates with p53 at later time points.

Our current understanding of Mdm2 tells us that the presence of Mdm2 with p53 on chromatin should inhibit p53 transactivation activity. However, we found Mdm2 and p53 co-localized on genes that were transcriptionally active. This caused us to consider that the dissociation of Mdm2 from p53 on chromatin was an early event in p53-dependent transactivation. Following the initiation of transcription, Mdm2 might return to p53 on chromatin to repress further transactivitation from the locus. We examined this possibility by following the localization of Mdm2 on the *mdm2* and *waf1* genes, while p53 was induced over time in the D-A2 cell line. Low levels of DNA were immunoprecipitated with p53-specific antibodies and modest levels of DNA containing the p53 responsive elements from both genes was immunoprecipitated with Mdm2-specific antibodies prior to the removal of doxycycline (Figures 3.4A & B, lane 1). Interestingly, while the level of DNA immunoprecipitated with p53-specific antibodies remained high, the amount of DNA immunoprecipitated with Mdm2-specific antibodies decreased following doxycycline removal, with the lowest quantity evident after 1.5 hrs (-DOX) (Figures 3.4A & B, lane 3). These data suggest that Mdm2 began to dissociate from the chromatin on *mdm2* and *waf1* as early as 30 minutes following the induction of p53, while p53 association with the chromatin increased slightly over time (Figures 3.4A & B, lane 2). The p53 induced after 1.5 hrs of DOX removal was practically free from bound Mdm2 protein (Figures 3.4A & B, lane 3). These data, taken together, suggest that Mdm2 co-localized with p53 on chromatin prior to transcriptional activation and then upon the activation of p53 it dissociates alleviating repression of p53-dependent

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transcription. The presence of Mdm2 with p53 on these enhancer elements after 6 hrs of p53 expression (Figure 3.4A & B, lane 5) also suggests that after a period of transcriptional activity, Mdm2 again co-localizes with p53 on chromatin to yield an inhibitory complex. The reformation of this inhibitory complex may serve to downregulate the p53 response after its initiation.

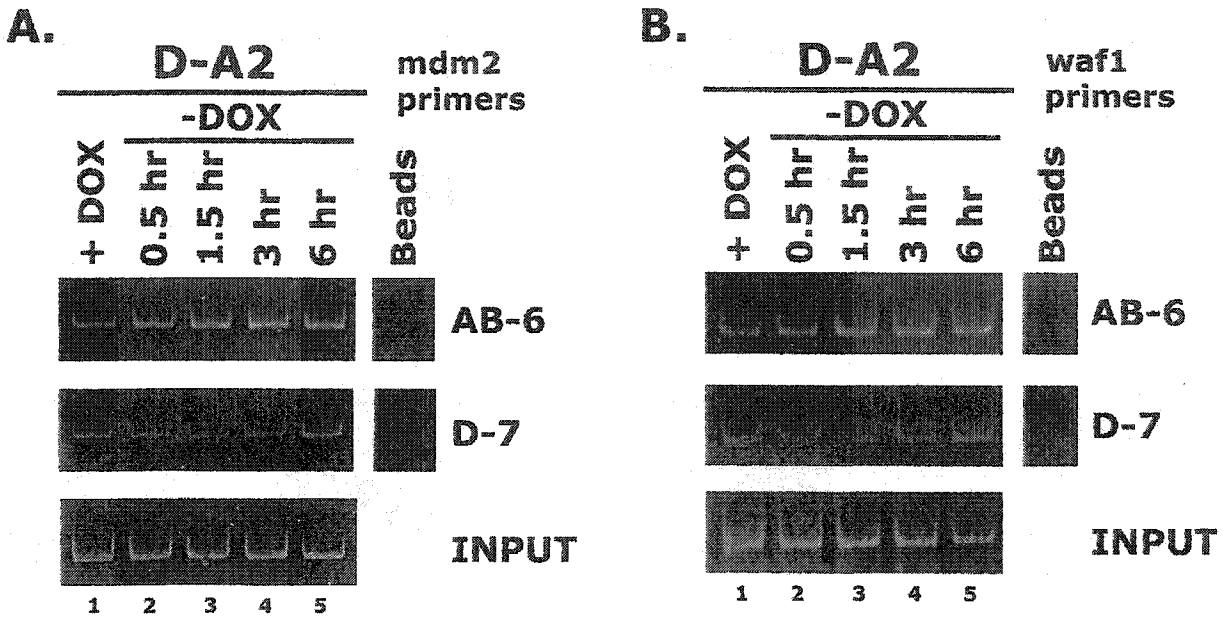

Figure 3.4 MDM2 dissociates from p53 early on following the induction of p53. *Chromatin immunoprecipitations were carried out on D-A2 cells grown in the presence of doxycycline (+DOX, lane 1) or for increasing times in the absence of doxycycline (-DOX, lanes 2 though 5) using the p53 specific antibody AB-6 (upper panel), the Mdm2 specific antibody D-7 (second panel from the top), or with beads alone (second panel from the bottom). Primers specific for the mdm2 (A) or waf1/cip1 (B) promoters were utilized to analyze the resulting DNA for 33 and 30 cycles, respectively. To show equal loading between lanes these samples were also immunoblotted for actin (A, lower panel).*

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The re-association of Mdm2 with p53 on chromatin is followed by decreases in transcription.

If the reformation of the Mdm2-p53 inhibitory complex on the p53 response elements serves to downregulate the p53 response, then a decrease in p53-mediated transactivation should be evident after the re-association of Mdm2 with chromatin. To address the hypothesis, we used quantitative PCR to examine if the level of *waf1* and *mdm2* transcript decreased after the 6 hr time point where we saw Mdm2 association with p53 on chromatin. The level of *waf1* and *mdm2* transcript decreased after 6 hrs of p53 expression suggesting that oscillations in Mdm2-p53 association occur on chromatin, and that Mdm2 reforms its inhibitory complex with p53 in order to quell the already initiated p53 response (Figure 3.5C & D).

Figure 3.5 The re-association of Mdm2 with p53 on chromatin correlates with decreases in transcription. *Quantitative PCR was carried out on cDNA obtained from RNA isolated from D-A2 cells grown in the presence (+DOX) or absence (-DOX) of doxycycline for 3, 6, or 9 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of mdm2 (C) and waf1/cip1 (D) transcripts were calculated over the D-A2 (+DOX) sample. The results shown are representative of two independent experiments.*

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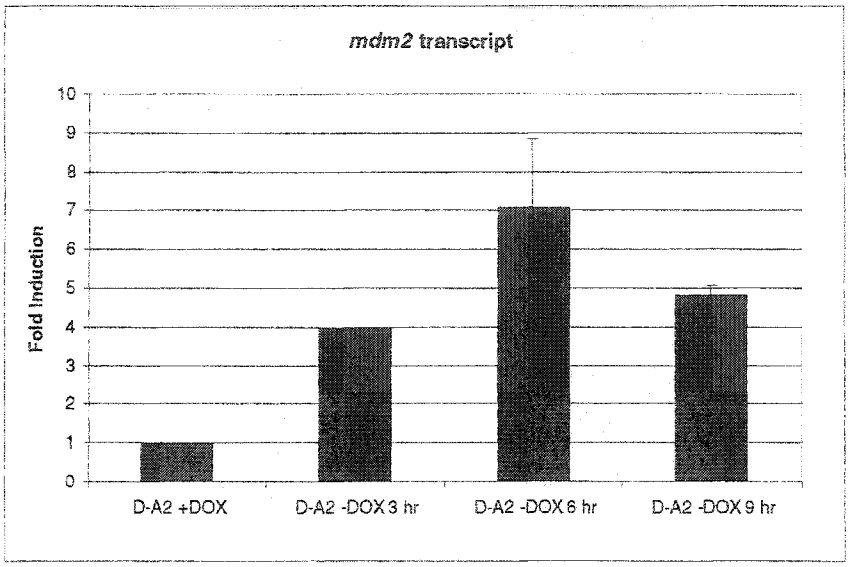
A.



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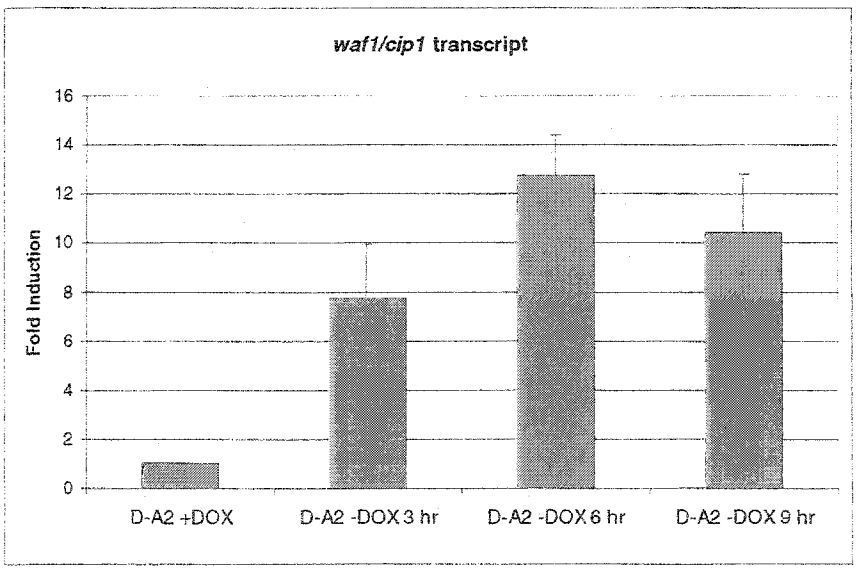

Figure 3.5 cont.

Similar levels of p53 protein also differentially regulate downstream target genes in cells that contain a natural p53 response.

Since the p53 expression system in D-A2 cells is artificial, we next looked at whether p53 associated with Mdm2 on chromatin in cells that have a well-documented, in tact p53 response. We were also interested in determining whether the presence of this

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complex on chromatin, if detected, correlated with the pattern of gene expression exhibited by p53 induced by various agents. The myeloid leukemia cell line, ML-1, has been used in numerous studies examining p53 activation following stress (Houser et al., 2001; Kastan et al., 1991; Nelson and Kastan, 1994; Zhan et al., 1994b). We used the proteasome inhibitor, N-acetyl-Leu-Leu-Norleu-al (LLnL), to induce high levels of p53 protein in the absence of a damage response. LLnL treatment of ML-1 cells facilitated the investigation of p53 and Mdm2 association on chromatin in the absence of DNA damage as determined previously in the D-A2 cell line. The p53 null erythroid leukemia cell line, K562, was used to examine the association of Mdm2 with chromatin in the absence of p53. ML-1 cells were treated with different dosages of LLnL, CPT, and ETOP to induce similar levels of p53 (Figure 3.6A). Although similar levels of ETOP and CPT generated p53 with calpain cleavage, this has been seen before (Kubbutat and Vousden, 1997). Using antibodies specific for phosphoserine-15 p53, we confirmed that CPT and ETOP treatment exhibited higher levels of p53 protein phosphorylated at serine 15 (Figure 3.6B). No p53 was detected in K562 cells, even in the presence of drug (data not shown).

In order to confirm that the p53 induced by these agents was transcriptionally active, quantitative PCR was performed on cDNA created from RNA isolated from drug treated ML-1 and K562 cells. ETOP treatment induced a form of p53 in ML-1 cells that exhibited the most transcriptional activity, promoting high levels of both *mdm2* and *waf1* transcripts (Figures 3.6C & D). In contrast to earlier findings in the D-A2 cell line, high levels of p53 induced in the absence of damage using LLnL only modestly induced the

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mdm2 and *waf1* genes (Figures 3.6C & D). Consistent with results obtained in the p53 regulatable cell line TR9-7 by Xiao et al (Xiao et al., 2000), CPT treatment promoted the induction of *waf1* transcripts, but repressed transcription from the *mdm2* gene (Figure 3.6C & D). The upregulation of *waf1* transcription in response to CPT may partly be due to a p53-independent mechanism, as the p53 null cell line K562 exhibited some induction of this gene, however, no induction of *waf1* was observed in K562 cells treated with ETOP or LLnL (Figure 3.6F). There was also no induction of *mdm2* in drug treated K562 cells (Figure 3.6E).

Figure 3.6 Similar levels of p53 protein induced by different drugs differentially regulate downstream target genes in ML-1 cells. *Nuclear extracts were prepared from ML-1 cells that were grown in the absence (lane 1) or presence of 20 uM LLnL (lanes 2 and 3), 0.5 uM CPT (lanes 4 and 5), or 8 uM ETOP (lanes 6 and 7) for 3 and 6 hrs. 100 ug of the resulting extract was electrophoresed on a 10% SDS-PAGE and immunoblotted for p53 using pAb1801 (A, upper panel) or anti-phosphoserine-15 p53 (B, upper panel). To show equal loading between lanes these samples were also immunoblotted for actin (A and B, lower panel). Quantitative PCR was performed on cDNA from RNA isolated from ML-1 (C and D) and K562 cells (E and F) grown in the presence or absence of 20 uM LLnL, 0.5 uM camptothecin (CPT), or 8 uM etoposide (ETOP) for 3 and 6 hrs and for the samples examined for mdm2 transcript level, 12 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of mdm2 (C and E) or waf1/cip1 (D and F) transcripts was calculated over the untreated sample. The results shown are representative of two independent experiments.*

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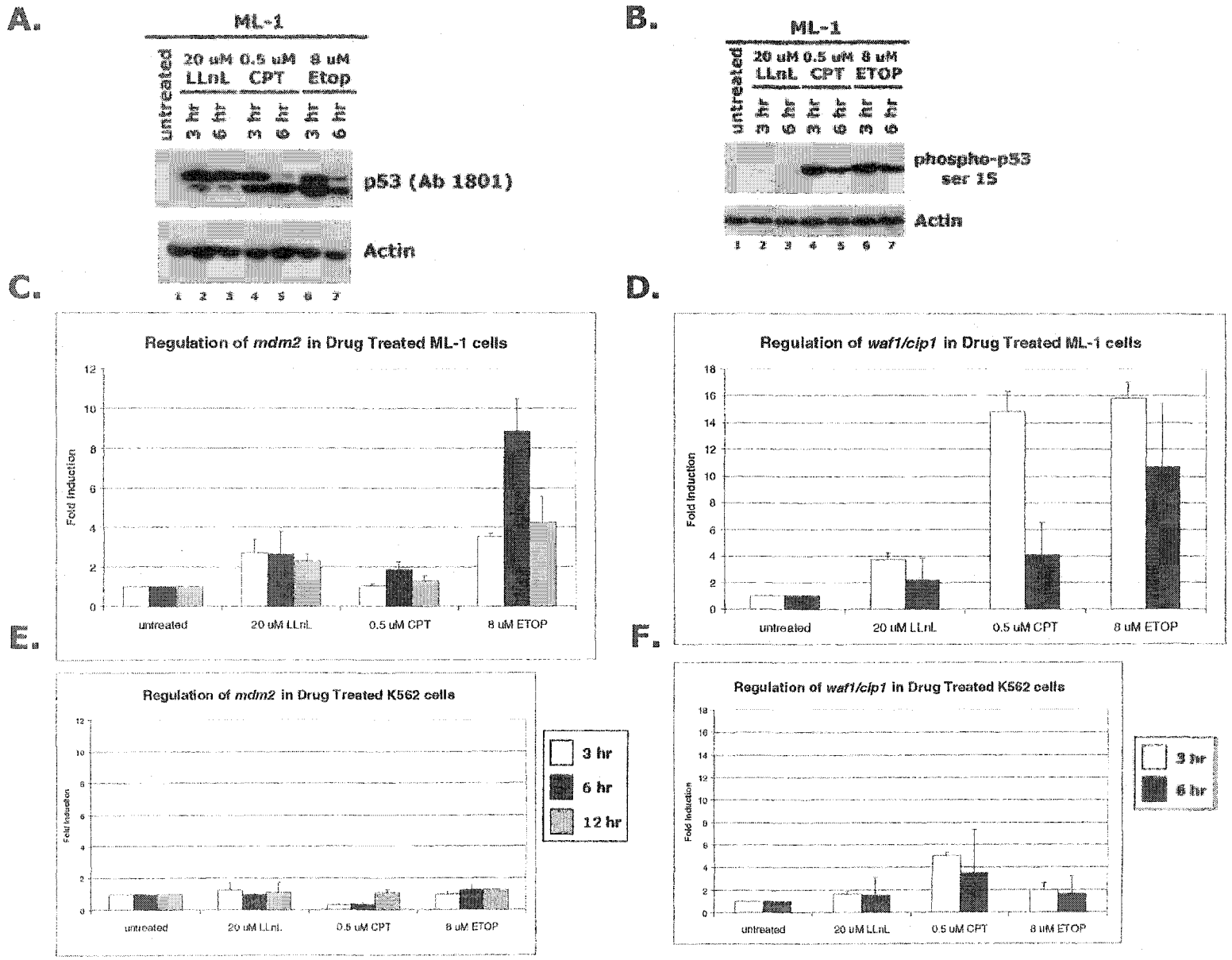

Figure 3.6 cont.

p53 induced by different drugs binds site-specifically to DNA *in vitro*.

In order to examine the capacity of the p53 stabilized by different signaling pathways to bind to DNA, electrophoretic mobility shift assays (EMSA) using the p53 super consensus sequence (SCS) were carried out. The super consensus sequence is an oligonucleotide containing 3 half sites from the p53 responsive element which has been optimized for binding by p53 (el-Deiry et al., 1992). The p53-specific antibody 421 was added to the binding reaction to promote binding *in vitro*, and to supershift the p53-specific binding species (Hupp and Lane, 1994). Interestingly, we found that the p53 421-induced binding extracts from LLnL treatment exhibited stronger DNA binding ability than the p53 induced by damage at the 3 hr timepoint (Figure 3.7A, compare lane 4 to lanes 6 and 8). The p53 induced by CPT exhibited less DNA binding ability at 6 hrs than the p53 induced by either LLnL or ETOP treatment (Figure 3.7B, compare lane 6 to lanes 4 and 8). Although these data determined that the p53 stabilized by these drugs were capable of binding to DNA *in vitro*, they did not address the ability of the p53 protein to interact with chromatin.

Figure 3.7 p53 induced by different drugs binds site-specifically to DNA *in vitro*. *The ability 5 ug of nuclear extract isolated from ML-1 cells grown in the absence (lanes 1 and 2) or presence of 20 uM LLnL (lanes 3 and 4), 0.5 uM CPT (lanes 5 and 6), or 8 uM ETOP (lanes 7 and 8) for 3 (G) and 6 hrs (H) to bind to a radiolabeled SCS oligonucleotide was determined in an EMSA. 2 ug of the p53 specific antibody was added to the binding reaction where noted. Reactions were electrophoresed on a 4%*

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nondenaturing acrylimide gel and the resulting bands were visualized by autoradiography.

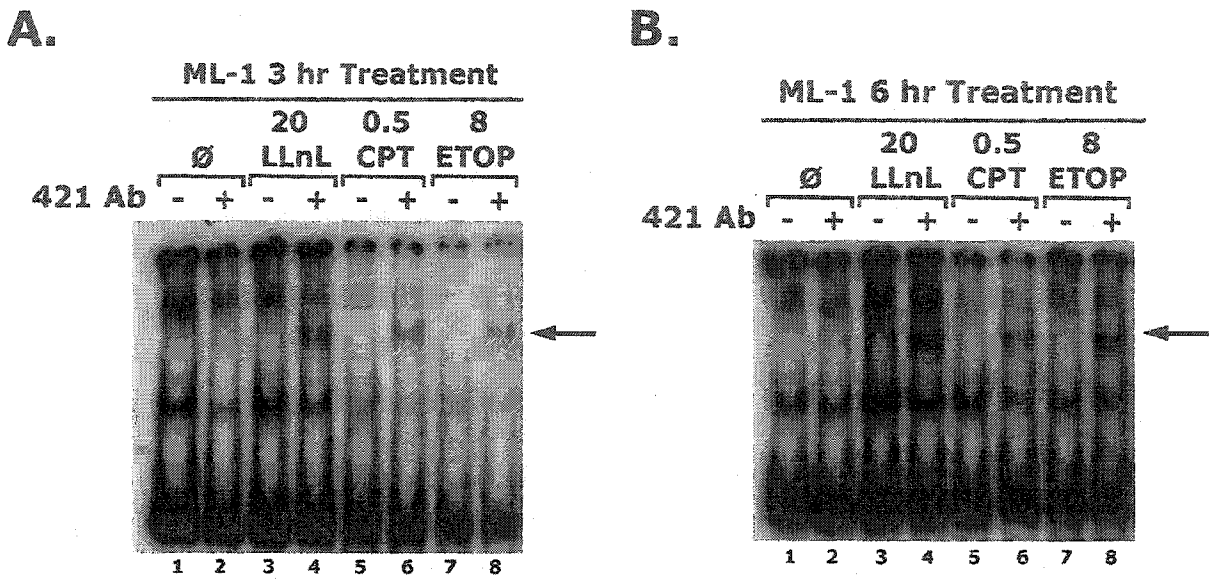

Figure 3.7 cont.

The differential regulation of p53 target genes was not due to differences in the ability of p53 to localize to chromatin.

Chromatin immunoprecipitations carried out in the D-A2 cell line showed that the high levels of p53 expressed upon doxycycline removal bound to their response elements on both target genes examined in this study. While the levels of induced p53 associated with chromatin remain unchanged, oscillations in the association of Mdm2 with this chromatin bound p53 were evident during the p53 response. Similar chromatin immunoprecipitation experiments were carried out on drug treated ML-1 cells in order to determine whether oscillations in Mdm2-p53 association also occurred in cells with a natural p53 response. Although drug treatment did not affect the ability of high levels of p53 to associate with chromatin, we first addressed whether the drug-induced p53 in ML-

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1 cells differentially associated with its binding sites on the *mdm2* and *waf1* genes. As demonstrated previously with chromatin immunoprecipitations of DLD-1 cells, no detectable DNA containing the p53 binding site from either the *mdm2* or *waf1* gene was immunoprecipitated from the K562 cell line using p53-specific antibodies (Figure 3.8A & B). In ML-1 cells, antibodies specific for p53, but not p53 phosphorylated at serine 15, immunoprecipitated low levels of DNA from both the *mdm2* and *waf1* genes prior to drug treatment (Figure 4.8). Following treatment with LLnL, greater quantities of DNA from both target genes were immunoprecipitated with the p53-specific antibody AB-6 (Figure 3.8C through G, top two panels). Unexpectedly, low levels of DNA from the *mdm2* p53 binding site, but not from the *waf1* gene, were also immunoprecipitated using a phosphoserine-15 p53 antibody after 3 hrs of LLnL treatment (data not shown). These data confirm our earlier data in the D-A2 cell line as well as other studies that demonstrated that high levels of p53 protein were capable of binding to p53 responsive elements in the absence of stress (Figure 3.2) (An et al., 2004; Kaeser and Iggo, 2002). In ML-1 cells treated with CPT and ETOP, decreases in the amount of *mdm2* and *waf1* DNA immunoprecipitated using the p53-specific antibody AB-6 were complemented by increases in the amount of detectable DNA immunoprecipitated with phosphoserine-15 p53 specific antibodies. These data suggest that the phosphorylation of p53 at serine 15 may recruit in factors at these time points that mask the AB-6 epitope (Figure 3.8B, C, F, and G, top two panels). No detectable levels of DNA were found to interact with the beads when p53 was present. The lowest panel from each ML-1 ChIP represents one set of INPUT samples. Similar levels of amplicons were obtained from each INPUT portion removed from the samples described previously. These data indicate that p53 was bound

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to its responsive element on both *mdm2* and *waf1* in ML-1 cells prior to treatment with these agents. Following treatment, higher levels of p53 become detectable on these responsive elements, regardless of the amount of transcription evident from these target genes. These data support an earlier study which suggested that the presence of p53 on its responsive element did not predict the level of transcriptional activity from that locus (Szak et al., 2001).

The association of Mdm2 with chromatin requires p53.

The Mdm2 specific antibodies D-7 and 4B2 were used to determine if Mdm2 protein localized on p53 responsive elements in drug treated ML-1 cells. No detectable DNA containing the p53 responsive element from *mdm2* or *waf1* was immunoprecipitated from K562 cells which lack p53 using Mdm2-specific antibodies (Figure 3.8A and B, lanes 4 and 5). These data suggest that Mdm2 cannot bind to chromatin without p53 also present. In contrast to the data obtained in the D-A2 cell line, the quantity of DNA from the *waf1* gene immunoprecipitated with Mdm2-specific antibodies was very low and difficult to detect above background. DNA from the *mdm2* gene, however, was detectable using this method. Interestingly, significant amounts of DNA containing the *mdm2* p53 responsive element was immunoprecipitated from ML-1 cells treated with ETOP for 1 and 6 hrs, but not at the 3 hr time-point, using Mdm2-specific antibodies (Figure 3.8E, D-7 and 4B2). These data resemble the oscillations in Mdm2-p53 association noted on chromatin from the D-A2 cell line. It is interesting to note that the oscillations in Mdm2 localization on chromatin occurred in ETOP treated cells where the induced a form of p53 exhibited the most transcriptional activity. Sustained levels of

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DNA containing the *mdm2* p53 binding site were immunoprecipitated with Mdm2-specific antibodies from cells treated with LLnL from 1 to 3 hrs (Figure 3.8C, D-7 and 4B2). These samples exhibited similar quantities of chromatin-bound p53, but only modest levels of p53 transcriptional activity (Figure 3.6C). Taken together, these data suggest that the sustained presence of Mdm2 protein with p53 on p53 responsive elements may decrease the amount of transcription evident from these loci. Interestingly, CPT treatment also exhibited sustained quantities of DNA containing the p53-responsive element on *mdm2* immunoprecipitated with these Mdm2-specific antibodies (Figure 3.8D, D-7 and 4B2). Like the LLnL treated samples, these data also correlated with low levels of p53-dependent transcription of *mdm2* (Figure 3.6C). It is unclear whether Mdm2 localization in CPT-treated ML-1 cells contributed to this lack of *mdm2* transcriptional activity. No Mdm2 was detected on the p53-responsive element from the *mdm2* gene in D-A2 cells when CPT-mediated repression of p53-dependent transcription was evident.

Figure 3.8 p53 co-localizes with Mdm2 on the *mdm2* promoter. *Chromatin immunoprecipitation was performed on formaldehyde crosslinked DNA isolated from the p53 null K562 cell line for mdm2 (A) and waf1/cip1 (B) from ChIP using antibodies specific for p53 (lane 2, upper panel), phosphoserine-15 p53 (lane 3, upper panel), and Mdm2 (lanes 4 and 5, upper panel). As a negative control, beads without antibody were also used (A and B, lane 1, upper panel). As an additional control, PCR was carried out from DNA isolated by a Bcl-XL ChIP of ML-1 cells treated for 1 hr with 8 uM ETOP (A and B, lane 6). One fifth of the K562 and ML-1 input DNA from each sample was also amplified using the same primers for mdm2 (F, lower panel) or waf1/cip1 (J, lower panel)*

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promoters for 34 and 31 cycles, respectively. PCR was also carried out on DNA isolated from ML-1 cells grown in the absence (lane 1) or presence of 20 uM LLnL (C and F), 0.5 uM CPT (D and G), or 8 uM ETOP (E and H) for 1, 3 and 6 hrs using antibodies specific for p53 (upper panel), phosphoserine-15 p53 (second highest panel), the Mdm2-specific antibodies D-7 (third highest panel), 4B2 (fourth highest panel) or no antibodies and beads alone (second lowest panel). PCR was then performed on the resulting DNA using mdm2 (C, D, and E) or waf1/cip1 (F, G, and H) specific primers from 34 to 30 cycles depending on the sample set. One fifth of the ML-1 input DNA from each sample was also amplified using the same primers for mdm2 (C, D, and E, lowest panel) or waf1/cip1 (G, H, and I, lowest panel) promoters. Only one set of input samples are presented in this figure. Similar levels of amplification were obtained from all input samples examined (data not shown).

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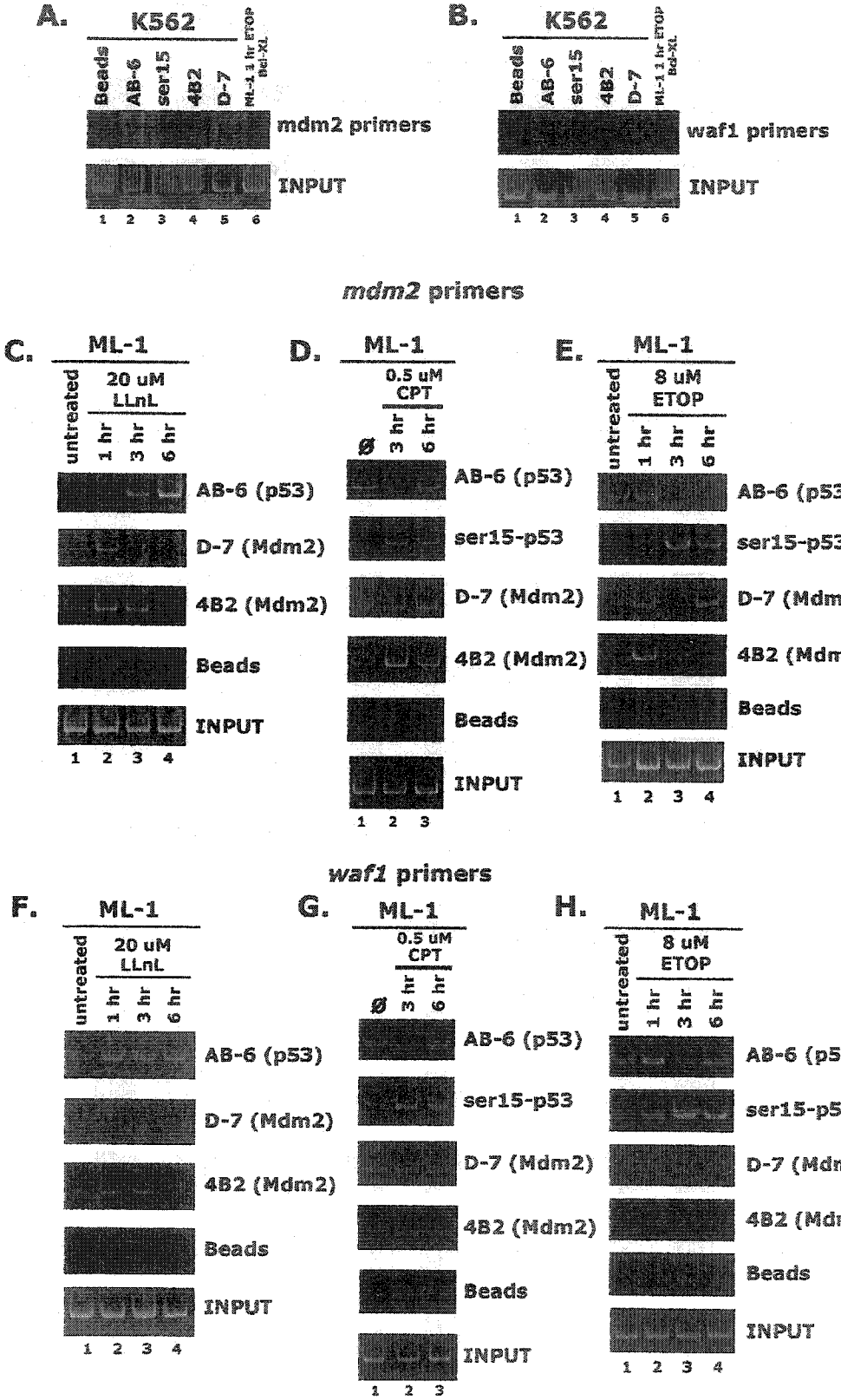

Figure 3.8 cont.

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3.3 Discussion

The interaction of p53 with chromatin has been demonstrated by both ChIP and *in vivo* footprinting (Chin et al., 1997; Espinosa et al., 2003; Szak et al., 2001; Xiao et al., 2000; Xiao et al., 1998). While this p53-chromatin interaction has been determined and an association between the proteins p53 and Mdm2 has been well described (Haines et al., 1994; Moll and Petrenko, 2003), the co-localization of these two proteins on chromatin required investigation. In order to determine if Mdm2 was able to co-localize with p53 on chromatin we examined two p53 responsive genes (*mdm2* and *waf1/cip1*) in four different cell lines under many different conditions. What was striking in our study was that Mdm2 only localized to chromatin if p53 was also associated with chromatin. Initially, this result was perplexing because Mdm2 co-localized with p53 on the promoters of genes that exhibited active transcription. Current evidence suggests that Mdm2 inhibits the ability of p53 to promote transcription (Barak et al., 1993; Oliner et al., 1993; Thut et al., 1997). We expected to see transcriptional repression from this complex. When we examined the kinetics of Mdm2-p53 chromatin association, we found that Mdm2 dissociated from the chromatin as early as 30 minutes following gene induction and then re-localized later. Our data suggests that Mdm2 can localize with p53 at the enhancer elements prior to the activation of transcription. Following the increase in p53 protein, we found that Mdm2 briefly disassociated from the chromatin and then returned at later time-points perhaps reforming an inhibitory complex. We observed similar phenomena on the p53 target genes in ML-1 cells and notably after etoposide treatment, which corresponded to the greatest gene activation and the least amount of Mdm2 protein was found to co-localize on the chromatin. This suggests that less Mdm2

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associated with the chromatin corresponds to greater gene activation. Our data suggests that this brief disassociation of Mdm2 from p53 may relieve the repression inherent at these loci, and thus facilitate p53-mediated transcriptional activation.

We were only able to find Mdm2 associated with chromatin when p53 was present. No Mdm2 was detected on the p53 responsive element on either *mdm2* or *waf1* in DLD1 or K562 cells, which lack wild-type p53. Recent work by Gu and colleagues (Gu et al., 2002) showed that Mdm2 can promote transcription from the *NFkappaB/p65* gene by associating with Sp1 binding sites. The *mdm2* primers used in this study amplified a 400 bp region of the *mdm2* P2 promoter containing not only the p53 responsive element but a number of Sp1 binding sites. Even with these sites present, we did not detect any Mdm2 binding in the absence of p53. Therefore if Mdm2 can interact with Sp1 binding sites, it requires p53 on the chromatin.

The co-localization of Mdm2 with p53 on the p53 binding sites on mdm2 and waf1 genes correlated with a decrease in the level of transcription evident from these loci. Yu and colleagues observed significant decreases in the level of *waf1* transcript in D-A2 cells after 6 hrs of p53 induction (Yu et al., 1999). In our study, we found that the level of *mdm2* and *waf1* transcript decreases in D-A2 cells 9 hrs after doxycycline removal. This decrease in p53-dependent transcription was preceded by an association of Mdm2 with the chromatin on the *mdm2* and *waf1* (5') genes. Mdm2 localization on *waf1* and *mdm2* was also observed in ML-1 cells following the induction of p53 by LLnL, CPT, but far less after ETOP treatment. In the case of ML-1 cells treated with ETOP, Mdm2

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re-association with p53 on the chromatin of the mdm2 promoter occurred at 6 hrs and was followed by slightly decreased mdm2 transcript levels. Taken together, these data suggest that the association of Mdm2 with p53 on chromatin represses p53-dependent transcription from loci after an initial period of activation.

Mdm2 association with the p53 protein has been shown to repress p53 transcriptional activity (Haines et al., 1994; Leng et al., 1995; Thut et al., 1997). LLnL and CPT treatment of ML-1 cells induced a form of p53 that exhibited specific DNA binding activity in vitro and in vivo. The p53 induced by these agonists, however, was not transcriptionally active. Using chromatin immunoprecipitation, Mdm2 was found to co-localize with p53 up to 3 hrs following LLnL treatment, and 6 hrs following CPT treatment. In the ML-1 cells treated with ETOP, where the resulting p53 induced exhibited high levels of transcriptional activity, no Mdm2 protein was observed in conjunction with p53 on chromatin after one hour. The early dissociation of Mdm2 from p53 may be a critical event in promoting the assembly of a transcriptional complex that exhibits the highest level of activity.

Although many reports have stated that such phosphorylation interrupts the interaction of p53 with Mdm2 (Chehab et al., 1999; Shieh et al., 1997), others have argued that this is not always the case (Blattner et al., 1999; Dumaz and Meek, 1999). The possibility exists that some p53 molecules in the tetrameric p53 are phosphorylated, while others are not. In addition, Mdm2 might then interact with the non-phosphorylated molecules of the tetramer. In our model depicted in Figure 3.9, we propose that some of

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the four molecules of tetrameric p53 bound to DNA were associated with Mdm2 protein prior to (Figure 3.9, panel 1) and following activation (Figure 3.9, panel 3). The transcriptional repression mediated by Mdm2 is relieved by the phosphorylation of p53 during activation (Figure 3.9, panel 2). Furthermore, Mdm2 cannot associate with the region encompassing the p53 responsive element without p53 bound to this site (Figure 3.9, bottom panel).

In our present study, we observed yet another facet of the intriguing interplay between p53 and its antagonistic inhibitor Mdm2. Whether Mdm2 serves to inhibit the transcriptional activity of p53 on chromatin, or target p53 for ubiquitination on DNA as well, has yet to be determined. The current dogma suggests that Mdm2 inhibits p53 function by multiple mechanisms, utilizing ubiquitination and transcriptional repression. Recent studies using ChIP by Szak and colleagues showed that acetylated p53 can be detected on p53 responsive elements following DNA damage (Szak et al., 2001). In addition, acetylation of p53 at its C terminal lysine residues following DNA damage has been shown to stabilize p53 by preventing ubiquitination by Mdm2 at these sites (Li et al., 2002; Luo et al., 2004; Nakamura et al., 2000). In order for chromatin bound p53 to be ubiquitinated by Mdm2, deacetylases would have to be recruited to these enhancer elements. The histone deacetylases HDAC1 and Sir2alpha have been shown to associate with p53 and repress transcription (Ito et al., 2002; Murphy et al., 1999; Smith, 2002). Additionally, HDAC1 has recently been found to associate with Mdm2 (Ito et al., 2002). It would be interesting to examine whether Mdm2 and HDAC1 co-localize with p53 on the p53 binding sites, and if the presence of this complex (if it exists on chromatin)

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correlates with decreased levels of acetylated p53 as well as lower levels of p53-dependent transcription. These data may then begin to shed useful insight into the kinetics of p53 ubiquitination and transcriptional repression by Mdm2, and whether these events occur on both "free-" and "chromatin-bound" p53 populations in the nucleus.

Figure 3.9 A model depicting the regulation of p53 transcriptional activity by Mdm2 on chromatin. *A summation of our hypothesis is depicted in a model where Mdm2 co-localizes with p53 at the p53 responsive element prior to transcription, dissociates from p53 following activation, and then reforms its inhibitory complex at a later time-point. In addition, our data suggests that Mdm2 protein cannot bind to the region encompassing the p53-responsive element without p53 present (lowest panel).*

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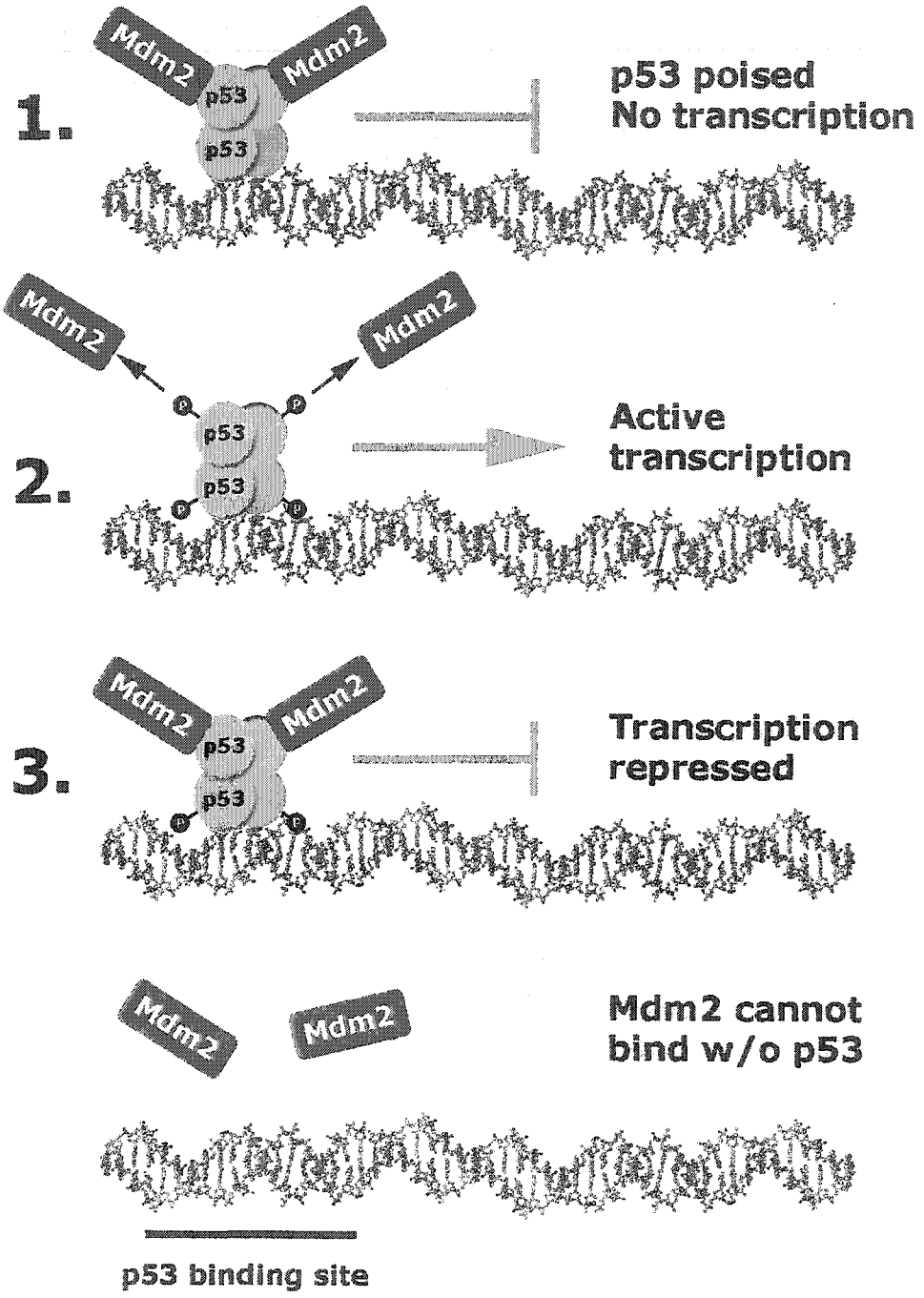

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Chapter 4. The activation of growth arrest or apoptotic pathways is not determined by the level of induced p53 protein or p53 target gene expression.

4.1 Introduction

Although it is clear that the p53 tumor suppressor protein protects against cellular transformation by mediating growth arrest and apoptotic pathways, it is unclear how p53 dictates a cell's fate (Oren, 1994). In response to numerous types of cellular stress, p53 protein becomes stabilized (Colman et al., 2000; Meek, 2004) and activates downstream target genes that promote either growth arrest or apoptosis (Colman et al., 2000). Numerous lines of evidence suggest that p53 may dictate the growth arrest and apoptotic pathways by differentially regulating its downstream target genes. Association of p53 with ASPP has been shown to be required for p53 to mediate apoptosis by promoting p53's ability to transcriptionally activate apoptotic target genes (Samuels-Lev et al., 2001). Additionally, clear differences in the components of p53-driven transcriptional initiation complexes have been found on the growth arrest promoting gene *waf1/cip1* and the apoptotic target gene *fas/apo1* (Espinosa et al., 2003). Other studies suggest that the fate of the cell is determined by the level of p53 protein stabilized following stress. In accordance with this hypothesis, it has been shown in numerous studies that lower levels of p53 protein promote growth arrest, while higher levels of p53 induce apoptosis (Chen et al., 1996; Inga et al., 2002; Zhao et al., 2000). Another factor that may contribute to the ability of p53 to promote apoptosis or growth arrest may be the underlying cellular context. Some cells have been shown to be predisposed to undergo growth arrest in

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response to stress, while other cells treated with the same agent may induce apoptosis (Oren, 1994; Polyak et al., 1996).

In this study we charectorized the p53 response in cells expressing similar levels of p53 protein induced by the topoisomerase poisons, camptothecin (CPT) and etoposide (ETOP), which promoted p53-dependent apoptosis, or the bleomycin analog, Zeocin (ZEO), which induced growth arrest in the myeloid leukemia cell line ML-1. Our goal was to determine if p53 directed the cell fate by differentially regulating its downstream target genes in cells undergoing growth arrest or apoptosis. By focusing on the regulation of p53 in one cell line, we excluded the affects of any intercellular variables that might arise from differences in cellular context. Additionally, by utilizing different drugs that induce similar levels of p53 protein, but promote different cellular outcomes, we exclude any variability that may occur because of differences in the p53 protein level. Surprisingly, there was no correlation between the level of growth arrest promoting or apoptotic target genes induced by each drug and the p53-dependent pathway that the drug promoted. These data suggest that the cell's fate in response to different drugs is not determined by the specific pattern of genes up-regulated by p53. To further confirm that the differential regulation of target genes by p53 did not play a role in directing cell fate, we treated ML-1 cells with increasing concentrations of ZEO to shift the cells from a growth arrest to apoptotic cellular outcome. Interestingly, the shift from growth arrest to apoptosis in response to higher concentrations of ZEO did not occur as a result of enhanced apoptotic gene expression but an overall increase in the p53 response. These data suggest that p53 does not direct the fate of the cell by differentially regulating the

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downstream target genes. These data also suggest that the induction of p53 and p53 target genes in not always sufficient to promote apoptosis. Furthermore, the level of p53 protein and target gene expression that is required to shift a cell from a growth arrested to an apoptotic cell fate differs according to the drug.

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4.2 Results

ETOP, CPT, and ZEO treatment stabilize similar levels of p53.

In order to address whether different types of damage promote alternate cellular outcomes through the differential regulation of p53 target genes, we first needed to confirm that similar levels of p53 protein were being induced by CPT, ETOP, and ZEO. Next, we needed to confirm that these similar levels of p53 were directing different cell fates, ie. growth arrest or apoptosis. By having similar levels of p53 induced by these agents, we exclude any variability that might occur in the regulation of the target genes which could be attributed to higher or lower levels of p53. The myeloid leukemia cell line, ML-1 has a well documented wild-type p53 response (Kastan et al., 1991; Nair et al., 2000; Zhan et al., 1994b). Similar levels of p53 were induced in ML-1 cells grown in the presence of 0.5 uM CPT, 8 uM ETOP, and 50 ug/ml ZEO for 3 and 6 hrs (Figure 4.1A). To determine if these DNA damaging agents were signaling to p53 in a similar manner, we analyzed the phosphorylation status of p53 at serine 15. Previous studies have shown that the phosphorylation of p53 at this site is important in the activation of p53 following damage (Bean and Stark, 2001; Shieh et al., 1997). Similar levels of serine-15 phosphorylation on p53 were observed in response to all of the agents used in this study, although the level of phosphorylated p53 in response to ZEO was slightly less (Figure 4.1B). These data suggest that all three drugs were capable of eliciting a DNA damage response and that some elements of these pathways were signaling to p53. The p53 null erythromyeloid leukemia cell line K562 was used as a negative control. As expected, p53 protein was not detected in extracts from this cell line in the presence or absence of drugs (Figures 4.1A & B, lanes 1 and 2).

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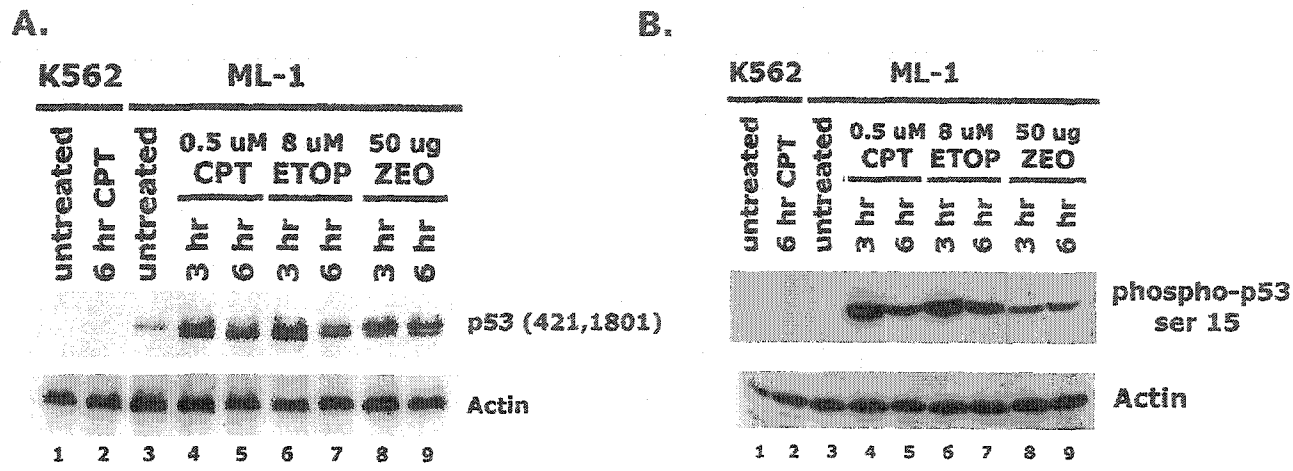

Figure 4.1 ETOP, CPT, and ZEO treatment stabilize similar levels of p53 protein, and promote the phosphorylation of p53 at serine 15. *100 ug of nuclear extract from ML-1 or K562 cells grown in the presence or absence for 3 and 6 hrs with 0.5 uM CPT, 8 uM ETOP, or 50 ug/ml ZEO were electrophoresed on a 10% SDS-PAGE. The resulting gel was electrotransferred to nitrocellulose and this membrane was immunoblotted with a mixture of 421 and 1801 antibody (A, top panel), phosphoserine-15 p53 specific antibody (B, top panel), or anti-actin (A & B, lower panel).*

Similar levels of p53 protein induced by different drugs promote different cellular outcomes.

The ability of these drugs to induce growth arrest or apoptosis at these chosen dosages was then confirmed by multiple methods. CPT and ETOP have been shown to induce apoptosis in numerous cell lines (Abbas et al., 2002; Han et al., 2002; Kaneko and Tsukamoto, 1995). CPT and ETOP, but not ZEO, treatment promoted the cleavage of PARP-1 in ML-1 cells (Figure 4.2A). Additionally, no activation of caspase-8 or 9 was observed in the ML-1 cells treated with 50 ug/ml ZEO, while ETOP and CPT treated

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exhibited activation of these apoptotic initiators (Figure 4.2C). No PARP-1 cleavage was evident in drug treated K562 cells, even after 24 hrs of treatment (Figure 4.2B). These data suggest that CPT and ETOP treatment induced p53-dependent apoptosis, while ZEO at this concentration did not promote cell death in either cell line. To determine if ML-1 cells were undergoing growth arrest in response to Zeocin treatment, FACS analysis was used to examine the cell cycle profile of ML-1 and K562 cells grown in the absence or presence of CPT, ETOP, or ZEO for 24 hrs. In accordance with previous results obtained in our lab (Abbas et al., 2002; Houser et al., 2001), CPT treatment of ML-1 cells exhibited an increase in sub-G1 populations. ETOP treatment also exhibited a significant increase in the sub-G1 population in this cell line. While treatment of ML-1 cells with ZEO did not exhibit any notable increase in the sub-G1 populous, FACS analysis displayed significant enrichment in G1 and G2/M subpopulations compared to the control (Figure 4.2D). These data indicate that CPT and ETOP treatment induce apoptosis in ML-1 cells, while ZEO treatment induces growth arrest. K562 cells did not exhibit sub-G1 populations in response to CPT, ETOP, or ZEO after 24 hrs. ETOP and ZEO treatment, however, displayed enrichment in the G2/M population as well as a decrease in the G1 population (Figure 4.2E), which may be p53-independent G2 arrest. In order to confirm that these enhanced cell cycle subpopulations were indeed caused by growth arrest in response to Zeocin treatment, growth curves were carried out on drug treated ML-1 and K562 cells. In both ML-1 and K562 cell lines, drug treatment drastically decreased the growth rate of the cell population over the 24 hr treatment period (Figure 4.2F and G). Although the K562 cells treated with CPT did not exhibit an abnormal cell cycle profile as determined by FACS analysis, it is clear that the cell growth was stunted

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by treatment with this agent (Figure 4.2G). Taken together, these data confirm that CPT, ETOP, and ZEO differentially affect the ability of similar levels of p53 to regulate downstream effector pathways. CPT and ETOP promoted p53-dependent apoptosis, while low doses of ZEO induced growth arrest.

Figure 4.2 Similar levels of p53 protein induced by different drugs promote different cellular outcomes. *Nuclear extract isolated from ML-1 and K562 cells treated with CPT, ETOP, or ZEO for 3, 6, and 24 hrs was electrophoresed on a SDS-PAGE and then transferred to nitrocellulose. The resulting membrane was immunoblotted for PARP-1 (A & B, upper panel). The level of actin was also determined as a loading control (A & B, lower panels). Cytoplasmic extracts were also isolated from ML-1 cells treated with CPT, ETOP, or ZEO for 3 and 6 hrs, electrophoresed on a SDS-PAGE, and then transferred onto nitrocellulose. The resulting membrane was immunoblotted for procaspase-9 (C, top panel), procaspase-8 (C, middle panel), and actin (C, lower panel). The cell cycle profile of ML-1 (D) and K562 (E) cells grown in the absence or presence of CPT, ETOP, or ZEO for 24 hrs was determined by FACS analysis. A profile of the cell growth over the 24 hr period of treatment was also determined by counting these samples after 4, 7, 10, and 24 hrs of treatment using a hemacytometer (F & G). The data is representative of three independent experiments.*

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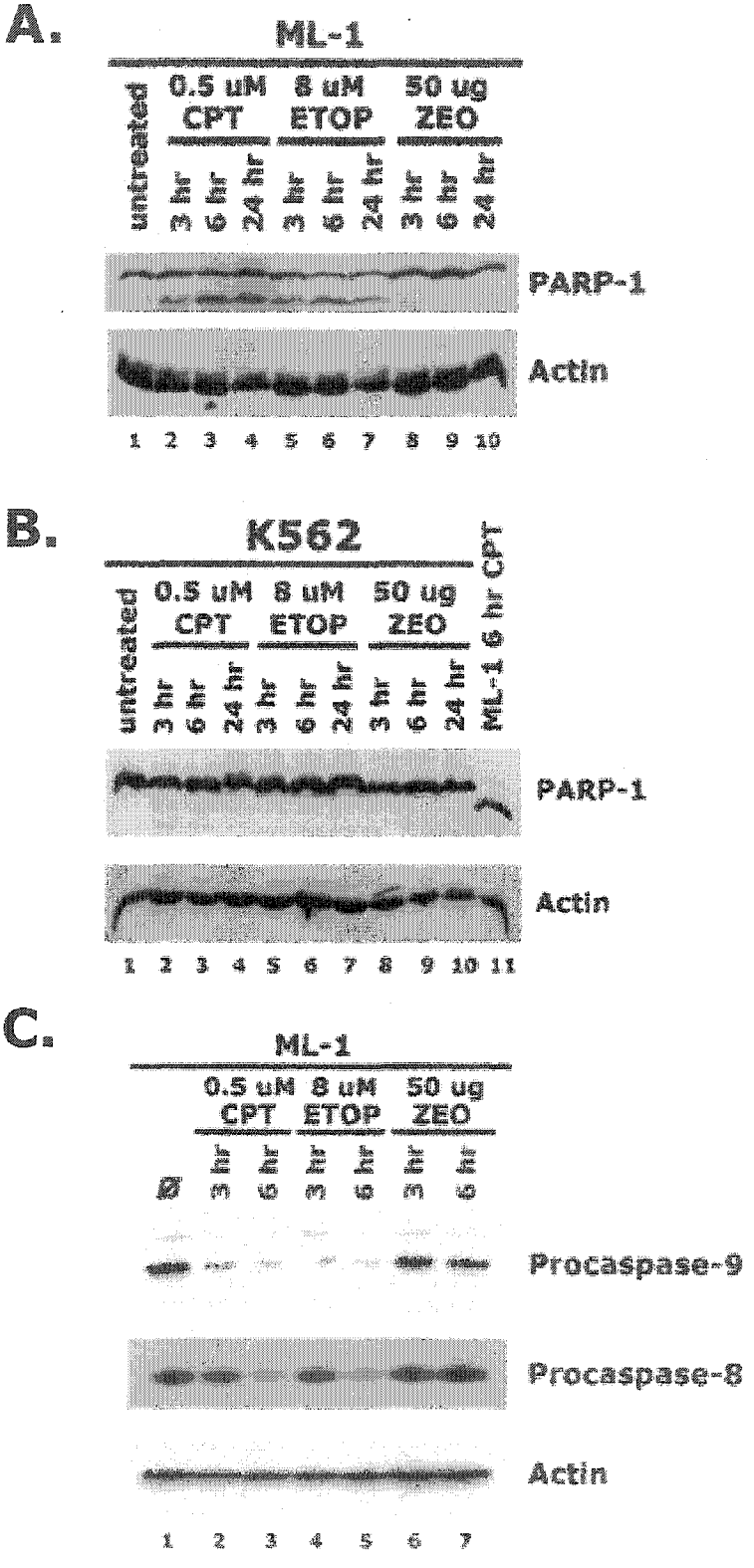

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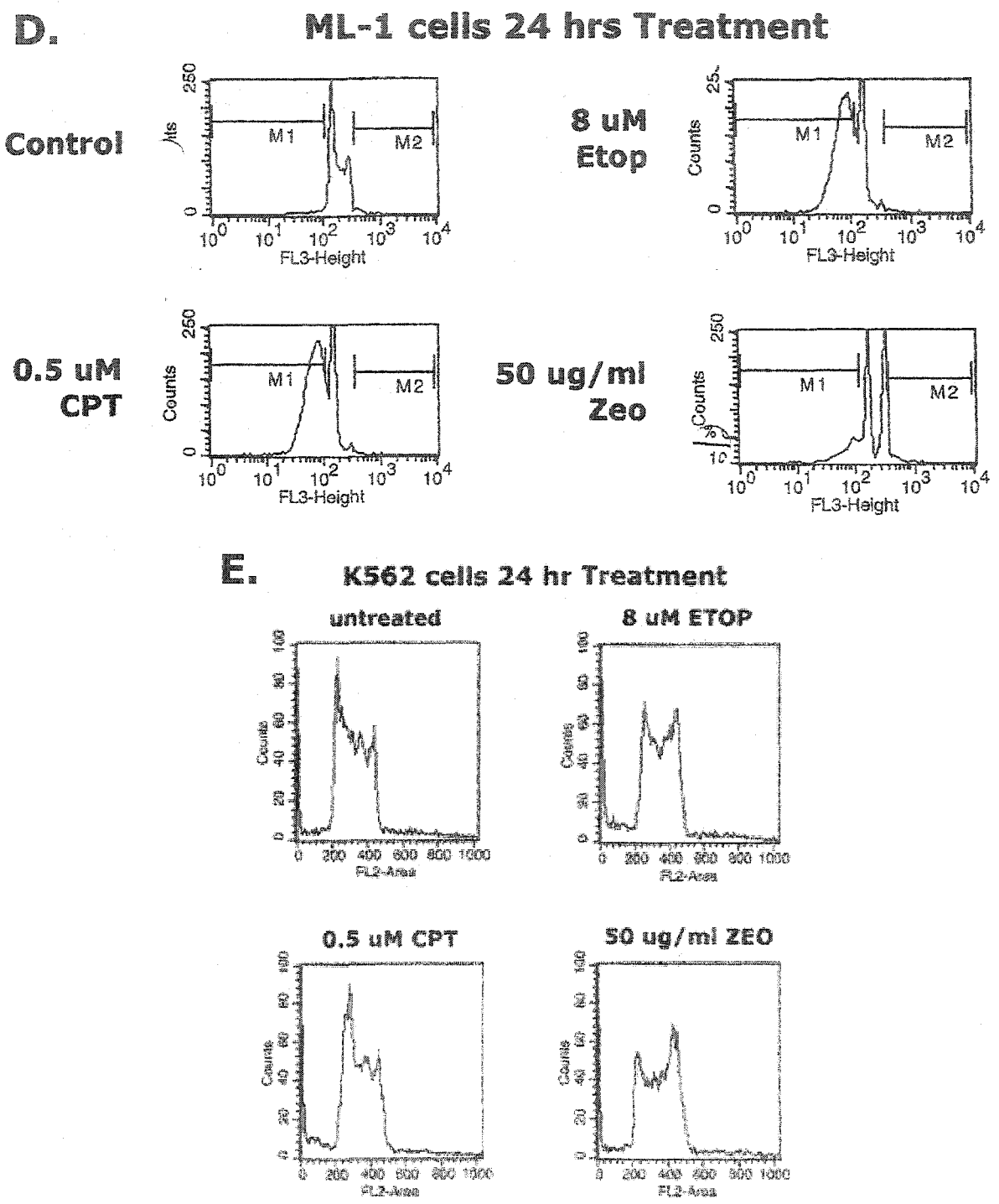

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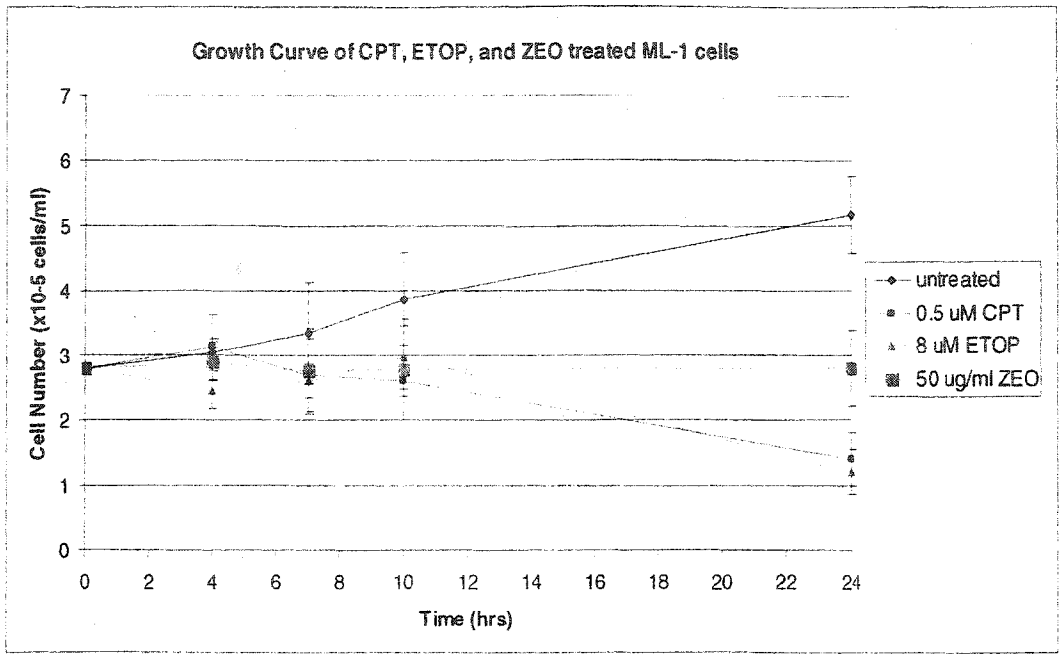

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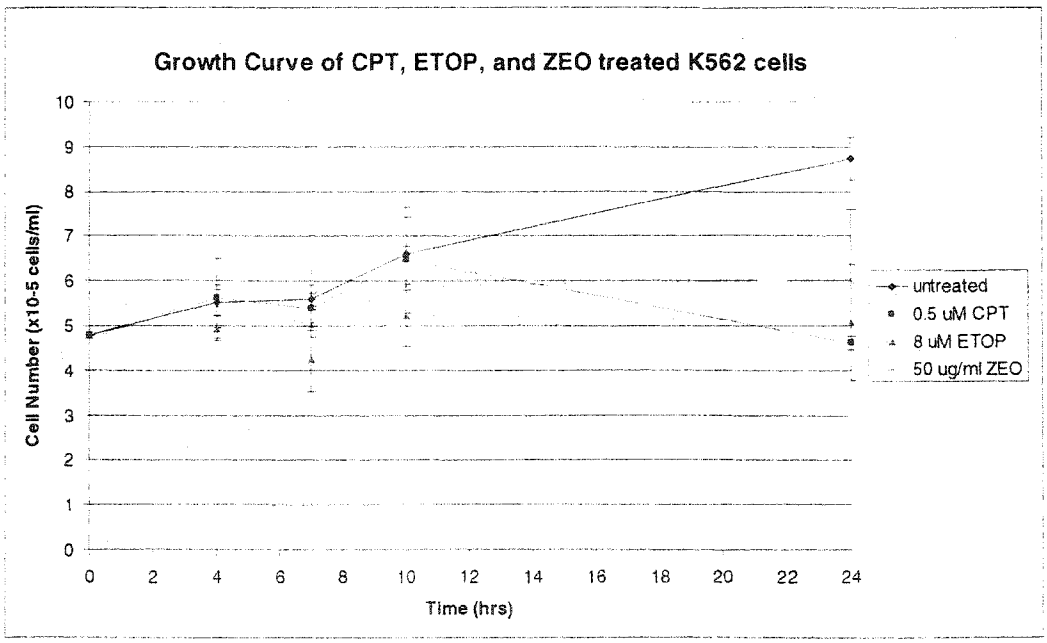

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The differential regulation of p53 target genes in response to CPT, ETOP, and ZEO does not correlate with the cell fate induced by these drugs.

We next sought to address whether these similarly induced levels of p53 protein provoke growth arrest verses apoptosis by differentially activating p53 target genes known to mediate these pathways. Quantitative PCR was carried out on cDNA synthesized from total RNA isolated from drug treated ML-1 and K562 cells. Surprisingly, p53 induced by low doses of ZEO transcriptionally activated all p53 target genes tested as effectively as p53 induced by CPT. In fact, ZEO treatment (50 ug/ml) induced similar levels of the p53 apoptotic target genes, *fas/apo1*, *bax*, *pig-3*, and *noxa*, as CPT (Figure 4.3A), but unlike CPT, ZEO treatment did not promote apoptosis (Figure 4.2). Furthermore, ZEO treatment (50 ug/ml) did not activate higher levels of growth arrest-promoting target genes than CPT or ETOP treated samples (Figure 4.3A). p53 stabilized by ETOP treatment was the most transcriptionally active, exhibiting the greatest induction of all of the p53 target genes analyzed in this study (Figure 4.3A). Conversely, p53 responsive genes were not upregulated in drug treated K562 cells (Figure 4.3B) suggesting that the induction of these genes in ML-1 cells treated with these agents was p53-dependent. Taken together, these data suggest that the induction of growth arrest or apoptosis by similar levels of drug induced p53 does not lie in the ability of this activated p53 to differentially regulate downstream target genes.

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Figure 4.3 The differential regulation of p53 target genes in response to CPT, ETOP, and ZEO does not correlate with the cell fate induced by these drugs. *Quantitative PCR was performed on cDNA from RNA isolated from ML-1 (A) and K562 cells (B) grown in the presence or absence of CPT, ETOP or ZEO for 3 and 6 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of mdm2, waf1/cip1, gadd45, fas/apo1, bax, noxa and pig-3 transcripts was calculated over the untreated sample.*

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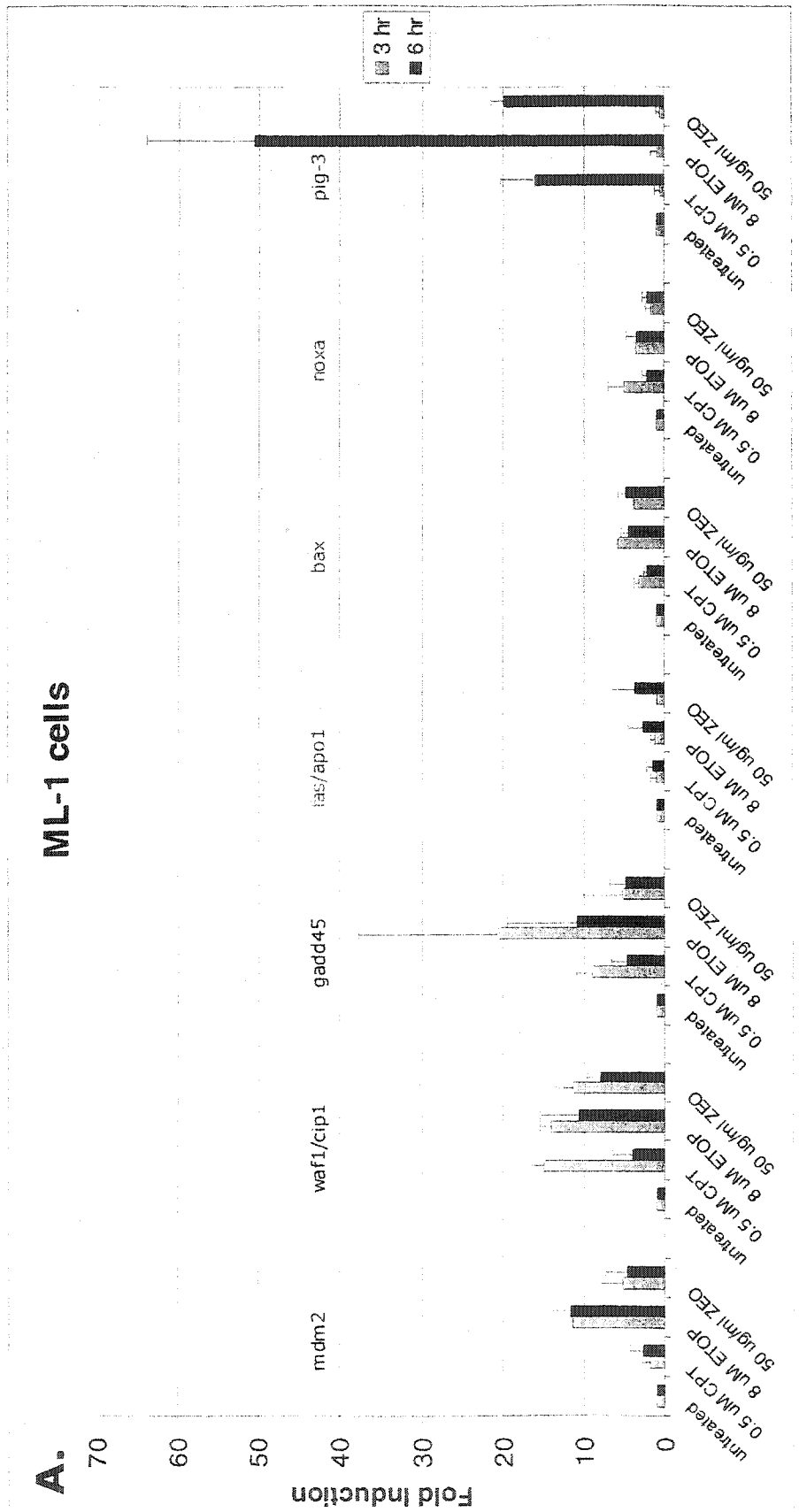

Figure 4.3 cont.

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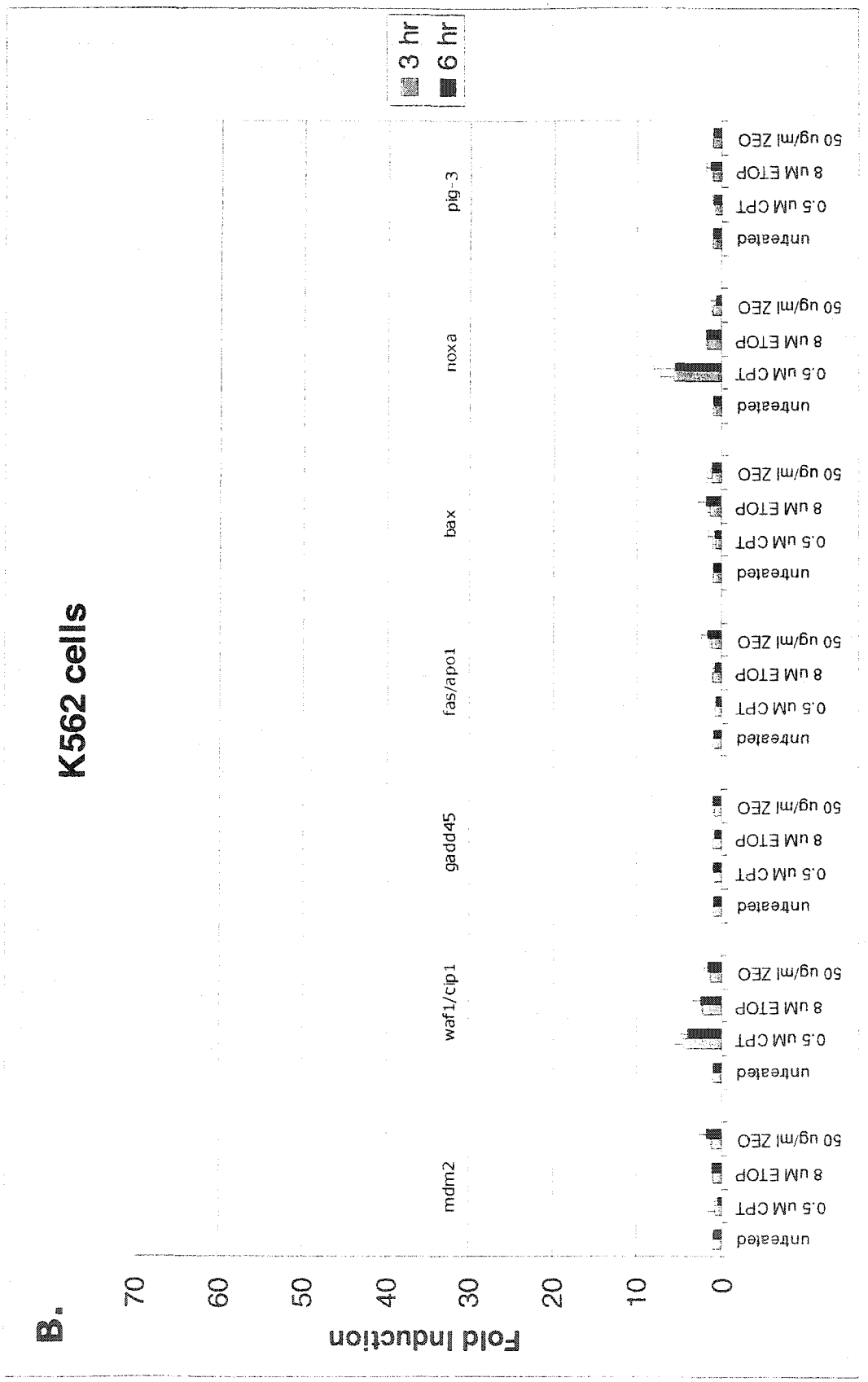

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The shift from growth arrest to apoptosis in response to higher doses of Zeocin correlates with an enhancement in the p53 response.

Since the growth arrest promoted by p53 in response to ZEO treatment exhibited similar levels of apoptotic target genes as the other drugs that induced apoptosis, we next sought to determine if higher doses of ZEO treatment would induce apoptosis by increasing the levels of apoptotic gene expression or by generally enhancing the p53 response. Treatment of ML-1 cells for 6 hrs with increasing doses of ZEO (50 – 600 ug/ml) induced cleavage of PARP-1 and activation of caspases-9, both hallmarks of apoptosis (Figure 4.4A & C). Caspase-8 was only modestly activated by these higher doses of Zeocin (Figure 4.4C). No PARP-1 cleavage was evident in similarly treated K562 cells (Figure 4.4B). These data suggest that ZEO treatment can promote p53-dependent apoptosis at higher dosages (~250 ug/ml). A dose-dependent increase in nuclear p53 protein level was also evident in ZEO treated ML-1 cells. Additionally, these ZEO treated cells exhibited a dose-dependent increase in all p53 target genes examined in this study. Surprisingly, the expression of p53 responsive genes increased overall by 2.5 fold over the lowest dosage of ZEO. In fact, the level of target gene expression at 600 ug/ml of ZEO is greater than what was observed in ETOP treated cells (Figure 4.3A). No significant dose-dependent increase in p53 target genes was observed in K562 cells treated with ZEO (Figure 4.5B-H). Taken together, these data suggest that for ZEO treatment to induce p53-dependent apoptosis, higher levels of p53 protein and p53 target gene expression are required. Lower levels of apoptotic gene expression may only be sufficient for driving p53-dependent apoptosis in response to some genotoxic agents, like CPT, but not others, like ZEO or ETOP. CPT treatment may activate other

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factors that may contribute to lowering the cell's apoptotic threshold and coordinate with p53 to induce cell death. ETOP and ZEO-mediated apoptotic pathways, on the other hand, may rely more heavily on p53 activity.

Figure 4.4 Higher doses of ZEO shift the p53 response from growth arrest to apoptosis. *Nuclear extract isolated from ML-1 and K562 cells treated with increasing concentrations of ZEO for 6 hrs was electrophoresed on a SDS-PAGE and then transferred to nitrocellulose. The resulting membrane was immunoblotted for PARP-1 (A & B, upper panel). The level of actin was also determined as a loading control (A, lower panel). Cytoplasmic extracts were also isolated from ML-1 cells treated with CPT, ETOP, or increasing concentrations of ZEO for 6 hrs, electrophoresed on a SDS-PAGE, and then transferred onto nitrocellulose. The resulting membrane was immunoblotted for procaspase-9 (C, top panel), procaspase-8 (C, middle panel), and actin (C, lower panel).*

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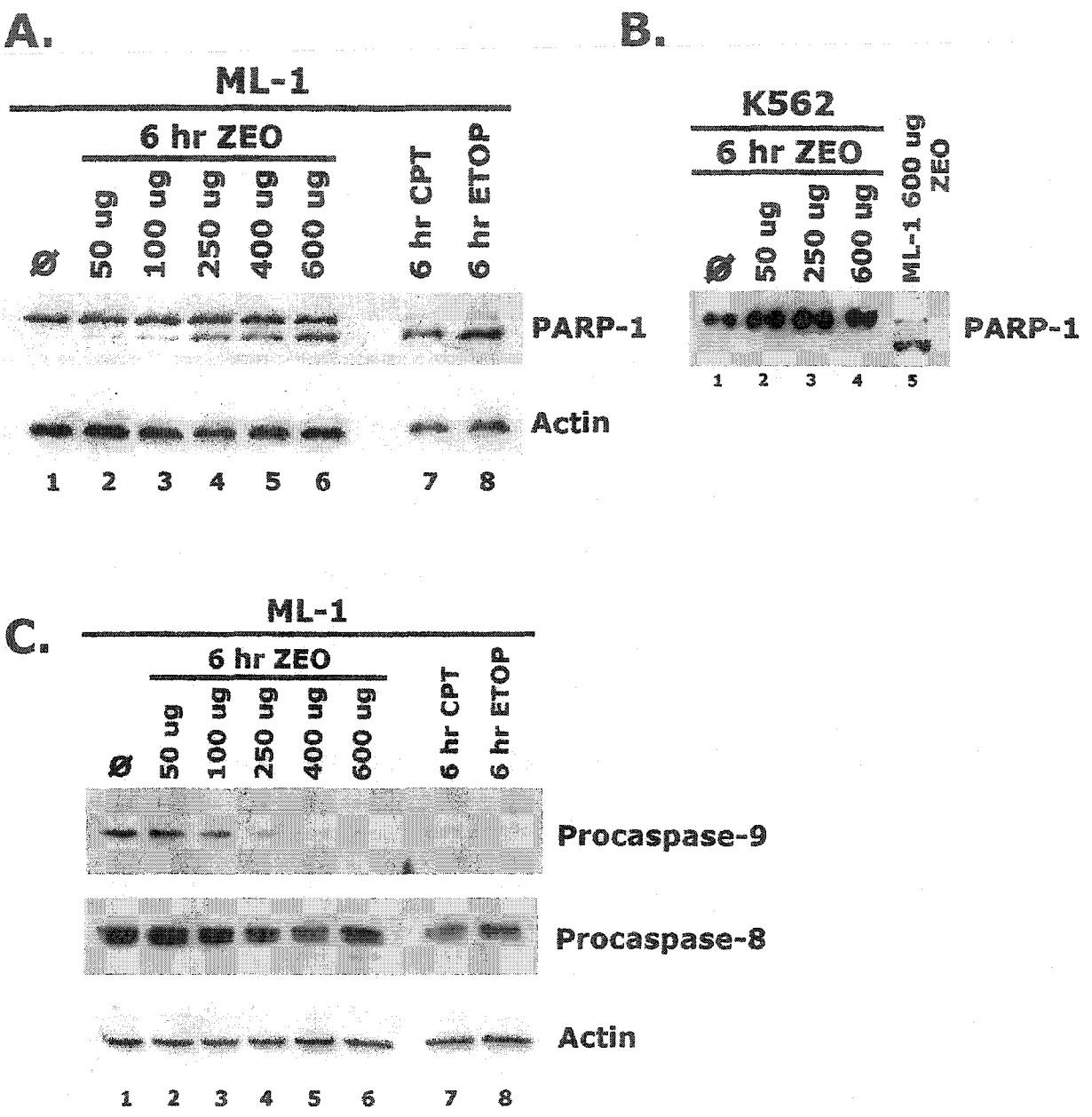

Figure 4.4 cont.

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Figure 4.5 The onset of apoptosis in response to Zeocin coincides with a dose-dependent increase in nuclear p53 and p53-dependent transcription of target genes. *Nuclear extract isolated from ML-1 and K562 cells treated with increasing concentrations of ZEO for 6 hrs was electrophoresed on a SDS-PAGE and then transferred to nitrocellulose. The resulting membrane was immunoblotted for p53 (A, upper panel). The level of actin was also determined as a loading control (A, lower panel). Quantitative PCR was performed on cDNA from RNA isolated from ML-1 (blue diamonds) and K562 (pink squares) cells grown in the presence of increasing concentrations of ZEO for 6 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of mdm2 (B), waf1/cip1 (C), gadd45 (D), fas/apo1 (E), pig-3 (F), bax (G), and noxa (H) transcripts was calculated over the untreated sample.*

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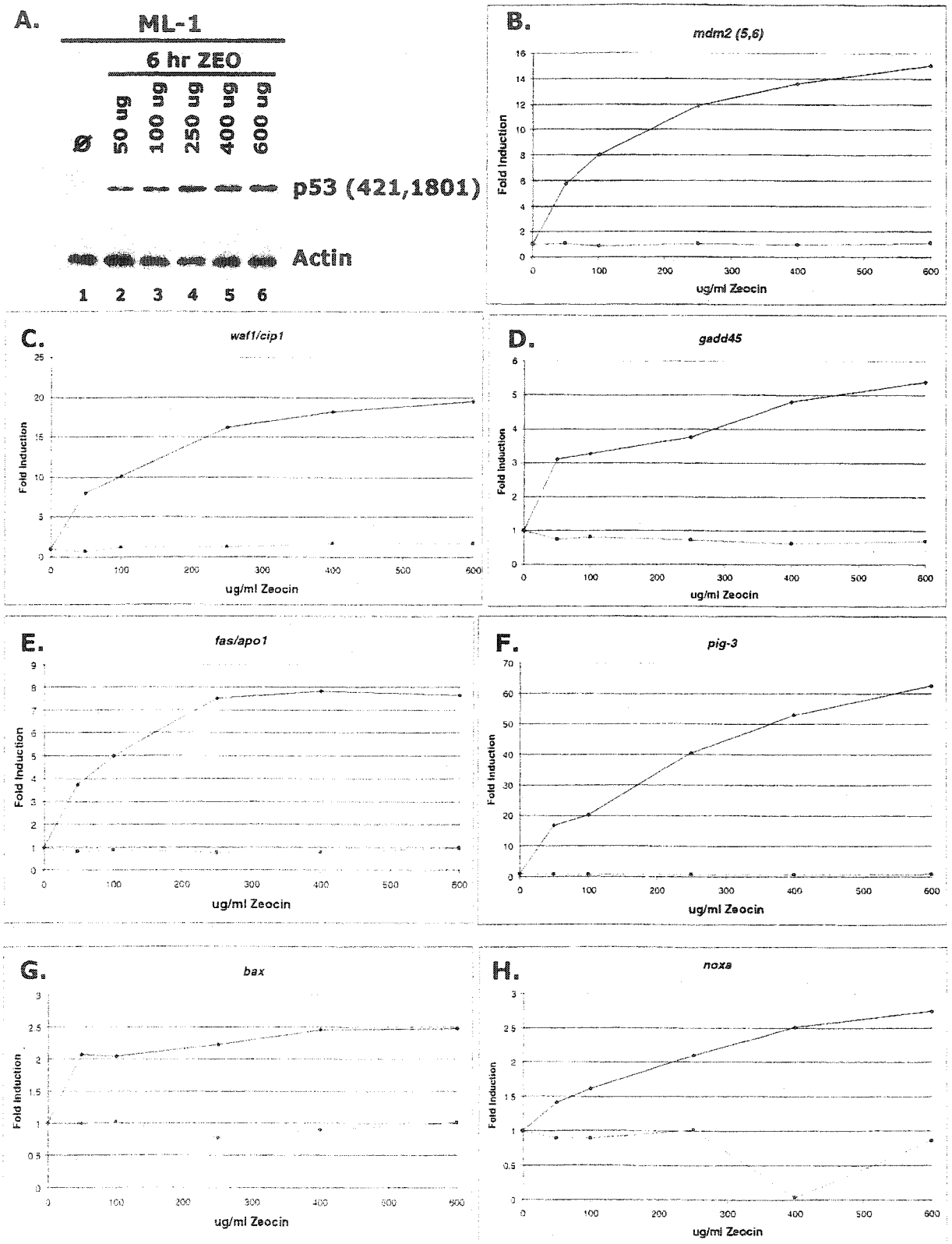

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4.3 Discussion

The mechanisms that enable p53 induced by genotoxic stress to discriminate between growth arrest and apoptotic outcomes remain poorly understood. There are a number of variables that contribute to p53 function once it is activated, including the cellular milieu (Oren, 1994), the type and amount of genotoxic stress exerted on the cell (Arriola et al., 1999; Ashcroft et al., 2000), the duration of exposure (Blagosklonny et al., 2002), and the level of stress-induced p53 protein (Chen et al., 1996; Zhao et al., 2000). In this study, we determined whether p53 promoted growth arrest and apoptosis by differentially regulating its downstream target genes. Similar levels of p53 were stabilized in ML-1 cells treated with ETOP and CPT and ZEO (50 ug/ml), however ETOP and CPT induced apoptosis while ZEO induced growth arrest. In examining the induction of numerous downstream target genes by p53, we found that ETOP and CPT did not promote the up-regulation of solely apoptosis-inducing genes. Furthermore, ZEO treatment (50 ug/ml) did not promote the activation of solely growth arrest-promoting genes. In fact, lower doses of ZEO activated apoptotic genes to a level that was equivalent to CPT treatment. Additionally, ETOP treatment resulted in a more robust activation of growth arrest promoting genes *waf1/cip1* and *gadd45* than ZEO treatment. These data support earlier studies that dispute the "differential regulation" model of apoptosis and cell cycle arrest by p53 (Inga et al., 2002; Robinson et al., 2003). Taken together, these data suggest that high levels of p53 and p53 target gene expression are not always sufficient to promote apoptosis.

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Another hypothesis used to describe p53's ability to direct cell fate predicts that lower levels of p53 protein induce growth arrest while higher levels of p53 promote apoptosis. Our data demonstrated that the level of p53 protein induced in cells undergoing p53-dependent growth arrest or apoptosis can be similar. The amount of stabilized p53 and induced p53 target gene expression required to shift a cell from a growth arrest to an apoptotic cell fate differs according to the drug. When we treated ML-1 cells with higher doses of ZEO (over 250 ug/ml), we found that the shift that occurred from p53-dependent growth arrest to apoptotic pathways correlated with dose-dependently increased levels of p53 protein and increased growth arrest and apoptotic promoting target gene expression. The higher levels of p53 protein and target gene expression correlated with the induction of apoptosis in response to this drug. Although the doses of CPT and ETOP used in this study induced p53-dependent apoptosis, both drugs have also been shown to induce growth arrest in other cell lines that expressed wild type p53 (Jaks et al., 2001; Malcomson et al., 1995). These data suggest that the ability of a DNA damaging agent to promote apoptosis through p53 does not solely rely on p53 level or target gene expression. The shift from growth arrest to apoptosis may rely on the ability of a DNA damaging agent to activate cooperative pathways that contribute to the p53 response.

Although p53 induced by CPT up regulated target genes as effectively as p53 induced by lower doses of ZEO (50 ug/ml), ZEO treatment promoted growth arrest. If apoptosis were solely dependent upon the level of apoptotic target gene expression, then ZEO treatment (at 50 ug/ml) should have induced apoptosis. CPT most likely activated

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additional pathways that contributed to the apoptotic response. CPT and ETOP have been shown to promote apoptosis through the activation of JNK, p38, and ERK pathways (Gibson et al., 1999; Hayakawa et al., 2003; Lee et al., 2002; Seimiya et al., 1997; Tang et al., 2002). Activation of these additional pathways lowers the apoptotic threshold and may enable lower levels of p53 and p53 target gene expression to promote apoptosis. In the model we proposed in Figure 3.6, higher doses of Zeocin (Figure 3.6, upper panel) are required to generate levels of p53 protein (and target gene expression) high enough to cross the apoptotic threshold and shift the p53 response from growth arrest to apoptosis. The apoptotic threshold is dependent in part on the ability of the damaging agent to activate other damage response pathways which converge on p53 (X axis). The stabilization of p53 protein and the induction of p53 target genes are depicted as a linear event. In our model CPT treatment more efficiently activates these damage responsive pathways and as a result the apoptotic threshold is lower. Therefore, the shift towards apoptosis occurs in the presence of lower levels of p53 (Figure 3.6, bottom panel). Although it is not currently known if ZEO treatment activates these stress responders, it is clear that the similar levels of p53 and p53 target genes expression induced by ZEO cannot promote apoptosis in the same manner as CPT. A greater understanding of how these drugs differentially lower the apoptotic threshold would be useful in gauging the effectiveness of these and other agents in clinical settings.

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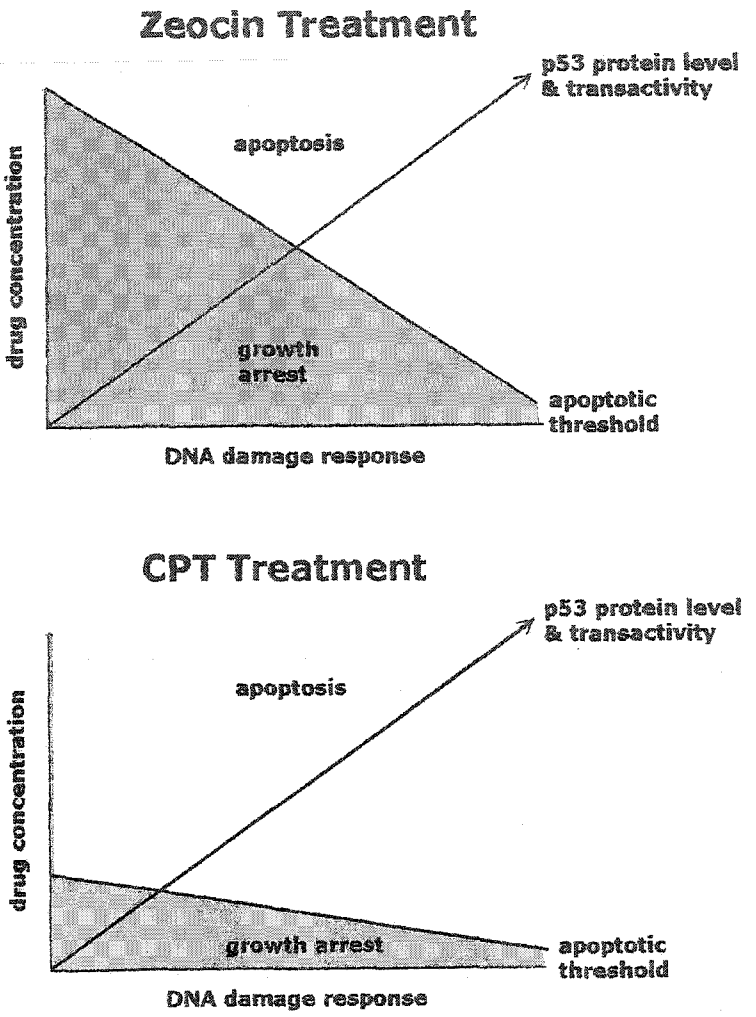

Figure 4.6. Model depicting the effect of drug treatment on apoptotic threshold.

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Chapter 5. 10-decarbamoyl Mitomycin C; a DNA alkylating and cross-linking agent with novel p53-dependent and p53-independent apoptotic activity

5.1 Introduction

One technique that is often used by scientists to improve the potency of a drug is to slightly alter its chemical structure. The alkylating agent mitomycin C (MC) is one of the more commonly used cancer drugs (Kelly et al., 2000; Verweij and Pinedo, 1990). Upon entering the cell, MC is enzymatically reduced into a highly reactive electrophile that targets DNA (Suresh Kumar et al., 1997). MC covalently bonds to guanine residues forming monoadducts as well as intra- and interstrand crosslinks (Palom et al., 1998; Tomasz and Palom, 1997). The monoadducts produced by the tautomerization of MC into 2,7-diaminomitosene (2,7-DAM) (Figure 5.1A) which is only capable of associating with one guanine residue. Although significant quantities of this DNA monoadducts formed by 2,7-DAM are present in the cell following MC treatment, it is not the source of MC cytotoxicity. In fact, 2,7-DAM was incapable of inducing p53 (Abbas et al., 2002). Although some reactive oxygen species are also created during its reduction, the principle cause of MC cytotoxicity has been shown to be the interstrand MC-DNA crosslinks (Palom et al., 2001) . Under the hypoxic conditions found in most solid tumors, greater cytotoxicity was achieved with MC by removing the carbamoyl group from its carbon at position 10 (Palom et al., 2002). In addition to forming similar quantities of the DNA adducts formed by MC, the resulting 10-decarbamoyl-mitomycin C (D-MC) was found to produce very high levels of a beta-interstrand crosslink (Figure 5.1B) not formed by MC

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(Palom et al., 2002). This beta-interstrand crosslink may be the source of its increased cytotoxicity. When cells containing or lacking p53 were treated with D-MC under aerobic conditions, both MC and D-MC were cytotoxic in ML-1 cells expressing wild type p53, but only D-MC was cytotoxic in K562 cells that lacked p53. Both MC and D-MC signal to p53, and have been shown to induce p53-mediated apoptosis. Surprisingly, D-MC, but not MC, is capable of inducing cell death in cells that lack p53, which suggests that D-MC may be promoting an alternate p53-independent apoptotic pathway (Abbas et al., 2002). The increased potency of this new MC analog D-MC under the hypoxic conditions found in tumors, as well as its ability to kill cells lacking an in tact p53 response suggest that it may have therapeutic potential, especially since over 60% of cancers harbor mutations in the p53 gene (Hollstein et al., 1991). In order to better understand the mechanisms by which D-MC promotes cell death, we charectorized the apoptotic pathways utilized by MC and D-MC in cells expressing or lacking wild type p53.

Figure 5.1. MC and D-MC form different DNA adducts. *(A) The chemical structures of mitomycin C, its major metabolite 2,7-diaminomitosene, and its chemical analog 10-decarbamoyl mitomycin C are depicted. (B) The various adducts formed when MC and D-MC complex with DNA are depicted. Since MC and D-MC differ in chemical structures at one location, this location is represented by the denotation, R. These different "R groups" are presented in the inset box.*

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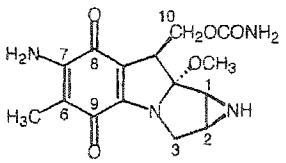
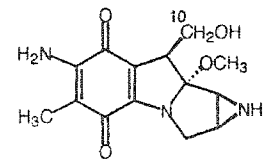
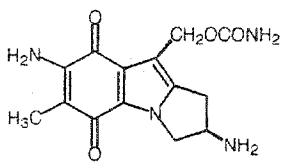
A. Structures of mitomycins



mitomycin C (MC)



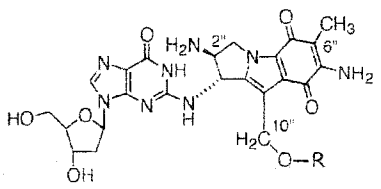
10-decarbamoyl mitomycin C (DMC)



2,7-diaminomitosene (2,7-DAM)

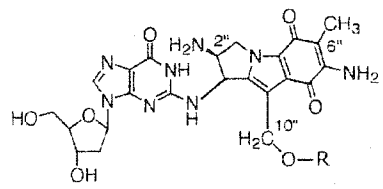
B. The major DNA adducts formed in MC-treated mouse mammary tumor cells.

D-MC and MC

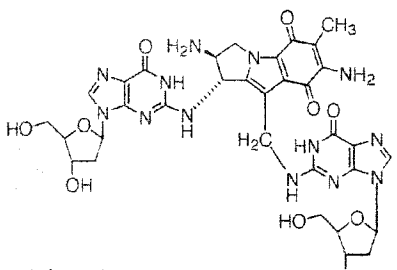


alpha -**(monoadduct)**

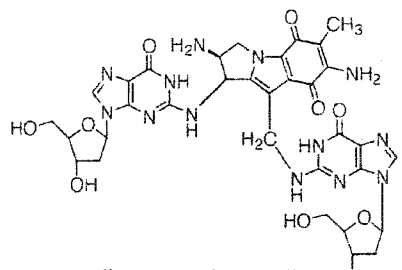
D-MC only



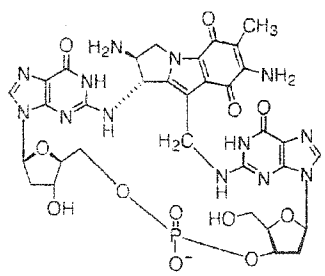
beta - **(monoadduct)**



alpha -**(interstrand cross-link)**



beta - **(interstrand cross-link)**



(intrastrand cross-link)

MC: R = $CONH_2$
D-MC: R = H

Figure 5.1 cont.

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One common protein that has been shown to contribute to both p53-dependent and independent apoptotic pathways is the p53 homolog, p73α. While MC requires wild-type p53 to induce apoptosis (Abbas et al., 2002; Xu et al., 1995), D-MC does not (Abbas et al., 2002). The two p53 homologues p63 and p73 have been shown to be crucial for the ability of p53 to promote apoptosis (Irwin et al., 2000; Urist and Prives, 2002). Using chromatin immunoprecipitation (ChIP), Flores and Jacks were able to show that p53 was unable to bind to its apoptotic target genes *perp*, *bax*, and *noxa* in cells that lack p63 and p73 (Reczek et al., 2003). It has not been determined whether p73 is induced in response to the alkylating agents MC and D-MC in cells that express wild type p53. To address how D-MC mediates apoptosis in cells lacking p53, we hypothesized that p73 may promote p53-independent cell death in response to D-MC treatment by transcriptionally activating apoptotic target genes of p53. p73α protein has been shown to be induced, in a c-Abl dependent manner, by several types of DNA damage including cisplatin and IR (Agami et al., 1999; Toh et al., 2004; Zhu et al., 2001a). Overexpression of p73α in cells lacking p53 activates p53 target genes and p53-independent apoptosis (Lee and La Thangue, 1999; Zhu et al., 1998; Zhu et al., 2001a). Fontamaggi and Bandino showed using ChIP that p73α could localize to the p53 responsive elements on a number of p53 target genes, including *pig-3*, *waf1/cip1*, and *14-3-3σ* (Fontemaggi et al., 2002).

Another protein shared by p53-dependent and independent apoptotic pathways is member of the family of cysteine proteases, the caspase. MC has been shown to mediate p53-dependent apoptosis primarily through the activation of the intrinsic pathway and

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caspase-9 (Kobayashi et al., 2000; Micheau et al., 1999; Park et al., 2000; Vit et al., 2001). Caspase-8 has also been shown to be activated by MC, but this activation occurs independently of the death receptor, Fas/Apo1 (Engels et al., 2000; Park et al., 2000; Pirnia et al., 2002; Wesselborg et al., 1999). Since MC does not induce cell death in the absence of p53, this is the only known apoptotic pathway mediated by MC. D-MC, however, has been shown to induce apoptosis in the presence or absence of p53 (Abbas et al., 2002). It has not been determined whether D-MC utilizes the same apoptotic pathway as MC for mediating cell death in the presence and absence of p53.

In this study, we begin to elucidate the mechanisms by which D-MC mediates apoptosis in cells containing or lacking wild type p53. We found evidence that there are differences in the array of activated proteins that may contribute to p53-dependent apoptosis in response to MC and D-MC. Interestingly, p73α protein was stabilized in ML-1 cells treated with MC, but not with D-MC. Furthermore, while MC and D-MC-mediated apoptosis correlated with the activation of caspases-9, 7, and 3, caspase-8 was more robustly activated by MC treatment. These data suggest that the p53-dependent apoptosis in response to both MC and D-MC utilizes the intrinsic pathway, while only MC seems to also utilize the extrinsic pathway. In examining the mechanisms by which D-MC induces cell death in the absence of p53, we found that the default p53-independent apoptotic pathway is not mediated by p73. Additionally, we found that D-MC mediated apoptosis through the activation of caspases and serine proteases. These data suggest that in the absence of p53 D-MC utilizes a default pathway and that this

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pathway does not utilize p73 but requires the activation of both caspases and serine proteases.

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5.2 Results

While MC and D-MC are capable of inducing apoptosis in cell lines containing wild type p53, only D-MC is capable of promoting p53-independent apoptosis.

Mitomycin C (MC) was one of the first genotoxic agents used to generate a p53 response (Fritsche et al., 1993; Hess et al., 1994). Studies using mitomycin C have focused mostly on how this drug activates p53's DNA binding ability (Tishler et al., 1993), transcriptional activity (Beard et al., 1996), or promotes apoptosis (Abbas et al., 2002; Blagosklonny and El-Deiry, 1998). Like many DNA damaging agents, MC requires p53 to exude its cytotoxic affects (Abbas et al., 2002). Numerous drugs, like etoposide, UV, and methylmethane sulfonate (MMS), have been shown to be capable of promoting cell death in the absence of p53 (Cho et al., 2001; Haapajarvi et al., 1999; Lackinger and Kaina, 2000; Malcomson et al., 1995). Previous work from our lab have confirmed that the analog of MC, 10-decarbamoyl mitomycin C (D-MC), is capable of inducing p53-independent apoptosis in the p53 null erythromyeloid leukemia cell line, K562. Treatment of these cells with equimolar dosages of MC, however, did not result in cell death (Abbas et al., 2002).

For this study, we utilized the myeloid leukemia cell line ML-1, that expressed wild type p53, and the p53 null cell line K562 to charectorize the apoptotic pathways mediated by MC and D-MC. Previous work by our lab has shown that in K562 cells, D-MC, but not MC, treatment for 24 hrs promoted PARP-1 cleavage as well as an increase in sub-G1 populations as determined by FACS. ML-1 cells also exhibited PARP-1

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cleavage and sub-G1 populations in response to both drugs (Abbas et al., 2002). The results in the current study corroborate these data (Figures 5.2A - F). MC and D-MC treatment of ML-1 cells exhibited increases in the level of sub-G1 cell cycle population, as well as clear PARP-1 cleavage after 24 hrs (Figure 5.2A and C). Additionally, D-MC, but not MC, similarly increased the population of cells in sub-G1 and the level of PARP-1 cleavage in K562 cells after 24 hrs of treatment (Figure 5.2B and D). In the samples that exhibited these apoptotic markers, both ML-1 and K562 cells displayed the morphological characteristics of cells undergoing programmed cell death, including the granulation of the cell interior (Figure 5.2A and B). Interestingly, the cell cycle profile of K562 cells treated with MC exhibited an enhancement in G1 and G2/M populations, which is suggestive of cell cycle arrest (Figure 5.2B). As an additional indicator of apoptosis, we also looked at the number of MC and D-MC treated ML-1 and K562 cells that stained positively for Annexin. Annexin stains phosphatidyl serine residues which have flipped from the interior of the cell membrane to the cell's exterior, one of the hallmarks of apoptosis. As expected, ML-1 cells treated with either MC or DMC stained positive for Annexin (Figure 5.2E), while only D-MC treated K562 cells were capable of being stained by this agent (Figure 5.2F). These data further substantiate the ability of D-MC, but not MC, to induce apoptosis in K562 cells. As was observed before, the degree to which D-MC affected this p53 null cell population was less overall than in the wild-type p53 containing ML-1 cells.

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Figure 5.2 MC and D-MC induce apoptosis in cells expressing p53, while only D-MC is able to mediate cell death in the absence of an in tact p53 response. *ML-1 (A) and K562 (B) cells were grown in the presence or absence of 5 uM MC or D-MC for 24 hrs. Images were taken of these samples at the 24 hr time-point under 100x magnification (A & B, left panel). Treated and untreated ML-1 and K562 cells were fixed, stained with propidium iodide, and then their cell cycle profiles were determined via FACS analysis. 100 ug of nuclear extract isolated from ML-1 (C) and K562 (D) cells grown in the presence or absence of 5 uM MC or D-MC for 3, 6, and 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for PARP-1 (top panel), or actin (bottom panel). ML-1 (E) and K562 (F) cells grown in the presence or absence of 5 uM MC or D-MC for 24 hrs were fixed and stained for DNA content and Annexin and then analyzed via FACS analysis.*

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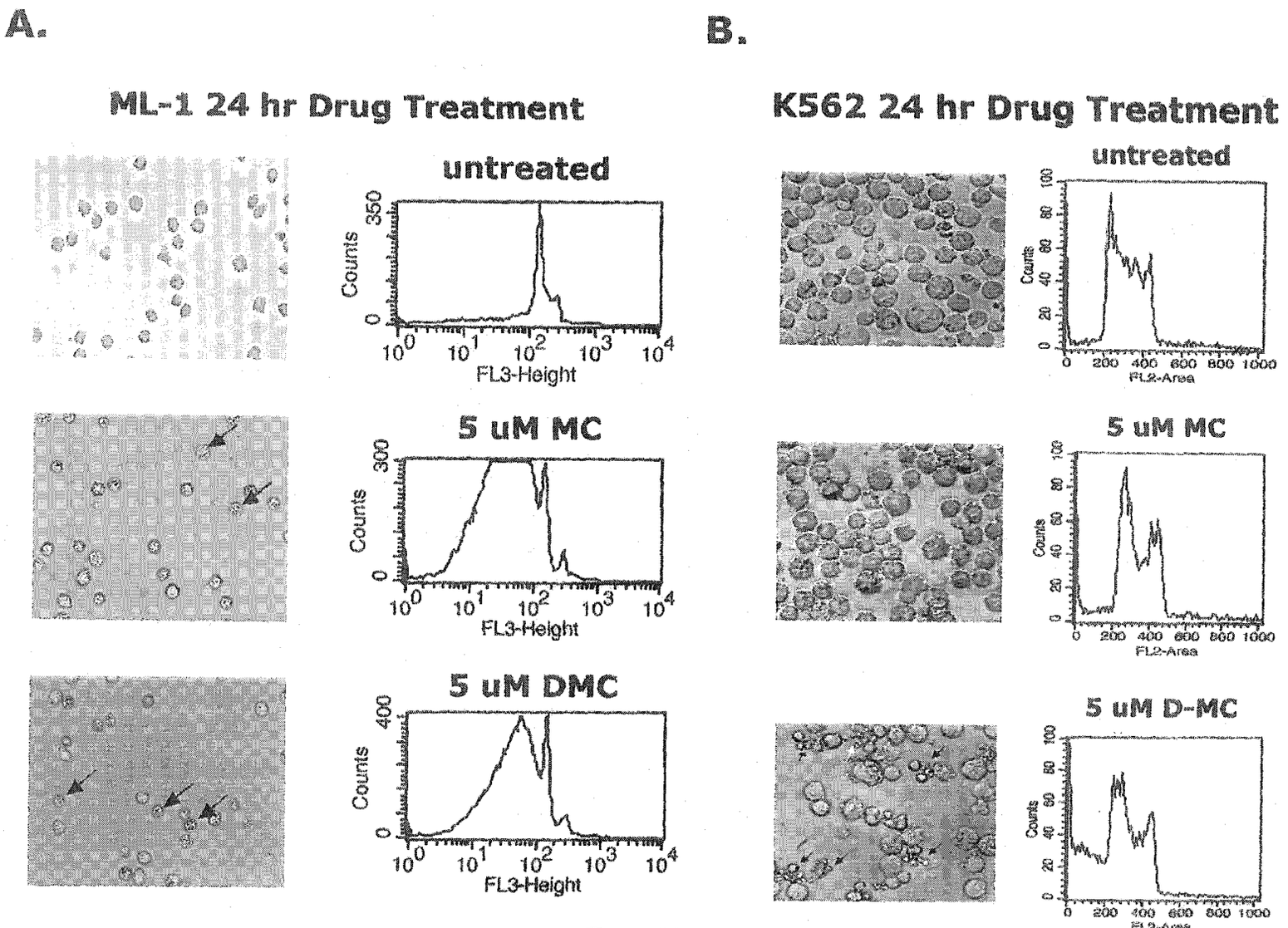

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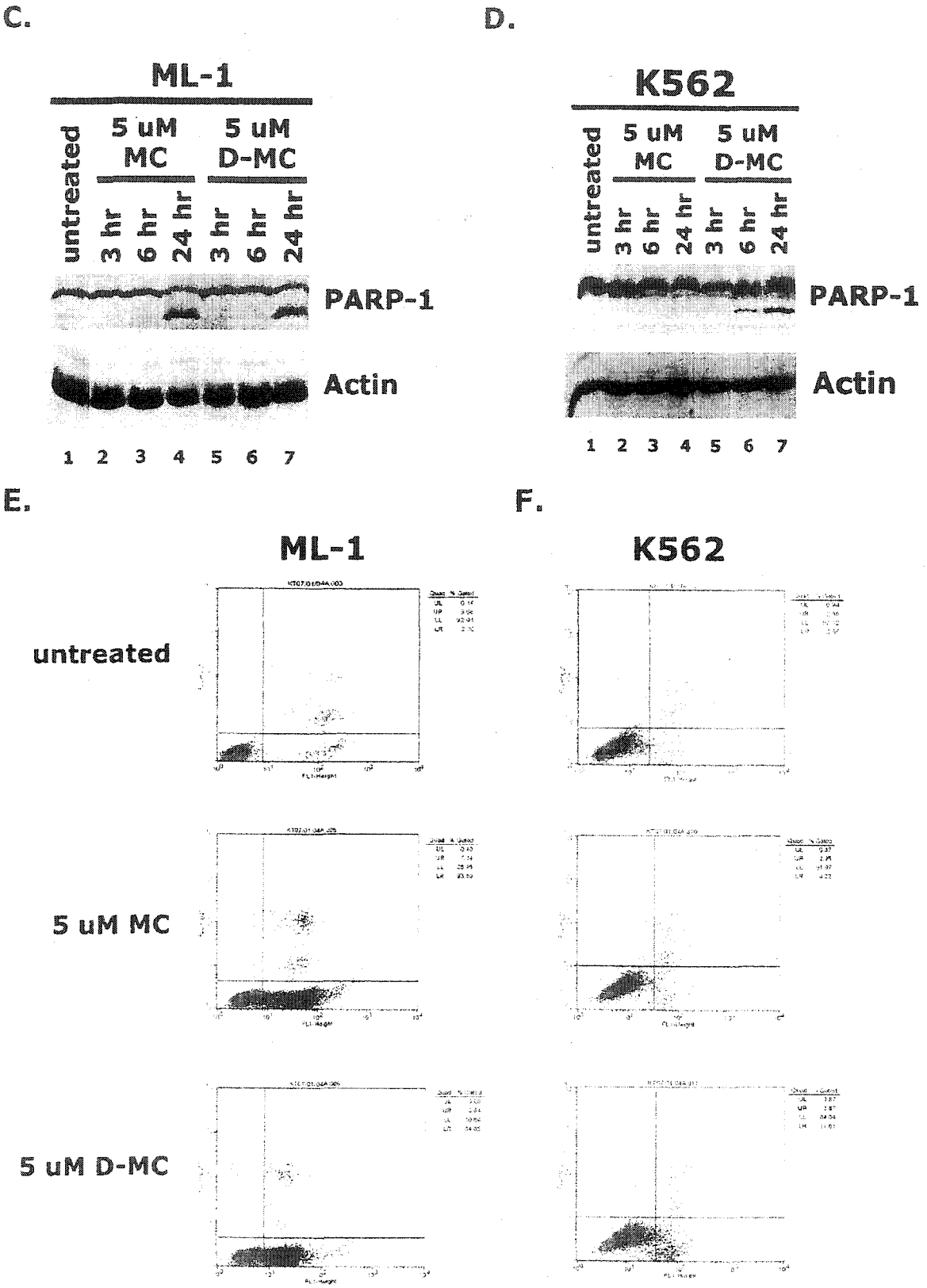

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MC induces cell cycle arrest, while D-MC induces cell death in K562 cells.

Analysis of MC treated K562 cells using flow cytometry displayed clear enhancements in the G1 and G2/M cell populations which is indicative of cell cycle arrest (Figure 5.2B). To address whether MC treatment induced p53 independent growth arrest, we examined the growth of MC and D-MC treated ML-1 and K562 cells over a 24 hr period. Interestingly, MC and D-MC more significantly impeded the growth of cells that contained wild-type p53. After 24 hrs of treatment, MC and D-MC decreased the cell number of ML-1 cells by more than half, from 5.17 x 10^5 cells/ml in the untreated sample to an average of 2.02 x 10^5 cells/ml and 2.46 x 10^5 cells/ml, respectively (Figure 5.3A). In K562 cells, MC and D-MC treatment was less effective decreasing the cell number from 8.73 x 10^5 cells/ml in the untreated sample to 5.6 x 10^5 cells/ml and 5.16 x 10^5 cells/ml, respectively (Figure 5.3B). The decreases in ML-1 cell population correlated with the higher levels of apoptosis in ML-1 cells observed via FACS analysis and Annexin staining (Figures 5.2A, B, E, and F).

We next addressed whether the p53-independent growth arrest was mediated by the potent Cdk inhibitor, p21. p53-independent growth arrest has been shown to occur in response to UV through the induction of p21 by the transcription factor, Sp1 (Haapajarvi et al 1999). Although p21 protein was clearly evident in MC and D-MC treated ML-1 cells after 24 hrs, no p21 was found in K562 cells treated with either drug suggesting that the p53-independent growth arrest promoted by MC was not mediated by p21 (Figure 5.3D). Additionally, MC-treatment did not significantly induce the level of *waf1* transcript. Interestingly, there was modest induction (3.5 fold) of *waf1* transcripts in

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K562 cells treated with D-MC (Figure 5.4B). These data suggest that although MC does not induce apoptosis in the absence of p53, it can still promote growth arrest. The ability of D-MC to induce apoptosis in the absence of p53, however, is indisputable.

Figure 5.3 MC induces p53-independent growth arrest. *ML-1 (A) and K562 (B) cells were grown in the presence or absence of 5 uM MC or D-MC for 24 hrs. Treated and untreated cells were counted after 4, 7, 10, and 24 hrs of growth. The growth curve is representative of three independent experiments. Nuclear extract isolated from ML-1(C) and K562 (D) cells grown in the presence or absence of 5 uM MC or D-MC for 3, 6, and 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for p21 (top panels), or actin (bottom panels).*

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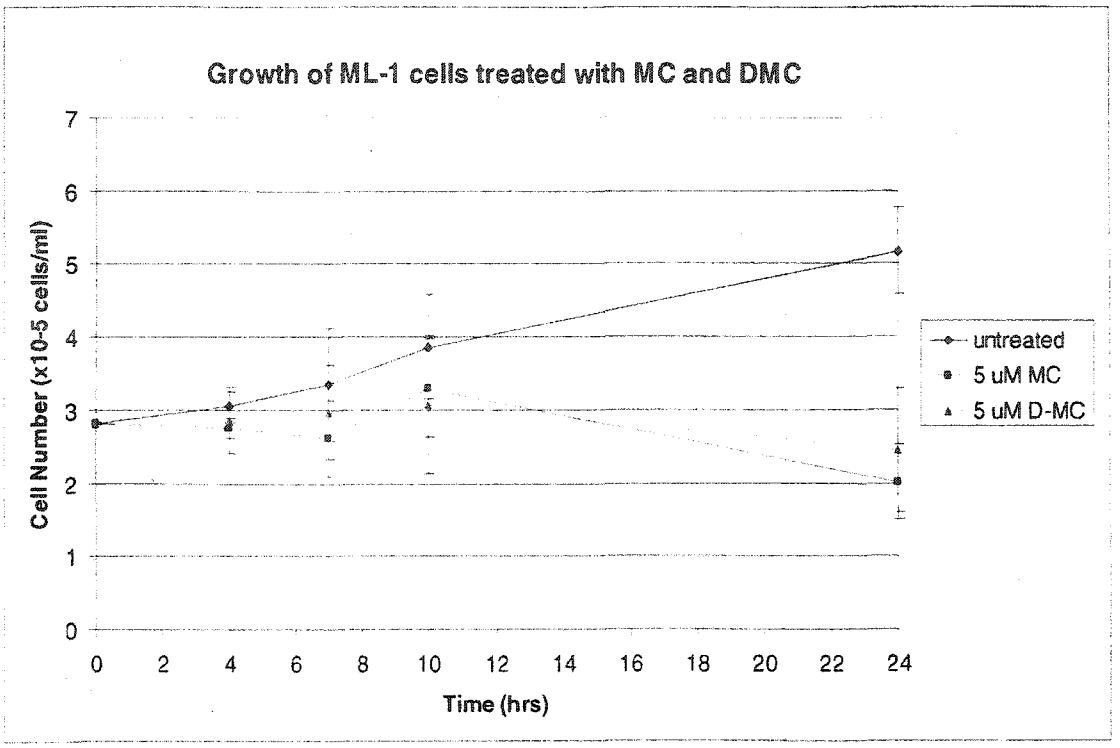

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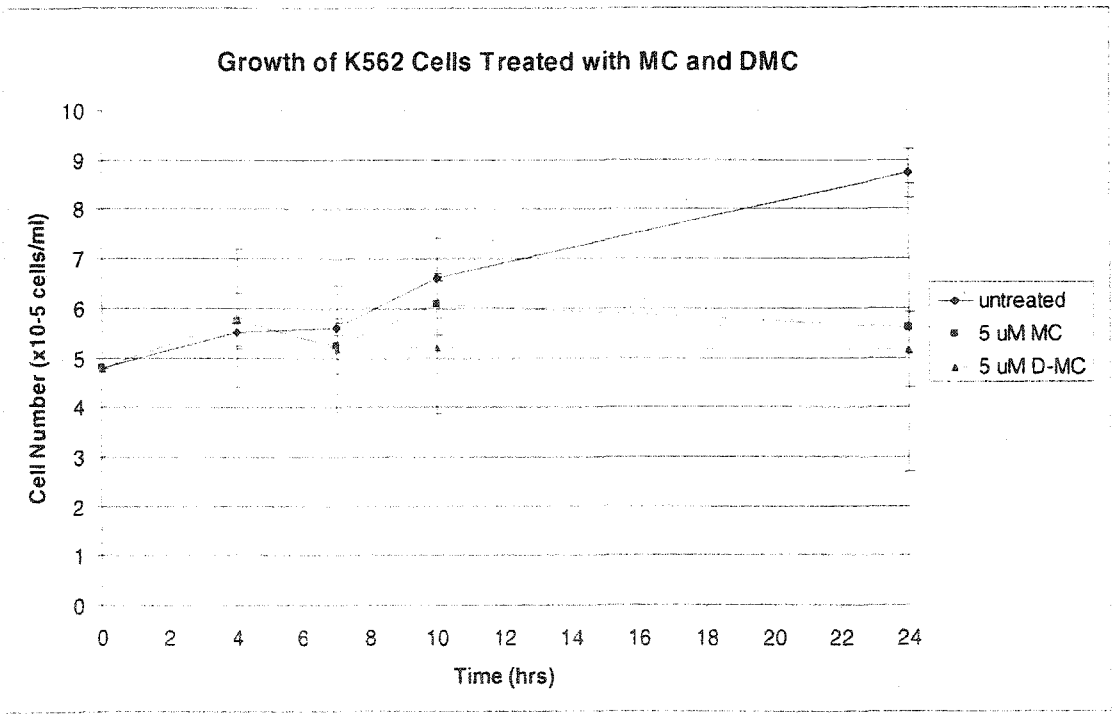

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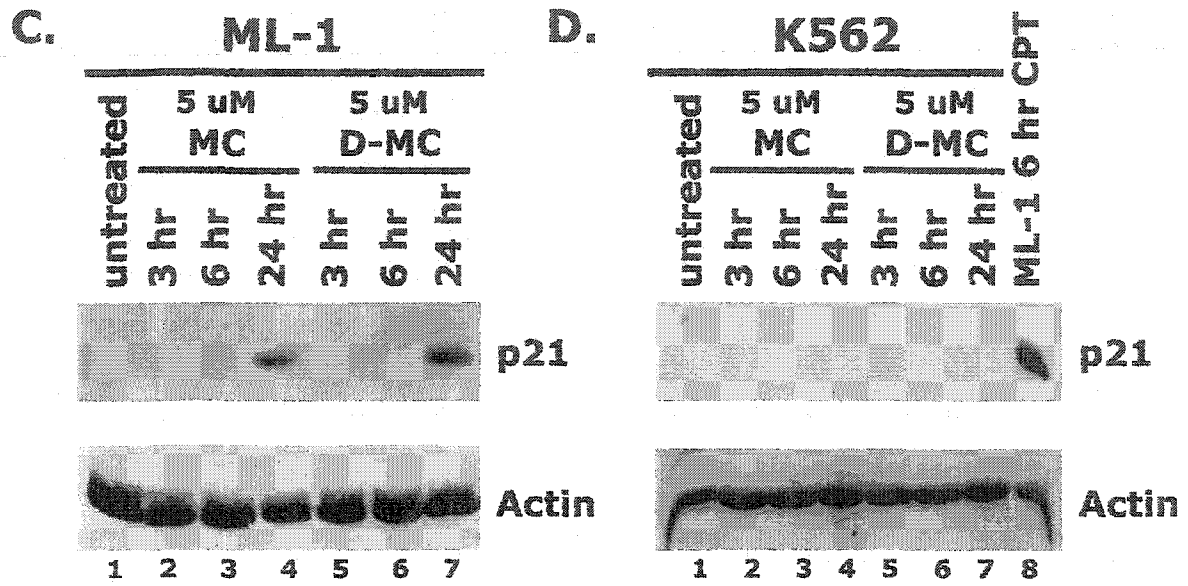

Figure 5.3 cont.

D-MC-induced p53-independent apoptosis is not due to the increased levels of p73α protein or the activation of p53 apoptotic target genes.

In order to determine if D-MC mediated apoptosis through similar pathways in the presence or absence of p53, we next determined if the p53-independent cell death occurred through the activation of the p53 family member, p73α. Preliminary work determined that K562 cells exhibited much higher levels of activated c-Jun than ML-1 cells in the presence and absence of MC or D-MC treatment (Figure 5.4A). To date, p73α has only been shown to be induced by a few DNA damaging agents, like doxyrubicin, IR, and cisplatin (Agami et al., 1999; Toh et al., 2004; Vossio et al., 2002). Previous work by Toh et al found that c-Jun was required for p73α stabilization in response to cisplatin, and was required (along with c-Abl) for p53-independent apoptosis by p73α and cisplatin (Toh et al., 2004). Numerous studies have shown that p73α can

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induce numerous p53 target genes and p53-independent apoptosis when overexpressed or induced by some types of DNA damage (Lee and La Thangue, 1999; Zhu et al., 1998; Zhu et al., 2001a). Not only did K562 cells have modest levels of phosphorylated c-Jun, but they also exhibited a modest level of *waf1/cip1* transcript in response to D-MC (3 fold after 6 hrs of treatment compared to 1.9 fold over untreated in MC samples) (Figure 5.4A and B). These data led us to speculate that D-MC may be inducing p73α protein in the K562 cells and that this activated p73α may be mediating p53-independent apoptosis by inducing apoptotic target genes of p53. To address this, we first examined the level of p73α protein in MC and D-MC treated K562 cells. However, no induction of p73α was observed in MC or D-MC treated K562 nuclear extracts (Figure 5.4C). To further confirm that our hypothesis was additionally invalid, we used quantitative PCR to examine the levels of *bax*, *noxa*, *fas/apo1*, and *pig-3* transcript in K562 cells treated with MC and D-MC. No induction of any of these p53-specific apoptotic target genes was found even after 12hrs of D-MC treatment when cell death was evident (Figure 5.4D). Taken together, these data suggest that the p53-independent apoptotic pathway induced by D-MC did not occur by the activation of p53 family members.

Figure 5.4 p73 is not activated in D-MC treated K562 cells. *(A) Nuclear extract isolated from ML-1 and K562 cells grown in the presence or absence of 5 uM MC or D-MC for 3, 6, and 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for phosphor-c-Jun (top panel), or actin (bottom panel). (B) Quantitative PCR was performed on cDNA from RNA isolated from K562 cells grown*

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in the presence or absence of 5 uM D-MC for 3 and 6 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of waf1/cip1 transcripts was calculated over the untreated sample. The results shown are representative of two independent experiments. (C) Nuclear extract isolated from ML-1 and K562 cells grown in the presence or absence of 5 uM MC or D-MC for 3, and 6 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for p73α (top panel), or actin (bottom panel). (D) Quantitative PCR was performed on cDNA from RNA isolated from K562 cells grown in the presence or absence of 5 uM D-MC for 3, 6, and 12 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of fas/apo1, bax, noxa, and pig-3 transcripts was calculated over the untreated sample. The results shown are representative of two independent experiments.

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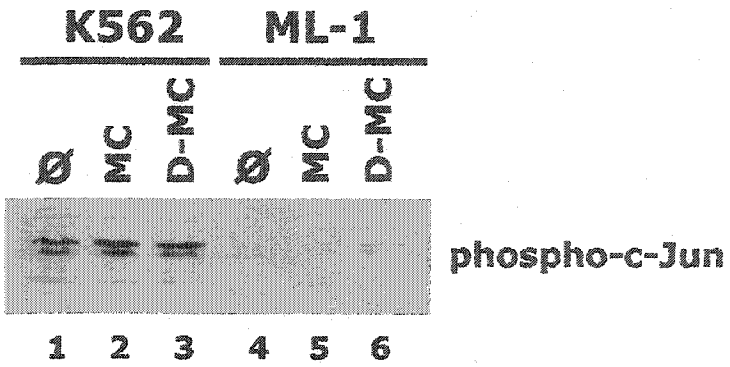

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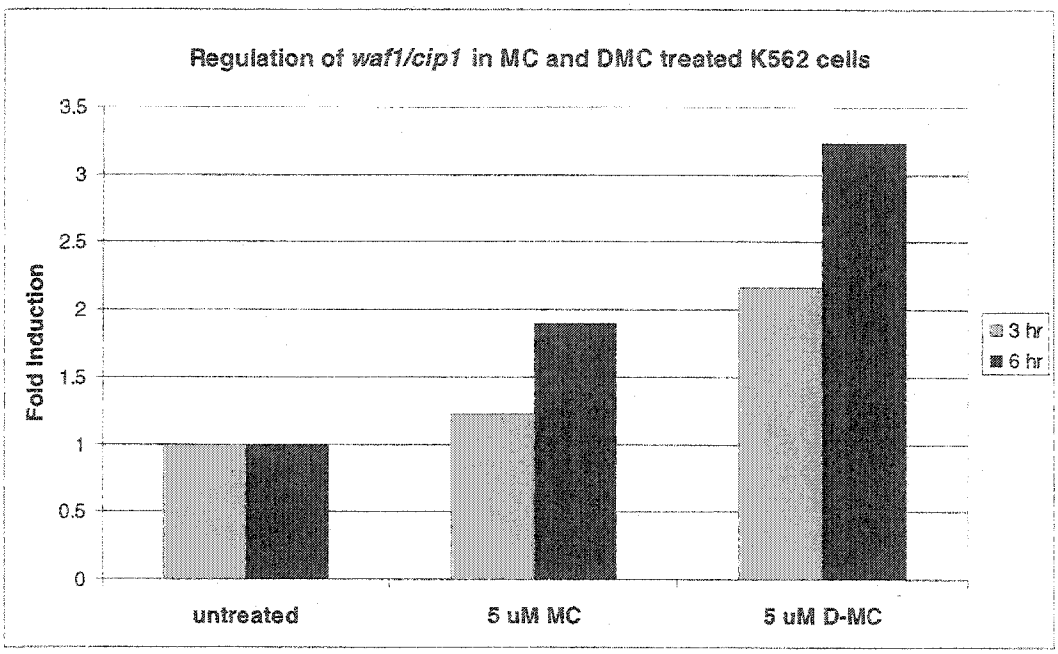

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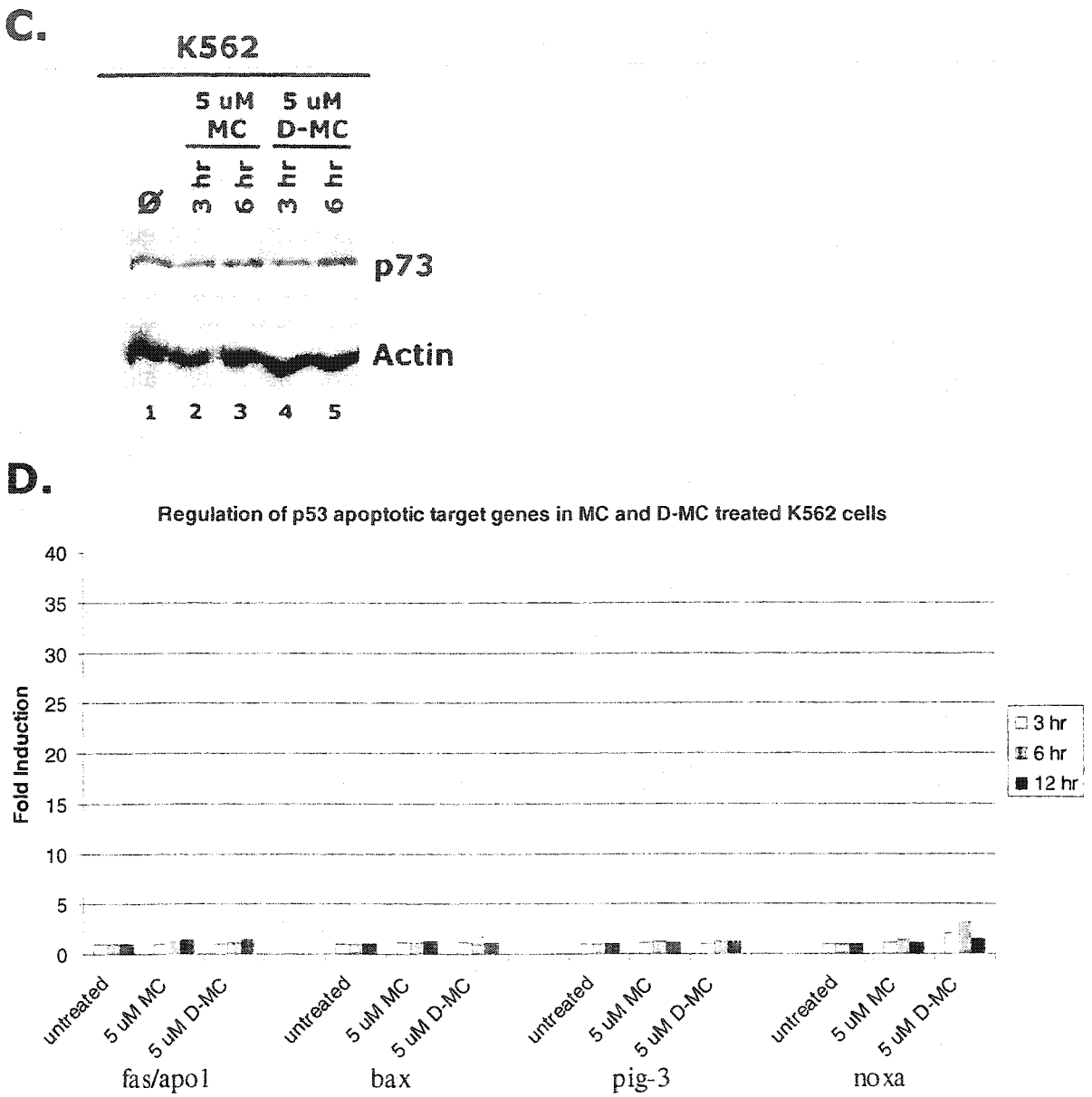

Figure 5.4 cont.

MC and D-MC differentially activate p73α and p53 target genes in ML-1 cells.

In addition to looking at the possible role of p73α in p53-independent apoptosis in response to D-MC, we also looked at how p73α was regulated by MC and D-MC in cells that express wild type p53. The p53 family members' p73 and p63 were shown to be

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required for the ability of p53 to transcriptionally activate apoptotic target genes and induce apoptosis in mouse embryonic fibroblasts (MEFs) infected with E1A (Flores et al., 2002). Additionally, p73 protein has been shown to be stabilized by numerous DNA damaging agents (Agami et al., 1999; Toh et al., 2004; Vossio et al., 2002). These data suggest that p73α may provide a critical role in facilitating p53-dependent apoptosis in response to genotoxic stress. Although we found that p73α was not upregulated in K562 cells treated with MC or D-MC (Figure 5.4C), it has not been determined whether p73α can be induced by either of these agents in cells with a wild-type p53 background. Furthermore, it is not known whether induced levels of p73α protein contribute to the transcriptional activity of p53. Similar levels of p53 protein were induced with similar kinetics in ML-1 cells treated with equimolar concentrations of MC and D-MC (Figure 7.1B). Interestingly, D-MC treated ML-1 cells exhibited significantly higher levels of *fas/apo1* and *pig-3* transcript, and a more modest increase in *bax* and *noxa* transcript levels than MC treated cells (Figure 5.5A). Taken together, these data suggest that D-MC induces a form of p53 that is very capable in activating target genes. In order to determine if D-MC treatment promoted higher levels of p53 target genes by also inducing p73α, the level of p73α protein in nuclear extracts from MC and D-MC treated ML-1 cells was examined via immunoblotting. Surprisingly, we found that p73α was induced by MC but not D-MC (Figure 5.5B). These data suggest that p73α induction is not required for p53 to produce the higher levels of activation of apoptotic target genes noted in ML-1 cells treated with D-MC. The difference in the response to MC and D-MC clearly indicates the activation of different stress pathways.

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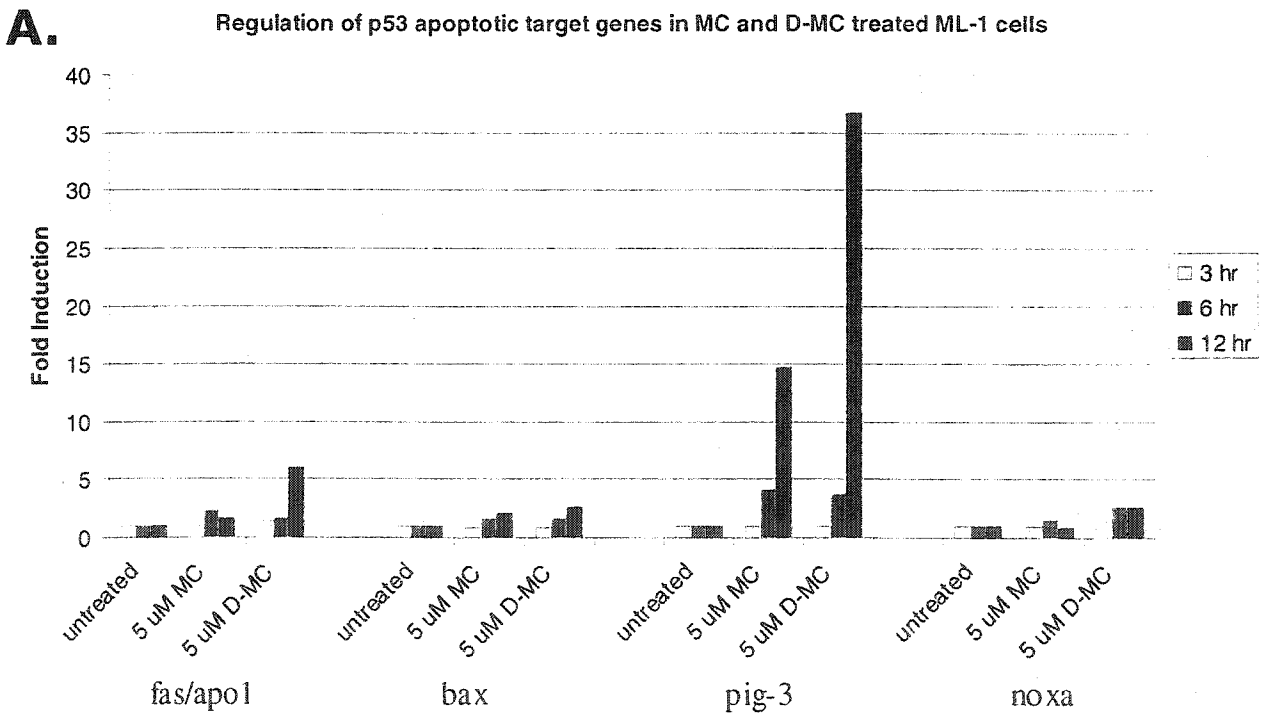
Figure 5.5 Although MC, but not D-MC, induces p73α protein, D-MC treatment induces higher levels of p53 apoptotic target genes. *(A) Quantitative PCR was performed on cDNA from RNA isolated from K562 cells grown in the presence or absence of 5 uM D-MC for 3, 6, and 12 hrs. The amount of RNA in each sample was normalized using TaqMan probes for gapdh. The fold induction of fas/apo1, bax, noxa, and pig-3 transcripts was calculated over the untreated sample. The results shown are representative of two independent experiments. (B) Nuclear extract isolated from ML-1 and K562 cells grown in the presence or absence of 5 uM MC or D-MC for 3, and 6 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for p73α (top panel), or actin (bottom panel).*



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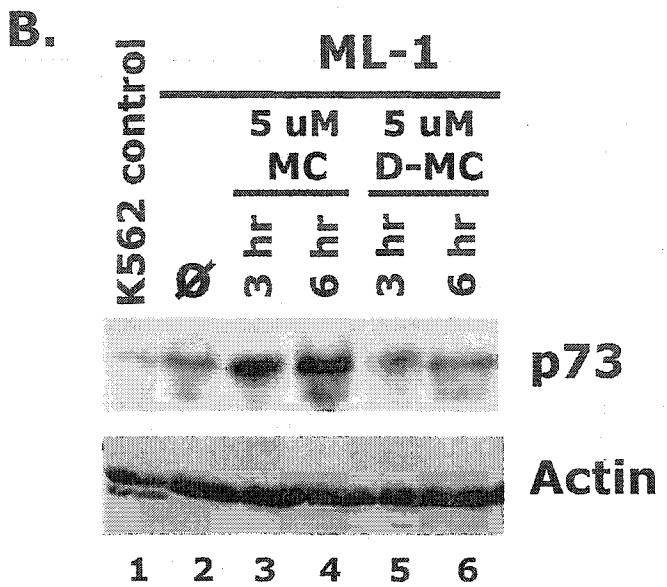

Figure 5.5 cont.

In the presence of p53, D-MC and MC treatment result in high levels of caspase activity, but in the absence of p53 D-MC promotes apoptosis through the activation of caspases and serine proteases.

Although numerous studies have shown the regulation of downstream caspases during apoptosis by MC (Guillouf et al., 1999; Pirnia et al., 2002; Wesselborg et al., 1999), it has not been clearly shown if these caspases are also activated in response to D-MC. To determine the caspases involved in cell death in response to MC and D-MC, the levels of procaspase 9, 8, 7, and 3 were analyzed in drug treated ML-1 and K562 cells using immunoblotting. Decreases in procaspase level have been shown to occur during caspase activation (Dubrez et al., 1998). In MC and D-MC treated ML-1 cells, we found that MC and D-MC activated caspases-9, 3, and 7, while only MC robustly activated caspase-8 as seen by the depletion of their procaspase counterparts (Figure 5.6A, lanes 5

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through 9). In K562 cells, however, we were unable to detect any procaspase depletion (Figure5.6A, lanes 1 through 4), even after 48 hrs of D-MC treatment (Figure 5.6B, lane 5). This result was unexpected since we detected proteolytic cleavage of PARP-1 in these cells treated with D-MC (Figure 5.2D, lane 7). It has been shown that PARP-1 is a target for caspases during the early stages of apoptosis (Datta et al., 1996). Additional analysis of caspase activity in MC and D-MC treated K562 cells using a multi-caspase assay exhibited modest levels caspase activity in response to 10 uM D-MC, but not in response to similar dosages of MC (Figure 5.5D, bottom panels). Conversely, very high levels of caspase activity in ML-1 cells treated with both MC and D-MC were observed using this method (Figure 5.6C). The activation of caspases is determined by the amount of cleaved caspase-specific peptides represented by a shift in detectable cells from the left to the right-hand quadrants of the data field (Figure 5.6E). These data, taken together, suggest that D-MC mediated apoptosis in K562 cells may utilize caspases, but at a significantly lower level than what was detected in MC and D-MC treated ML-1 cells.

Although the data suggested that D-MC mediated apoptosis in K562 cells was caspase dependent, the level of caspase activity noted in response to this agent was not strong enough to convincing state that caspases were integral parts of the p53-independent apoptotic pathway activated by D-MC. Alternate proteases involved in the D-MC mediated p53-independent apoptotic pathway were therefore examined. Serine proteases have been shown to play a role in mediating apoptosis (Philchenkov et al., 2004; Stenson-Cox et al., 2003). These data led us to hypothesize that serine proteases might be involved in the p53-independent apoptotic pathway induced by D-MC. To determine

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whether D-MC mediated apoptosis required the activity of caspases and serine proteases, K562 cells were treated with either the pan-caspase inhibitor zVAD-fmk or with the serine protease inhibitor 4-(2 aminoethyl)benzenesulfonyl fluoride (AEBSF) in addition to D-MC. Interestingly, inhibition of either caspases or serine proteases reduced the percentage of sub-G1 cells from 40.55% to 12.78% and 8.85%, respectively (Figure 5.7E). These data suggest that both caspases and serine proteases contribute to the apoptotic pathways in K562 cells initiated by D-MC treatment.

Figure 5.6 ML-1 cells exhibit more caspase activation in response to MC and D-MC than K562 cells. *(A) Nuclear extract isolated from ML-1 and K562 cells grown in the presence or absence of 5 uM MC or D-MC for 6, and 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for caspase-8, 9, 7, 3, or actin (bottom panel). (B) Nuclear extract isolated from K562 cells grown in the presence or absence of 5 uM MC and D-MC for 24 and 48 hrs, or 8 uM ETOP for 48 hrs, as well as from ML-1 cells treated with 5 uM MC or D-MC for 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for caspase-9 (top panel), or actin (bottom panel). Caspase activity was also examined in ML-1 (C) and K562 (D) cells treated with 5 or 10 uM MC and D-MC for 24 hrs using a Guava FACS machine. (E) A model of the data field from the Guava mult-caspase assay defining the quandrants from each sample set. (F) K562 cells grown in the presence or absence of 12 uM D-MC, 12 uM D-MC with 100 uM zVAD-fmk,*

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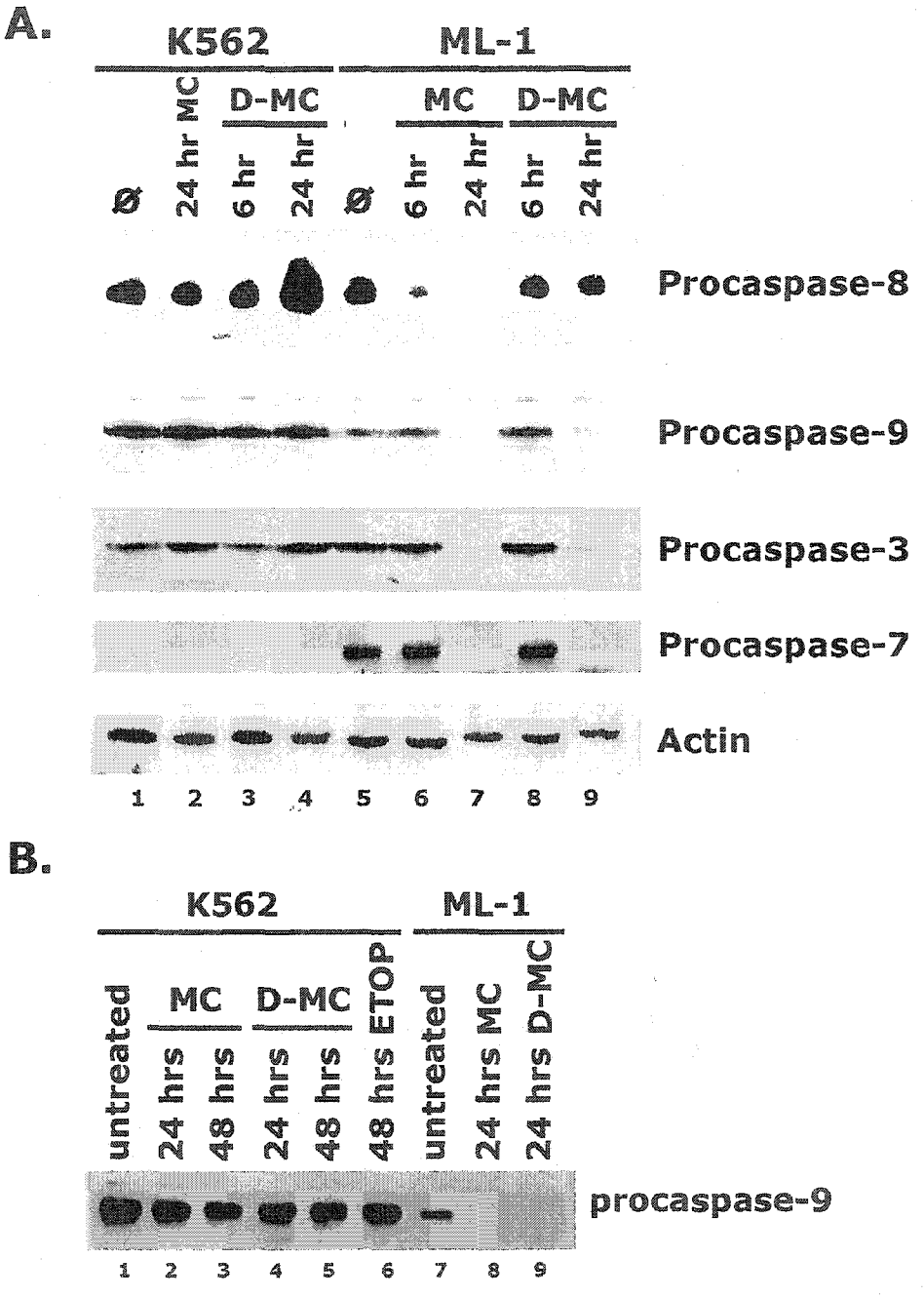
or 12 uM D-MC with 500 nM AEBSF for 24 hrs were fixed and stained with propidium iodide. The cell number and DNA content were then quantified via FACS analysis. The percentage of the total cell population in sub-G1 was then depicted in a histogram.



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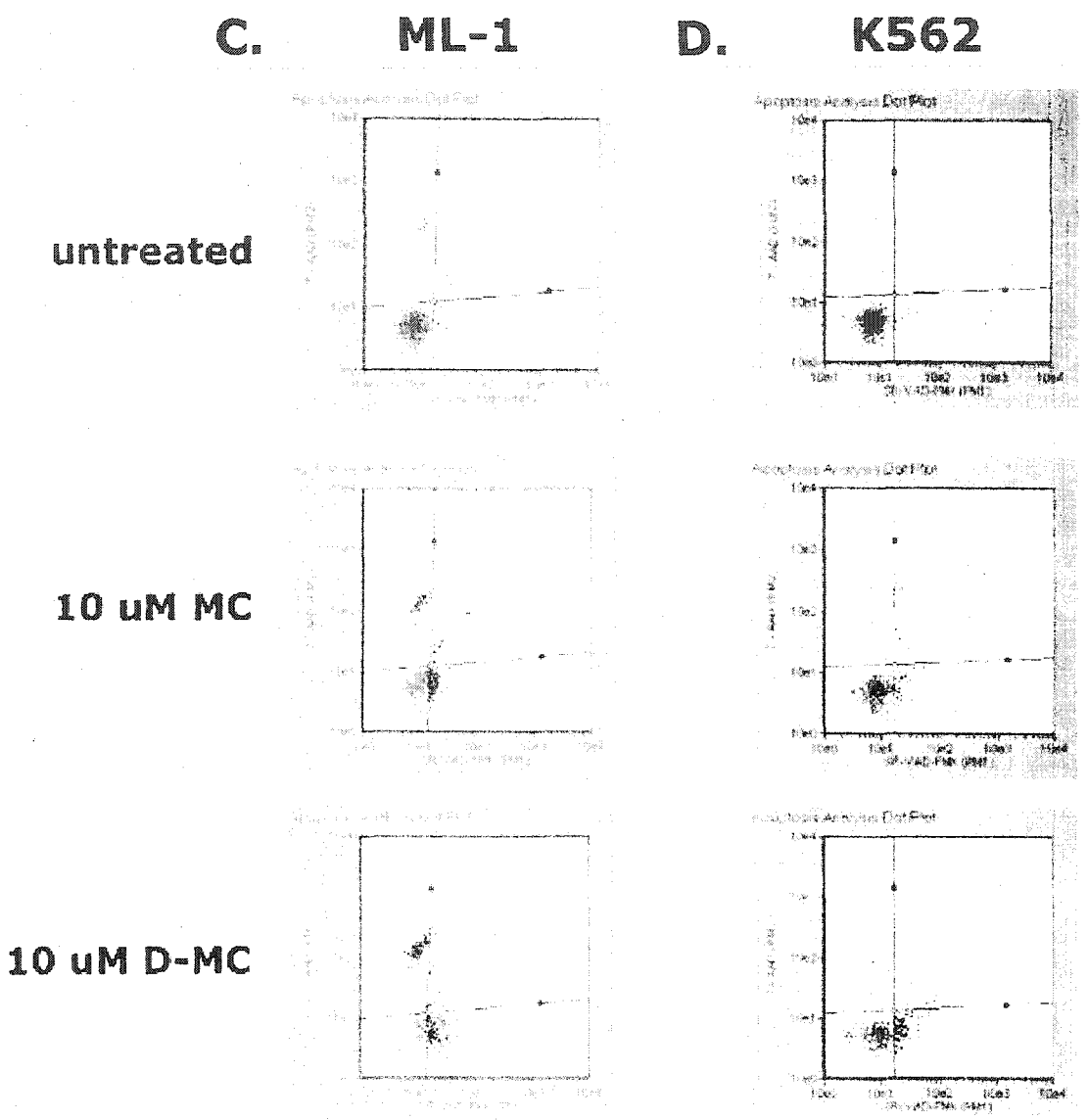

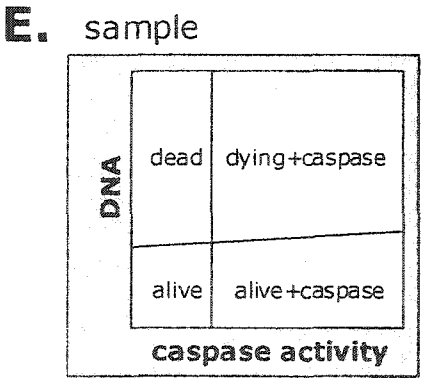

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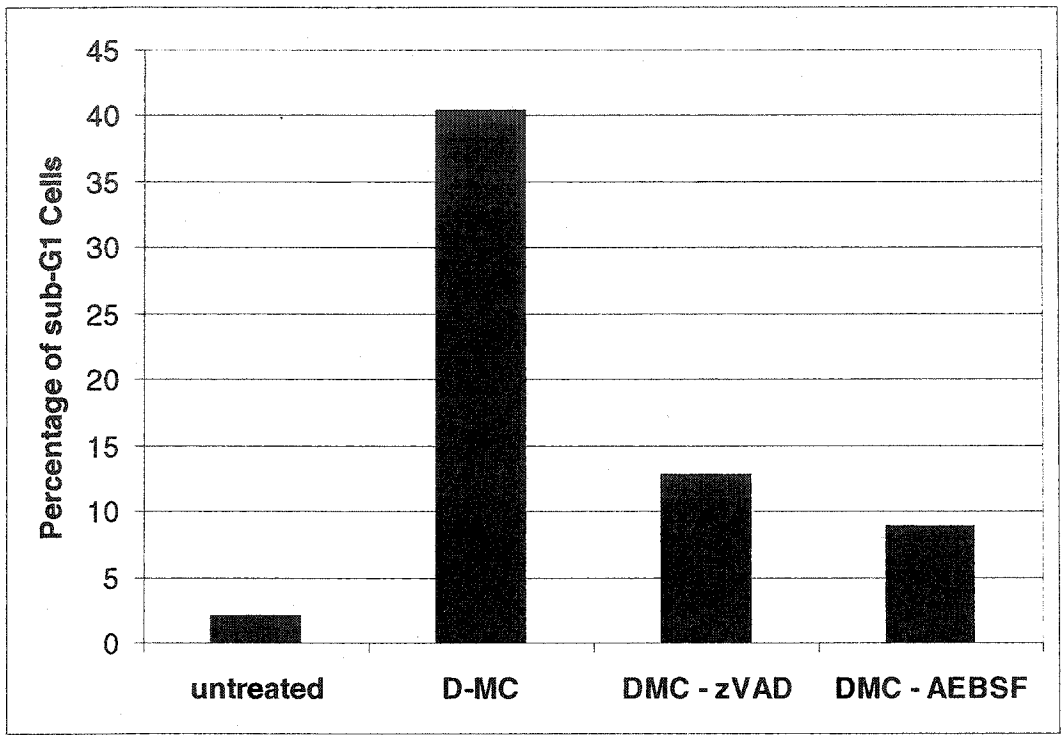

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5.3 Discussion

Although the structure of MC is very similar to its derivative D-MC, MC and D-MC act via different pathways. In cells that contain wild-type p53, MC-mediated apoptosis correlates with the stabilization of p73α protein and the activation of both extrinsic and intrinsic apoptotic pathways, while D-MC treatment does not stabilize p73α, and only robustly activates the intrinsic pathway during cell death. In cells that lack a functional p53 response, only D-MC is capable of promoting apoptosis. This D-MC mediated cell death did not involve p73α protein or the induction of p53 target genes, but does require that activity of caspases and serine proteases.

In this study we demonstrate that MC is capable of stabilizing p73α protein. Previous studies have shown increased levels of p73 in response to IR, doxyrubicin, and cisplatin (Agami et al., 1999; Toh et al., 2004; Vossio et al., 2002). Both MC and D-MC were equally capable of inducing apoptosis as long as p53 protein was present. The data suggest that the stabilization of p73α protein is not only drug specific, but also p53-dependent. Further studies need to be carried out in order to verify whether the induction of p73α by MC was specific to the ML-1 cell line. Furthermore, the requirement of p53 for MC-mediated p73α stabilization could be substantiated by infecting ML-1 cells with the viral oncoprotein E6 prior to treating these cells with MC. E6 has been shown to bind to p53 and target it for degradation (Scheffner et al., 1990).

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The differential activation of caspases by MC and D-MC treatment in the ML-1 cell line was also unexpected. D-MC treated cells did not exhibit any robust activation of caspase-8. In contrast, MC treated ML-1 cells displayed cleavage of procaspase-8. We cannot, however, rule out that lower levels of caspase-8 activity existed in response to D-MC treatment. Regardless, an earlier study by Vit and colleagues showed that caspase-8 was dispensable in the activation of apoptosis in response to IR and MC (Vit et al., 2001). Furthermore, both MC and D-MC induced similar levels of p53-dependent apoptosis. The p53-dependent apoptotic pathway utilized by D-MC is illustrated in Figure 5.7. D-MC mediated DNA damage promotes the induction of Fas/Apo1, Noxa, and Bax through the activation of p53. These pro-apoptotic proteins promote the activation of the intrinsic apoptotic pathway, although the activation of the extrinsic pathway (as stated earlier) cannot be ruled out.

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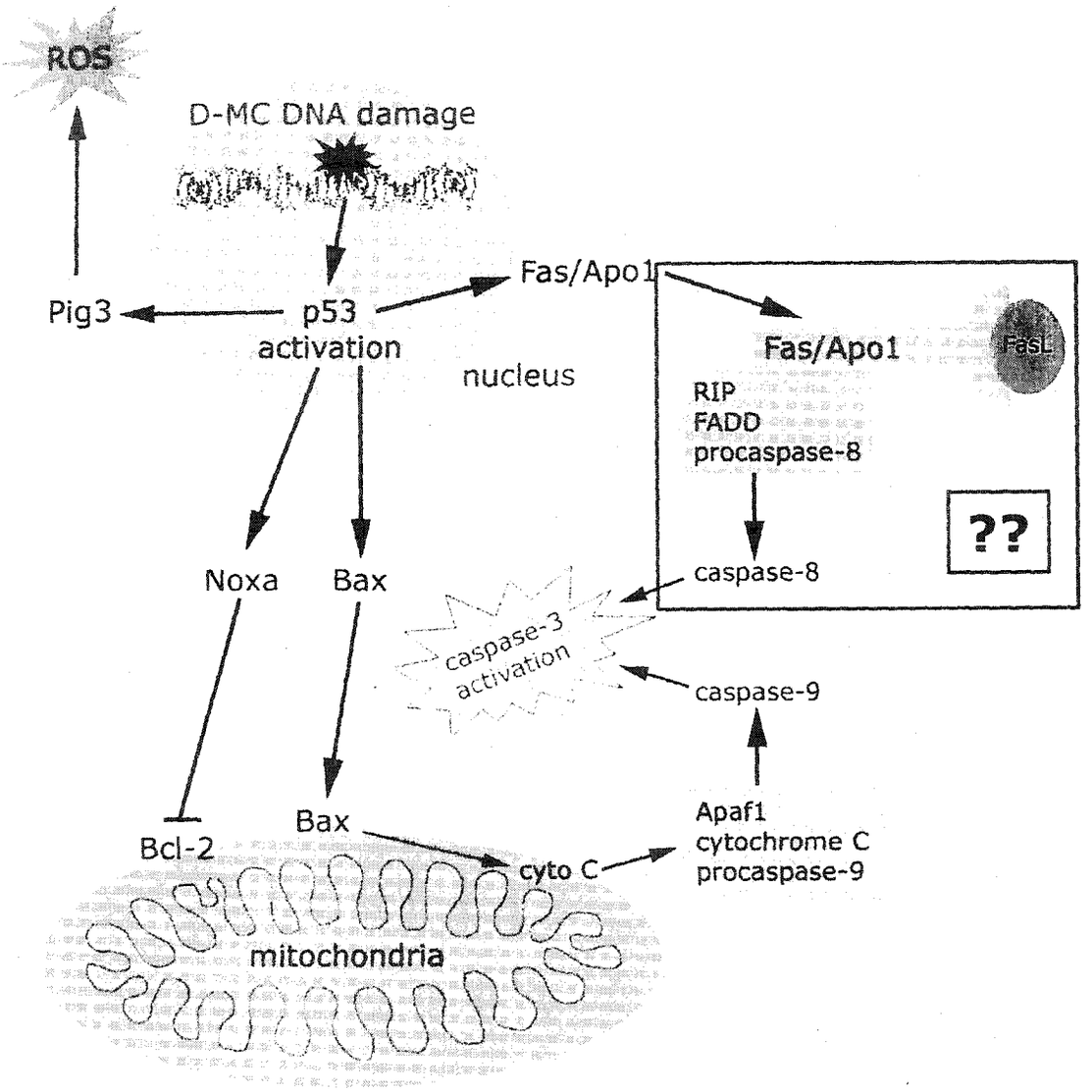

Figure 5.7 The p53-dependent apoptotic pathway utilized by D-MC in ML-1 cells.

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In this study, we also determined that p53-independent cell death in D-MC treated K562 cells occurred independently of p73α and required caspase and serine protease activity. Although the level of caspase activity detected was significantly less than what was observed in the ML-1 cell line, co-treatment of K562 cells with D-MC and the pancaspase inhibitor zVAD-fmk confirmed that caspases were involved. Furthermore, PARP-1 was cleaved in D-MC treated cells. A recent study by de Bruin and colleagues showed that the cleavage of PARP-1 in etoposide treated melanoma cells was caspase independent and was carried out by serine proteases (de Bruin et al., 2003). This led us to consider that serine proteases may also be involved in the D-MC mediated apoptotic pathway. Co-treatment of K562 cells with D-MC and the serine protease inhibitor AEBSF showed a marked decrease in sub-G1 populous suggesting that serine proteases played a role, in addition to caspases, in D-MC mediated apoptosis. Both caspase and serine protease inhibitors efficiently blocked cell death in response to D-MC, which suggests that both of these families of apoptotic proteases are required for this unique apoptotic pathway.

Recent studies by Yu et al have elucidated a p53-independent pathway that involves the activation of PARP-1. PARP-1 activation was shown to be an early event in the activation of apoptosis, resulting in the release of apoptosis inducing factor (AIF) from the mitochondria. Cytoplasmic AIF translocates to the nucleus and mediates the condensation of chromatin, as well as the flipping of phosphotidylserine on the plasma membrane (Yu et al., 2002). Further studies showed that AIF then triggers the release of cytochrome C from the mitochondria, which subsequently activates caspases. These

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caspases go on to cleave PARP-1, as well as numerous other survival proteins in the cell (review by Chiarugi and Moskowitz 2002, Cande et al 2002). It is possible that in cells that lack p53, D-MC promotes the translocation of AIF from the mitochondria to the nucleus, and that this event triggers the flipping of the phosphatidyl serine residues noted in our studies using Annexin. Furthermore, AIF translocation may promote a change in the mitochondrial membrane potential that facilitates the release of cytochrome C and serine proteases from the mitochondria. These mitochondrial proteins may then promote the activation of caspases and the cleavage of PARP-1 in the nucleus. We illustrate this hypothetical pathway in a model shown in Figure 5.8.

D-MC may be a potentially valuable tool in fighting cancer. With more than 60% of cancers containing mutations in the p53 gene, it is important to find new drugs that are capable of killing cells with these deficiencies. Not only does D-MC exhibit a similar ability to promote apoptosis as its source compound, MC, but it is clear from our studies in K562 and our current work with P1299 cells (see section 7.2) that it can effectively initiate cell death in cell lines that lack p53. The ability of D-MC to mediate apoptosis in other p53 null and mutant p53 containing cell lines needs to be explored to further substantiate the efficiency of this drug. In addition, it may be beneficial to carry out additional alterations in the structures of mitomycin C and 10-decarbamoyl mitomycin C in order to create even more effective cancer therapeutics for the future.

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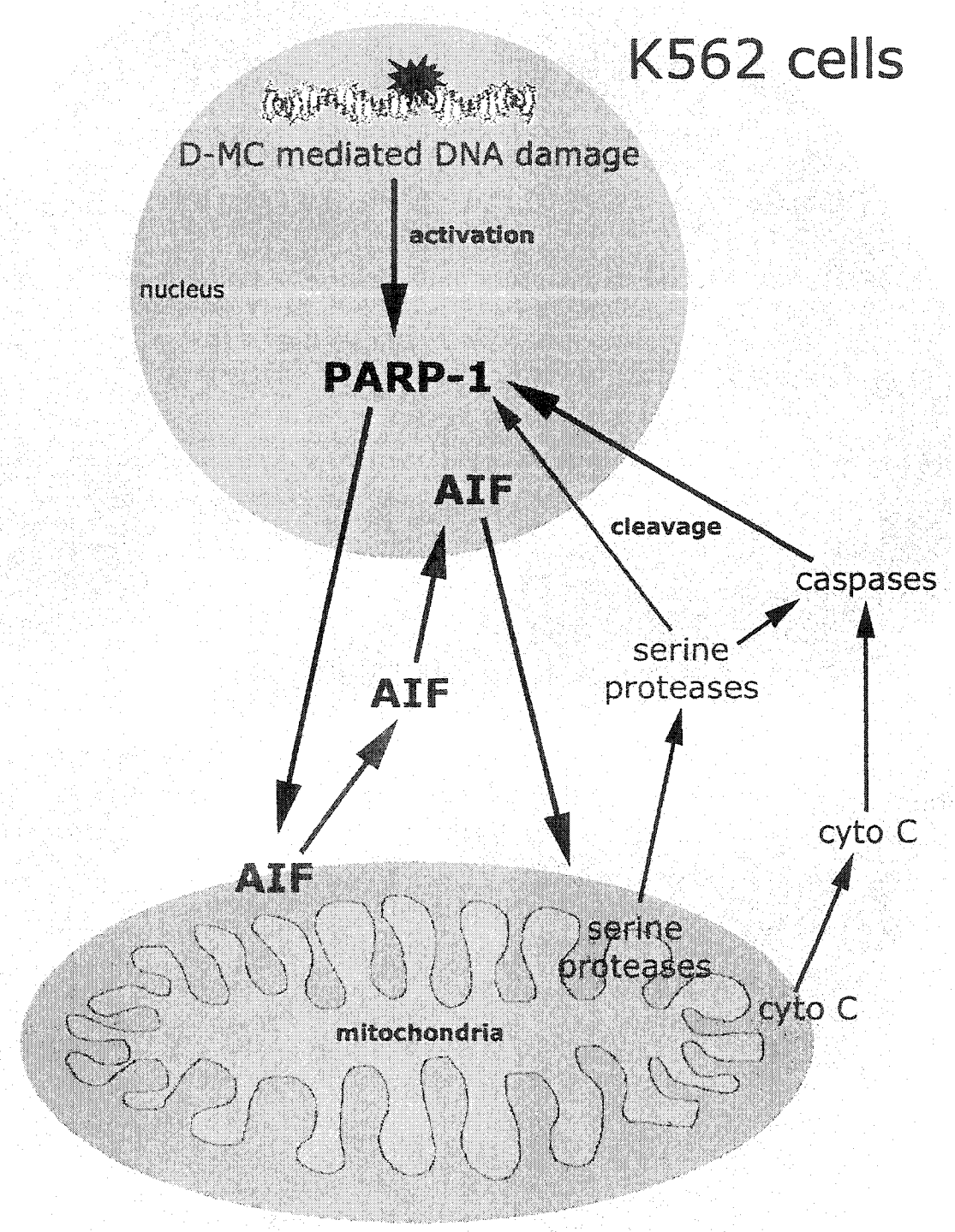

Figure 5.8 D-MC promotes apoptosis in cells lacking p53 through the translocation of AIF from the mitochondria to the nucleus.

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Chapter 6. Summary

The p53 is a multi-layered mechanism that results from the convergance of numerous pathways following stress. At the level of the p53 responsive element, we found that p53 becomes activated on chromatin through the dissociation of its inhibitory complex with Mdm2. Furthermore, Mdm2 was found to re-associate with p53 after an intial period of transcriptional activity. This reformation of the Mdm2-p53 complex on chromatin correlated with decreases in transcription. In examining the ability of p53 to regulate downstream pathways, we found that p53 does not dictate the fate of the cell by differentially regulating the downstream target genes. Induction of p53 protein and p53 target gene expression is not always sufficient to promote apoptosis. Each type of cellular stress has a minimum level of p53 activity required for its ability to shift the cell from a growth arrest to an apoptotic cell fate. Our data suggest that the ability of a DNA damaging agent to promote apoptosis through p53 relies on the ability of the drug to activate cooperative pathways that contribute to the p53 response. In the case of drugs with very similar chemical compositions, we found that mitomycin C (MC) and its derivative 10-decarbamoyl mitomycin C (D-MC) mediate p53-dependent apoptosis through slightly different pathways. While MC was only capable of mediating apoptosis in cells expressing p53, D-MC was capable of inducing apoptosis through distinct pathways in the presence or absence of p53.

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Chapter 7 Preliminary Data and Future Directions

7.1 Chk2 is not activated by MC and DMC in cells that lack wild-type p53.

Since the pathways that mediate MC and DMC mediated apoptosis in p53 containing cells, as well as the mechanism that promotes p53-independent apoptosis in response to DMC have not been elucidated, we looked at numerous upstream factors that might play a role in these phenomena. One of the principle DNA damage sensing kinases is the phosphoinositol-3-kinase (PI3K) family member, ataxia-telangiectasia-mutated (ATM). ATM has been shown to signal to p53 directly, through phosphorylation at serine 15, or indirectly, through the activation of Chk2, which phosphorylates p53 at threonine 18 and serine 20 (Hirao et al., 2002; Nelson and Kastan, 1994; Shieh et al., 2000). These phosphorylation events are critical for the activation of p53 because they serve to dissociate p53 from its negative regulator, Mdm2, following stress (Hirao et al., 2000). Phosphorylation of p53 by ATM and Chk2 has been shown to facilitate p53 dependent growth arrest or apoptosis (Dumaz et al., 2001; Unger et al., 1999b). Although it has been clearly shown that ATM is required for p53 dependent apoptosis in response to some types of DNA damage, Chk2 is capable of promoting p53 dependent apoptosis in the absence of ATM (Hirao et al., 2002). To examine the possible role of Chk2 in MC and DMC mediated DNA damage response, nuclear extracts from ML-1 and K562 cells treated with these agonists were analyzed by Western blot using a phosphorylated Chk2 specific antibody. The active form of Chk2 was only found in ML-1 cells treated with MC and DMC (Figure 7.1A). In addition, we found that p53 induced

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by MC and DMC was phosphorylated on serine 15, a known phosphorylation site targeted by activated ATM (Figure 7.1B, middle panel). These data suggest that ATM may be activated by MC and DMC treatment, and that as a result, both p53 and Chk2 are downstream effectors of its activity. Although Chk2 was not phosphorylated in K562 cells, we cannot rule out ATM in the response to either agent.

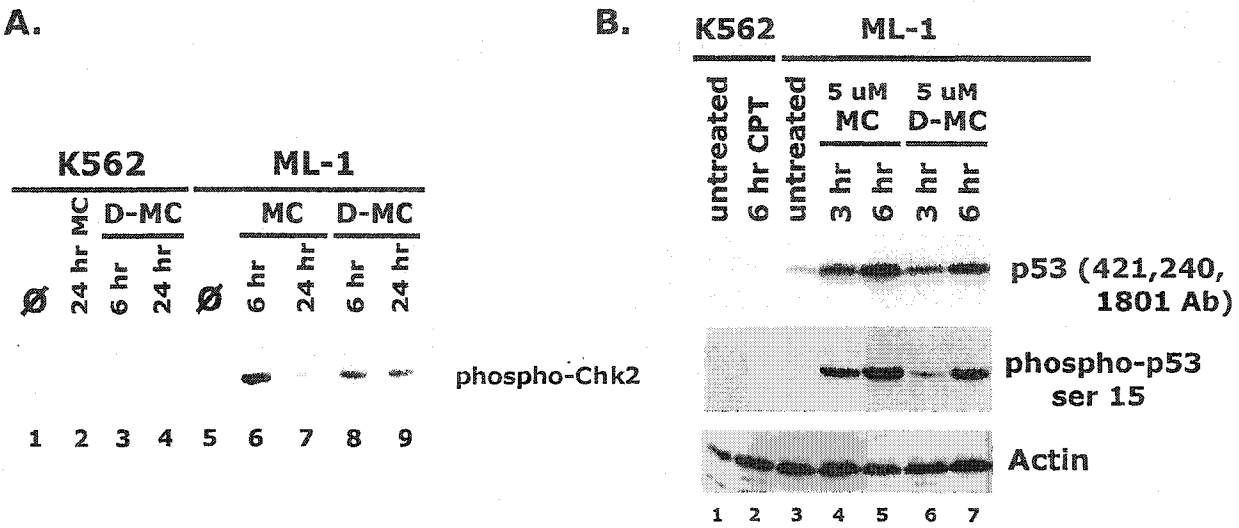

Figure 7.1 Chk2, and possibly ATM, is only activated in ML-1 cells that contain a p53 response. *Nuclear extract isolated from ML-1 and K562 cells grown in the presence or absence of 5 uM MC or D-MC for 3, 6, and 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for phosphor-Chk2 (A), p53 (B, top panel), phosphserine-15 p53 (B, middle panel), or actin (B, bottom panel). NOTE: Figure 7.1A was only done one time.*

MC and D-MC differentially activate stress responsive pathways. MC treatment was found to stabilize p73 protein in ML-1 cells. Additionally, the kinetics of Chk2

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activation differs between these mitomycins. Furthermore, the p53 protein induced by D-MC exhibited greater transcriptional activity than p53 stabilized in response to MC. Further studies analyzing the activity of the stress responsive kinases upstream of p53 in response to MC and D-MC may help elucidate the different response pathways utilized by these drugs. These studies would also aid in determining which proteins selectively activated by D-MC may aid in boosting the p53 transcriptional response.

7.2 D-MC also induces p53-independent cell death in P1299 cells

In order to further substantiate the effectiveness of D-MC in promoting cell death in cell lines lacking wild type p53, we treated two lung cancer cell lines, H460 which expressed wild type p53, and P1299 which were p53 null, with equimolar dosages of MC and D-MC. Several methods, including FACS analysis, Annexin staining, and the cleavage of PARP-1, were employed to determine whether apoptosis resulted from treatment. H460 cells exhibited an increase in the population of sub-G1 cells in response to both MC and D-MC. Additionally, drastic increases in the level of Annexin-stained cells were observed following treatment with both agents for 24 hrs (Figure 7.2A). Furthermore, cleavage of PARP-1 was also evident in this cell line treated with either drug (Figure 7.2C, lanes 5 through 8). Taken together, these data suggest that both MC and D-MC induce apoptosis in H460 cells. The p53 null cell line P1299 exhibited a significant increase in sub-G1 population following treatment with D-MC, but not MC, for 24 hrs. This correlated with an increase in Annexin staining noted in the D-MC, but not in MC, treated cells (Figure 7.2B). Modest levels of PARP-1 cleavage were also

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observed in P1299 cells treated with 10 uM D-MC, but not with similar doses of MC (Figure 7.2C, lanes 1 through 4). The amount of cleaved PARP-1 protein in D-MC treated P1299 cells was significantly less than what was observed in H460 cells, but these data correlated with the reduced levels of sub-G1 populous and Annexin staining also noted in this cell line (Figure 7.2A, B and C).

To determine whether MC and D-MC mediated cell death in H460 and P1299 cells involved activation of caspase-9, the level of procaspase-9 was examined via Western blotting. The levels of procaspase-9 was significantly less in P1299 cells treated with 10 uM D-MC, which suggested that caspase-9 may be active during D-MC-mediated p53-independent cell death (Figure 7.2D, lane 4). These supported earlier findings in the K562 cells that showed low levels of caspase activity following D-MC treatment (Figure 7.2D). Procaspase-9 was also cleaved in H460 cells in response to both MC and D-MC (Figure 7.2D, lanes 7 through 9). These data correlated with the activation of caspase-9 in ML-1 cells treated with these agents, as well as with earlier data that showed the activation of caspase-9 in MC-mediated cell death (Park et al., 2004). These data confirmed that D-MC induces cell death in other cell lines regardless of their p53 status.

Figure 7.2 D-MC promotes cell death in other cell lines regardless of their p53 status. *H460 (A) and P1299 (B) cells were grown in the presence or absence of 5 uM MC or D-MC for 24 hrs. Samples were fixed and either stained with propidium iodide (left panel) or stained with Annexin (right panel) and then analyzed with a flow cytometer.*

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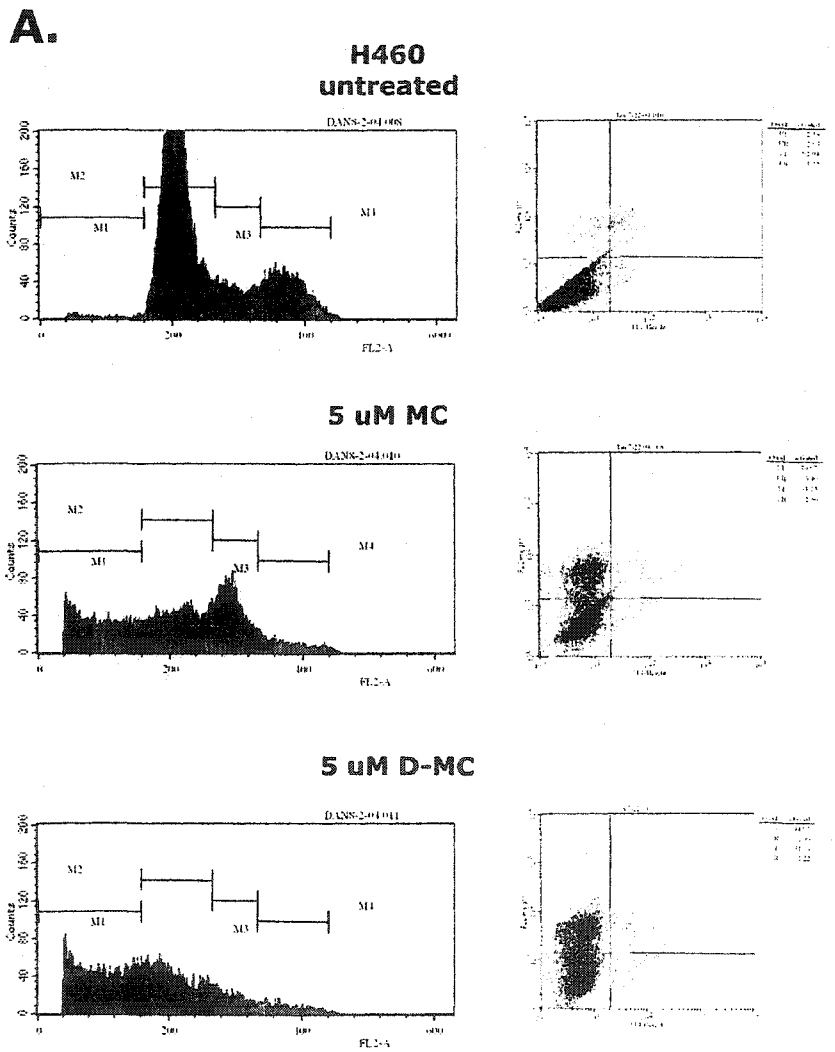
Nuclear extract isolated from H460 and P1299 cells grown in the presence or absence of 5 or 10 uM MC and D-MC for 24 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. This membrane was probed with antibodies specific for PARP-1 (C). Cytoplasmic extract from the same cells were similarly electrophoresed and the resulting gel was electrotransferred to nitrocellulose. This membrane was probed for pro caspase-9 (D, top panel), or actin (D, bottom panel). NOTE: The data in this figure was only done one time.



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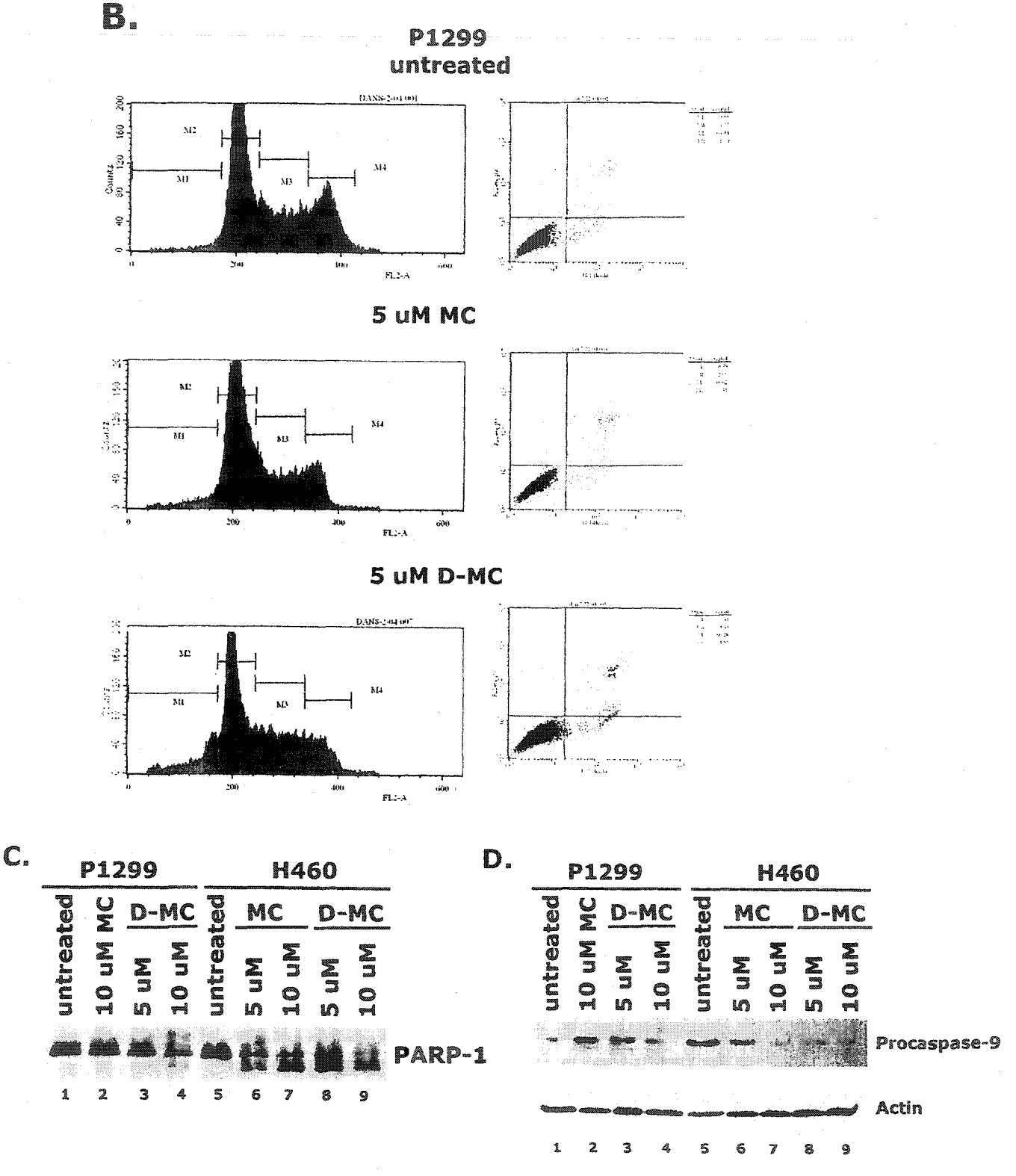

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7.3 Inhibition and depletion of PKC delta does not inhibit the stabilization of p53 in wild type p53 and p19arf null MEFs.

Although p19arf, the murine homologue of the human p14arf, has been shown to play a role in stabilizing p53 protein in response to oncogenic stimulation, the role of p19arf in p53's DNA damage response is still being elucidated. Recent work by Khan and colleagues showed that p14arf played a role in stabilizing p53 protein following treatment with ionizing radiation (IR) (Khan et al., 2004). This contradicted earlier work which suggested that p14arf did not play a role in the response of p53 to DNA damage (Stott et al., 1998). To examine whether the loss of p19arf affected the ability of p53 protein to be stabilized, mouse embryonic fibroblasts that were either wild type for both p53 and p19arf, wild type for p53 but p19arf null, or null for both p53 and p19arf were treated with increasing concentrations of the DNA damaging agent camptothecin (CPT) or the proteasome inhibitor LLnL. To determine if this stabilized p53 protein elicited a normal downstream response, we also examined the level of p21 protein. Surprisingly, the loss of p19arf did not affect the ability of p53 to be stabilized by either agent. Furthermore, the higher levels of p53 protein were capable of inducing p21 protein (Figure 7.3A and B). These data support earlier findings that p19arf is not required for the induction of p53 protein in response to stress.

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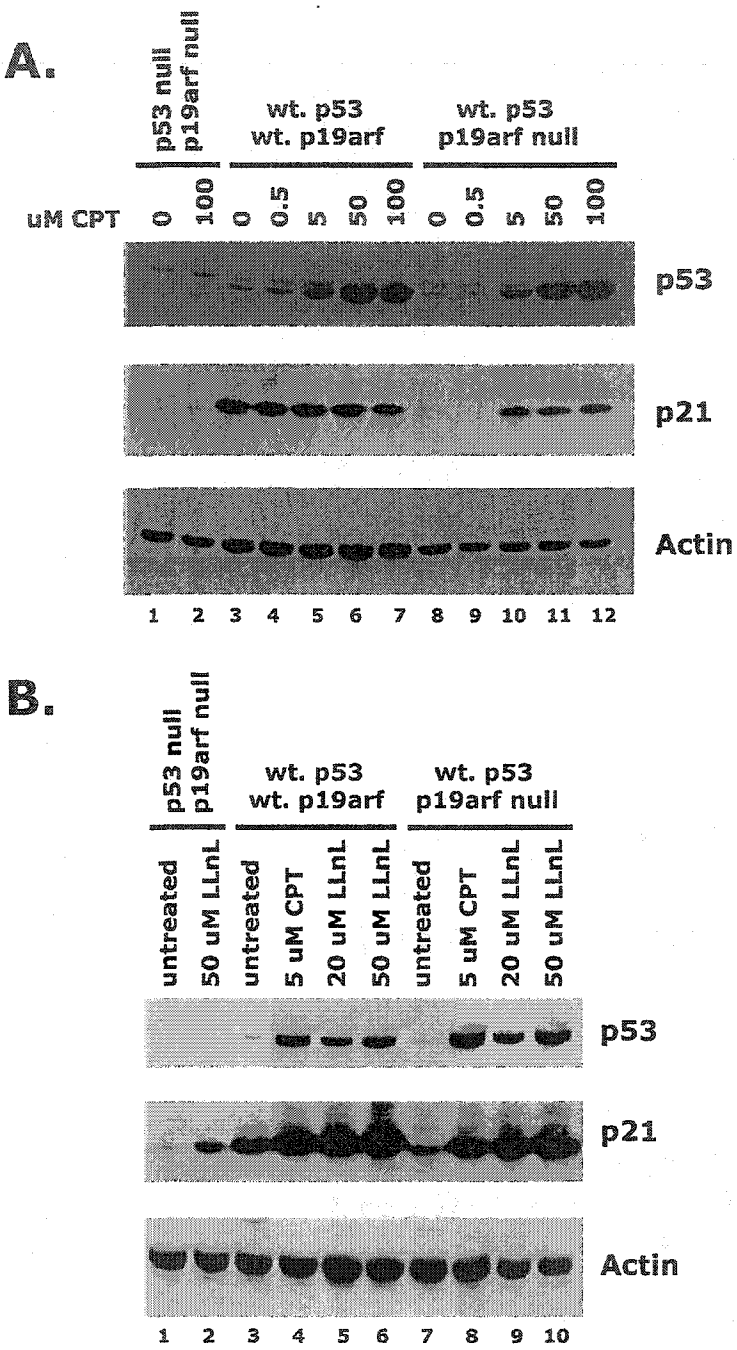

Figure 7.3 p19arf is not required for the activation of p53 protein in response to stress. *Nuclear extract isolated from p53 and p19arf wild type and null MEFs grown in the presence or absence of increasing concentrations of either CPT (A) or LLnL (B) for 6 hrs was electrophoresed on a 10% SDS-PAGE, and the separated protein was*

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electrotransferred onto a nitrocellulose membrane. These membranes were probed with antibodies specific for p53 (Ab 240 and 421, top panel), p21 (AB-4, middle panel), or actin (bottom panel).

Previous work in our lab showed that the downregulation of PKC delta resulted in decreased levels of p53 transcript. Repression of p53 transcription inhibited the stabilization of p53 protein following treatment with DNA damaging agents or proteasome inhibitors. Additionally, the lack of p53 induction also prevented the p53-dependent upregulation of target genes and prevented apoptosis in drug treated cells. In this study, two methods were employed to downregulate PKC delta. The first method was to pre-treat cells with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), which initially activates PKC. Following activation by TPA, however, the levels of PKC decrease in the cell. The specific inhibitor of PKC delta, rottlerin, was also employed to inhibit PKC activity (Abbas et al., 2004). Similar levels of p53 protein were induced in wild type p53 containing MEFs regardless of their p19arf status treated with 5 uM CPT and 50 uM LLnL (Figure 7.3A and B). To determine if PKC delta was required for the stabilization of p53 in response to these agents, MEFs were pretreated with TPA or rottlerin. Surprisingly, increasing dosages of rottlerin, from 6 uM (data not shown) to 24 uM did not block the induction of p53 protein in response to either CPT or LLnL treatment (Figure 7.4A). Rottlerin treatment also did not interfere with the ability of p53 to promote p21 induction (Figure 7.4A, middle panel). Furthermore, the loss of p19arf did not affect the p53 response (Figure 7.4A, lanes 11 through 20). Like rottlerin, pretreatment of MEFs with TPA failed to block p53 stabilization in response to CPT and

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LLnL (Figure 7.4B). Additionally, p21 protein was efficiently induced by these higher levels of p53 protein (Figure 7.4B, p21). Consistent with previous results, the loss of p19arf did not affect induction of p53 protein in response to drug or the ability of this p53 to induce p21 protein (Figure 7.4B, lanes 13 through 18). Surprisingly, TPA treatment did not decrease the levels of nuclear PKC delta (Figure 7.4B, PKC delta). To determine if TPA treatment failed to downregulate PKC delta in the cytoplasm as well, the level of PKC delta protein was examined in drug treated cytoplasmic extracts (Figure 7.4C). Interestingly, PKC delta was effectively downregulated in the cytoplasm, but not in the nucleus (Figures 7.4B and C). These data are inconclusive, and could suggest that PKC delta is not required for the regulation of p53 protein. More work needs to be carried out in order to determine if the failure of these downregulators of PKC delta activity to block p53 stabilization was not due to insufficient inhibitor dosage or to some unknown compensatory mechanism. The use of small interfering RNA (siRNA) to block PKC delta expression may be a more effective tool for examining the role of PKC delta in murine p53 regulation.

Figure 7.4 Inhibition and depletion of PKC delta does not prevent the p19arf-independent induction of p53 in response to CPT and LLnL treatment. *Nuclear extract isolated from p53 and p19arf wild type and null MEFs grown in the presence or absence of 5 uM CPT or 50 uM LLnL and pretreated with increasing concentrations of either Rottlerin (A) or 400 nM TPA (B) was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. These membranes were probed with antibodies specific for p53 (Ab 240 and 421, top panel),*

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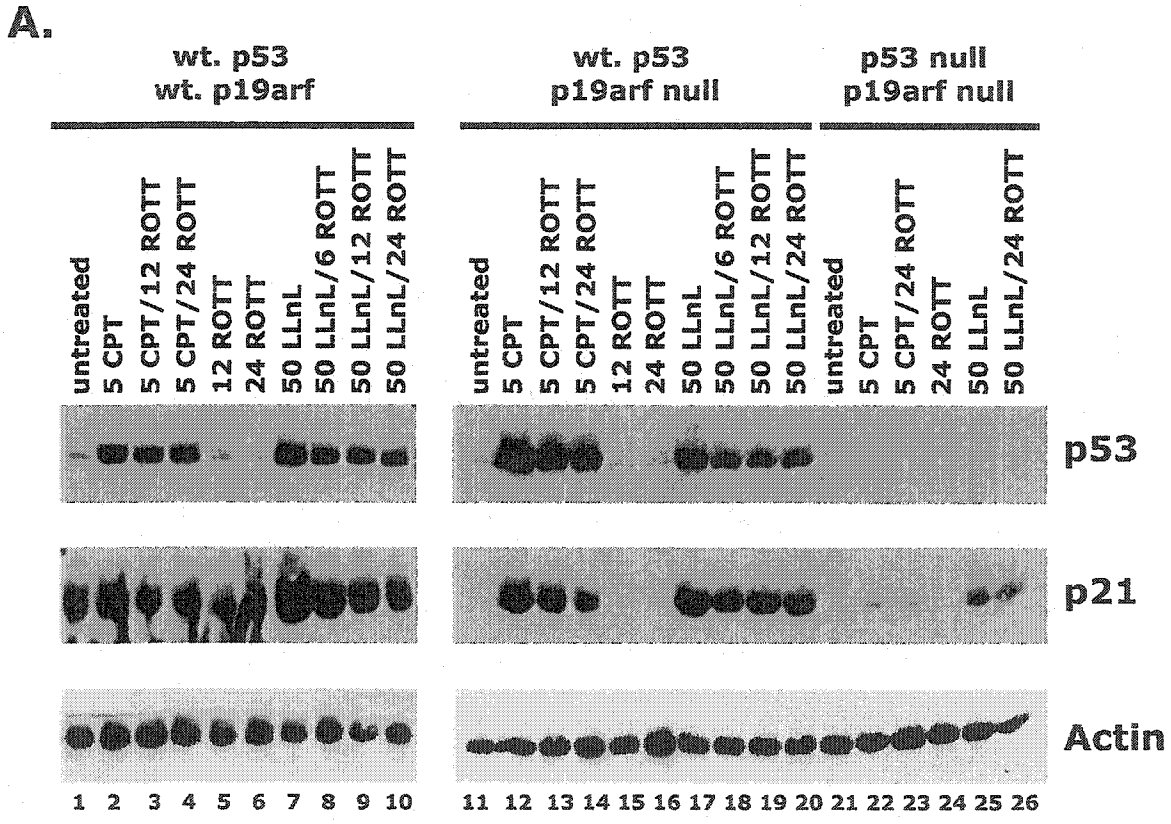
p21 (AB-4, middle panel), or actin (bottom panel). The level of nuclear PKC delta was examined in B, second lowest panel using the PKC delta specific antibody sc-213. (C) Cytoplasmic extract was also isolated from p53 and p19arf wild type and null MEFs grown in the presence or absence of 5 uM CPT or 50 uM LLnL and pretreated of 400 nM TPA was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. These membranes were probed with antibodies specific for PKC delta (sc-213, top panel), or actin (bottom panel).



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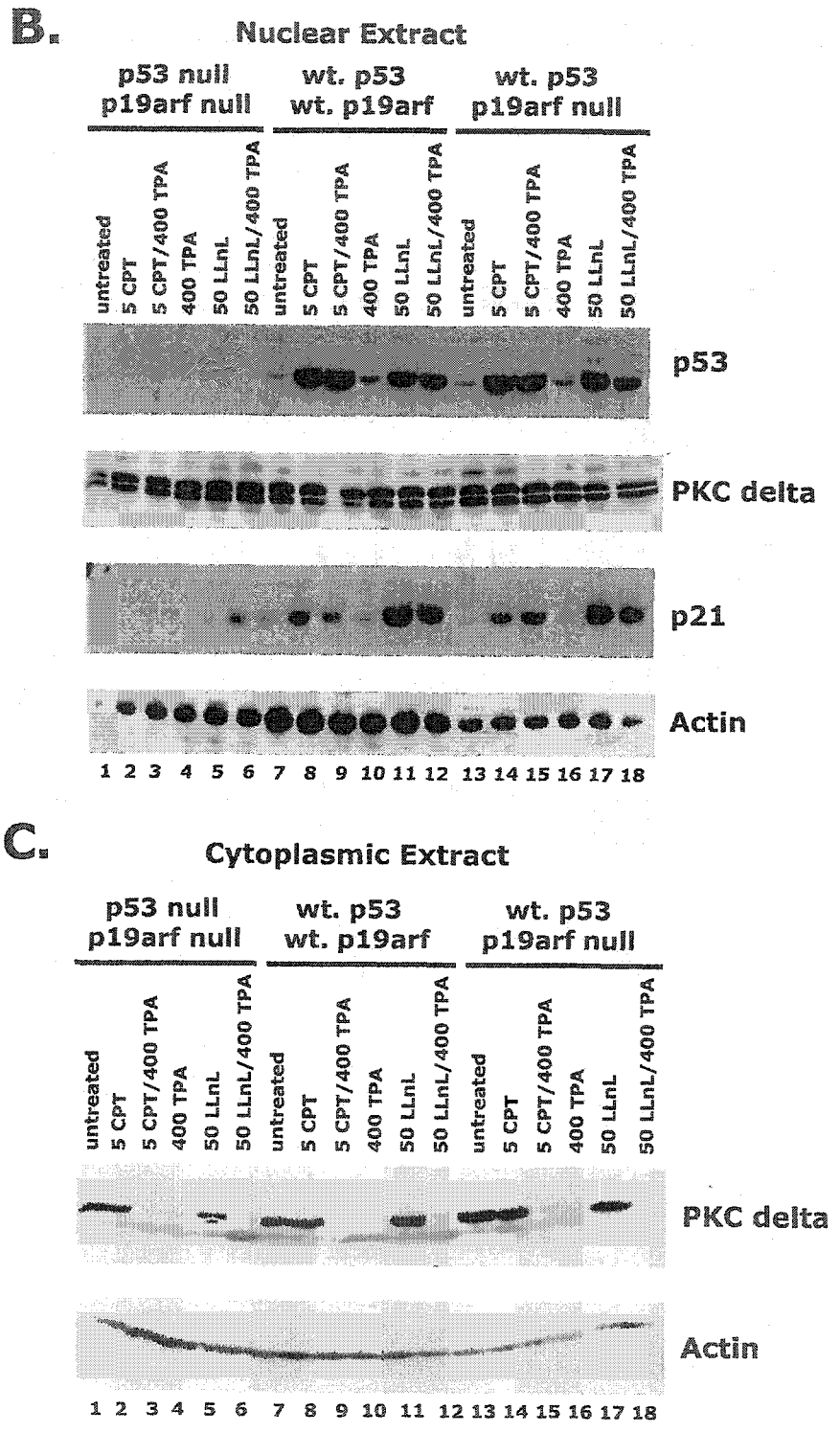

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7.4 Cycloheximide treatment increases p53 target gene expression

p53 differentially regulates its downstream target genes (Abbas et al., 2002; Szak et al., 2001; Wu et al., 1999). Previous work by Xiao et al in our lab showed that high levels of p53 protein expression in the absence of damage were capable of inducing *mdm2* and *waf1* transcription, but not *gadd45*. In order for p53 to induce the *gadd45* gene, a DNA damage signal was required (Xiao et al., 2000). Additionally, Zheng and colleagues showed that BRCA-1 acts as a co-repressor with the zinc finger protein ZBRK1 at a region upstream of the p53 binding site on the 3rd intron of gadd45. Binding of the BRCA-1/ZBRK1 complex represses transcription of *gadd45* (Zheng et al., 2000). These data suggest that p53 may have been prevented from mediating transcription of *gadd45* because of the presence of this or another repressor complex. To address this hypothesis, we treated TR9-7 cells with the protein synthesis inhibitor cycloheximide (CHX) in order to prevent the synthesis of these repressor molecules thereby relieving the repression inherently present at this loci. If cycloheximide treatment induced *gadd45* transcription when p53 was present in the absence of damage, then that may suggest that a repressor complex was participating in preventing the regulation of this gene by p53. Furthermore, cycloheximide treatment must only induce *gadd45* transcription, and not promote the induction of other downstream target genes of p53, like *waf1*, known to be regulated without repressors.

To address whether a repressor may be preventing the upregulation of gadd45 in the absence of a damage signal, the p53 regulatable cell line TR9-7 was treated with

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increasing concentrations of CHX in the presence or absence of CPT (Figure 7.5A, lanes 3 through 5). The level of p53 protein expression was not affected by CPT treatment or pretreatment with CHX (Figure 7.5A). Inhibition of p53 protein expression in response to the lowest dosage of CHX after 25 hrs of treatment suggested that the lowest dosage of CHX was capable of effectively inhibiting protein synthesis (Figure 7.5B, lanes 6 through 9). Quantitative PCR using molecular beacons was then utilized to examine the level of *gadd45* and *waf1* transcripts in TR9-7 cells treated with CHX in the presence or absence of CPT. Surprisingly, CHX treatment not only induced *gadd45* transcription in the presence of high levels of p53 and no DNA damage, but it also robustly activated waf1. Lower levels of *gadd45* and *waf1* transcription were evident in the presence of both CHX and CPT (Figures 7.5C and D). These data suggest that the induction of *gadd45* by p53 in the presence of CHX was not due to a relief in repression, but a general enhancement of the p53 response. Further studies need to be carried out in order to determine if inherent repression exists on the *gadd45* gene that prevents its ability to be transcriptionally activated by p53 in the absence of stress. One possible way to address this is to determine if the ZBRK1 binding site is required for the repression of gadd45 transcription in the absence of damage. Transfection of TR9-7 cells (-TET) with a reporter construct containing either the full 3rd intron of the *gadd45* gene or just the p53 binding site upstream of a SV40 promoter may help delineate whether this repressor binding site is required for the inherent repression seemingly present at this loci.

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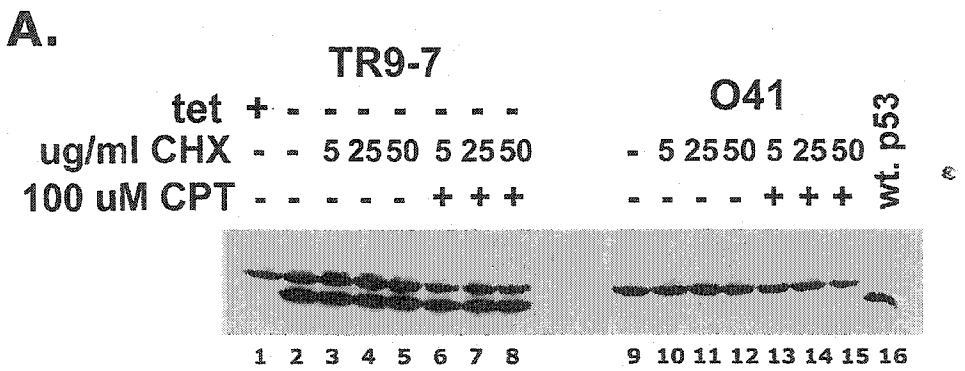
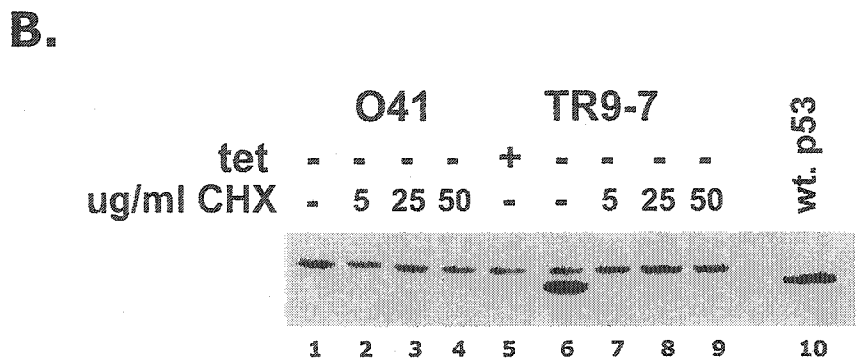
Figure 7.5 CHX treatment enhances p53-dependent *gadd45* and *waf1* transcription. *Nuclear extract isolated from O41 and TR9-7 cells grown in the presence or absence of tetracycline for 24 hrs and 100 uM CPT for 4 hrs. These cells were pretreated with increasing concentrations of either of CHX for 6 hrs (A) or 25 hrs (B) and the resulting nuclear extract was electrophoresed on a 10% SDS-PAGE, and the separated protein was electrotransferred onto a nitrocellulose membrane. These membranes were probed with antibodies specific for p53 (Ab 240, 1801, and 421). Quantitative PCR using molecular beacons specific for gadd45 and waf1 were utilized to analyze the expression of these p53 target genes in RNA isolated from TR9-7 and O41 cells treated with 100 uM CPT for 4 hrs and 5 ug/ml CHX for 6 hrs. Tetracycline was removed from TR9-7 cells for 18 hrs prior to CHX addition and 20 hrs prior to treatment with CPT.*





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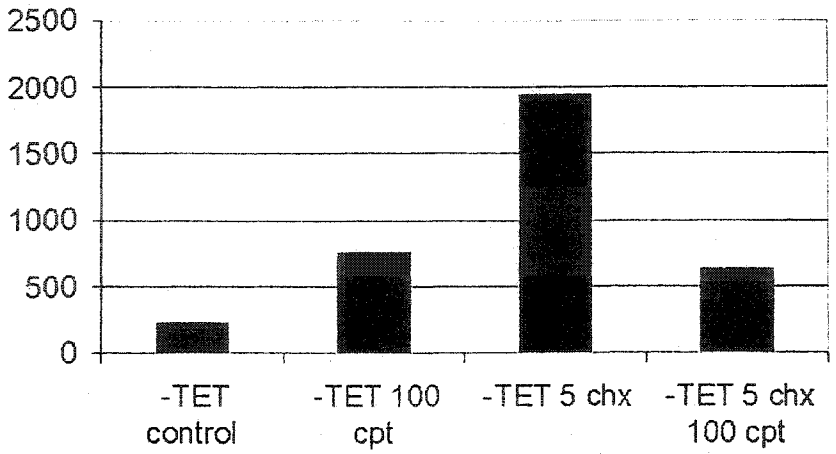
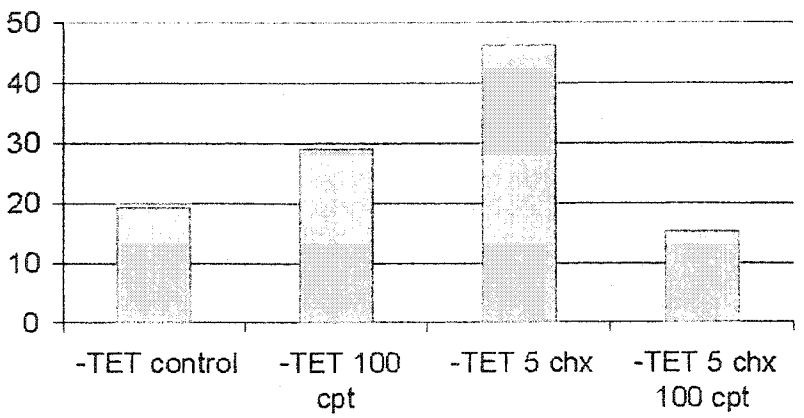
C.



gadd45

D.



waf1

Figure 7.5 cont.

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