ERK Activation Mediates Cell Cycle Arrest and Apoptosis After DNA Damage Independently of p53

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Running Title: Erk Activation Mediates Cellular DNA Damage Responses
Abstract

In response to DNA damage, ATM/ATR activate p53, resulting in either cell cycle arrest or apoptosis. We report here that DNA damage stimuli, including etoposide (ETOP), adriamycin (ADR), ionizing irradiation (IR), and ultraviolet irradiation (UV) activate Erk1/2 (Erk) mitogen activated protein kinase in primary (MEF and IMR90), immortalized (NIH3T3) and transformed (MCF-7) cells. Erk activation in response to ETOP was abolished in ATM-/- fibroblasts (GM05823) and was independent of p53. The MEK1 inhibitor, PD98059 prevented Erk activation but not p53 stabilization. Maximal Erk activation in response to DNA damage was not attenuated in MEFp53-/- . However, Erk activation contributes to either cell cycle arrest or apoptosis in response to low or high intensity DNA insults, respectively. Inhibition of Erk activation by PD98059 or U0126 attenuated p21CIP1 induction, resulting in partial release of the G2/M cell cycle arrest induced by ETOP. Furthermore, PD98059 or U0126 also strongly attenuated apoptosis induced by high dose ETOP, ADR or UV. Conversely, enforced activation of Erk by overexpression of MEK-1/Q56P sensitized cells to DNA-damage induced apoptosis. Taken together, these results indicate that DNA damage activates parallel Erk and p53 pathways in an ATM dependent manner. These pathways might function cooperatively in cell cycle arrest and apoptosis.

Key Words: apoptosis / cell cycle / DNA damage / mitogen activated protein kinase
Introduction

Eukaryotic cells employ multiple mechanisms to ensure accurate transmission of genetic information between generations. Critical surveillance of this transmission is provided by the DNA damage response, which may arrest the cell cycle to allow damage repair, or direct cells to apoptosis in situations of severe damage 1-4.

Our understanding of how cells sense DNA damage remains incomplete, but it is clear that two members of the phosphatidylinositol-3 (PI-3) kinase family, ATM and ATR, are major DNA damage signal transducers 4. Downstream of ATM/ATR lies p53, a central mediator of the response, which in turn induces cell cycle arrest by up-regulation of p21CIP1 and 14-3-3σ, activates DNA damage repair pathways, and induces apoptosis 5;6. p53 is normally rapidly degraded by Mdm2-mediated ubiquitin-dependent proteolysis 7;8. In response to DNA damage, p53 is stabilized by inhibition of this proteolytic process, partly through post-translational phosphorylation. Stabilization of p53 by phosphorylation on residues S15 and S21 by ATM and Chk2 kinase in response to DNA damage is well-described 9-11. Members of the mitogen activated protein kinase (MAPK) family have also been demonstrated to phosphorylate and stabilize p53 12-14. To wit, activation of JNK leads to p53 phosphorylation, thus interfering with the association of Mdm2 and p53 12. Furthermore, activation of p38 MAPK by UV was shown to phosphorylate p53 on S389 13;14.

The third member of the canonical MAPK family, Erk (extracellular-signal-regulated
kinase), is centered on multiple signal transduction pathways to accomplish a variety of functions. Activation of Erk through different pathways leads to fundamentally different cellular responses, including proliferation, differentiation, survival, and memory consolidation 15-19. Erk activation by extracellular growth signals is mediated through growth factor tyrosine phosphorylation and activation of a small G protein, Ras, and promotes cell proliferation 20;21. The importance of Erk in transduction of mitogenic signals is illustrated by the demonstration that activation of Erk is sufficient to transform NIH3T3 cells or MEF lacking either p53 or p16 22;23. In some circumstances, ligand interactions with growth factor receptors may result in Erk-mediated cell cycle exit. Indeed, Erk activation by nerve growth factor (NGF) drives PC12 cell differentiation 22;24.

Erk may function in the response to DNA damage. Erk activation was observed in response to cisplatin in ovarian cancer cells 25. Erk can phosphorylate p53 in vitro 25, but its role in vivo is unclear 25-27. The role of DNA damage-induced Erk activation in mediating apoptosis has also not been clarified 28;29. In this regard, however, tumors with constitutive Erk activation undergo apoptosis when Erk activity is blocked 30.

Consequently, we sought to explore Erk activation in response to DNA damage, and to outline the mechanisms and consequences of such activation. We report here that multiple DNA damage stimuli, including etoposide (ETOP), adriamycin (ADR), ultraviolet irradiation (UV) and ionizing radiation (IR) activate Erk in various cell lines. We show that this process depends on ATM and is independent of p53. Functionally, we demonstrate that Erk activation cooperates
with p53 to lead to apoptosis or cell cycle arrest. This represents the first report to fully outline an Erk-mediated DNA damage pathway and its functional importance.
Materials and Methods

Materials, cell lines, and plasmids.

Hoechst 33258, Propidium iodide (PI), ETOP, ADR, HU, and wortmannin were from Sigma. The MEK1 inhibitors, PD98059 and U0126 were from Calbiochem and Promega, respectively. Stocks of ETOP, ADR, wortmannin, PD98059, and U0126 were made in DMSO and used at the concentrations indicated. Hygromycin B was from GIBCO BRL.

NIH3T3 and MCF7 cells were cultured in DMEM supplemented with 10% fetal calf serum (Gibco BRL) at 37°C in a tissue culture incubator. MEF were isolated and cultured in DMEM, 10% FCS, 55µM β-mercaptoethanol and 10µg/ml gentamycin. IMR-90 was from ATCC and cultured in MEM and 10% FCS. The AT fibroblast line, GM05823, was from NIGMS Human Genetic Cell Repository and cultured in MEM, 10% FCS (uninactivated), with 2X concentrated essential and non-essential amino acids and vitamins.

The constitutively activated MEK1/Q56P cloned in the retroviral vector, pBabe, was kindly provided by Dr. Scott Lowe of Cold Spring Harbor Laboratories. pBabe/Bcl-2 and pBabe/Bcl-xL were constructed by insertion of human Bcl-2 and Bcl-xL into pBabe as we have published.

Retroviral Infection

Retroviral infection was performed as we have published. Constructs were subcloned into a retroviral vector. Plasmid DNA was isolated and used to generate retrovirus
using phoenix packing cells. Briefly, $5 \times 10^6$ phoenix cells were seeded in a 10 cm plate overnight before transfection with 15 $\mu$g retroviral vector for 48 hours. The virus-containing medium was harvested after 48 hours and filtered through a 0.45 $\mu$M filter. After addition of 10 $\mu$g/ml of polybrene (Sigma), the medium was used to infect NIH3T3 cells three times for 24 hours at 8 hour intervals to maximize transfection rate. Expression was confirmed with western blot.

**Cell lysis and western blot.**

After exposure to DNA damage agents as indicated, cells were lysed in a buffer containing 20mM Tris (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 25mM sodium pyrophosphate, 1mM NaF, 1mM $\beta$-glycerophosphate, 0.1mM sodium orthovanadate, 1mM PMSF, 2$\mu$g/ml leupeptin and 10$\mu$g/ml aprotinin. 50$\mu$g of total cell lysate was separated on SDS-PAGE gel and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked with 5% skim milk and then incubated with the indicated antibodies at room temperature for 1 hour. Signals were detected using an ECL Western Blotting Kit (Amersham). Primary antibodies and concentration used were: anti-ATM (Ab-3 at 2$\mu$g/ml, Oncogene); anti-p53 (FL-353 at 1$\mu$g/ml, Santa Cruz); anti-phospho-p53(S15) (Cell Signaling, 1:1000); anti-Erk (1:500, New England Biolabs); anti-phospho-Erk (1:500 New England Biolabs); anti-Actin (Santa Cruz); anti-p21CIP1 (1$\mu$g/ml, Santa Cruz).

**Apoptosis assay, viability assay, and determination of cell cycle distribution.**

DNA fragmentation and TUNEL assay were performed as we have previously published
31-33. For the cell viability assays, 2 x 10⁴ cells were seeded in 96 well plates and incubated at 37°C for 4 hours. Cells were then treated with DNA damage stimuli as indicated with or without PD98059 50µM or U0126 (10 or 50µM) for the indicated times. WST-1 cell proliferation reagent (Roche Diagnostics, Mannheim) 10µl/100µl medium was added and cells incubated for 30 minutes at 37°C. Viable cell numbers were determined at 450nm with a microplate reader (Fisher Diagnostics). Cell cycle distribution was determined by staining NIH3T3 cells with PI solution in the presence of RNase A overnight at 4°C followed by detection of signals using fluorescent automated cell sorting (FACS) as we have published 11.

**Mitotic Index**

Cells were grown to 80% confluence on a glass slip. After treatment with ETOP at the indicated concentrations for 30 minutes, nocodazole (200ng/ml) was added. After 12, 18, 24 and 30 hours cells were fixed in 3.7% formaldehyde and stored at 4°C for 24 hours. Cells were then permeabilized in a Triton X-100 containing solution and stained with Hoechst 33258 DNA stain (10µg/ml; Molecular Probes). The mitotic index was scored under a fluorescent microscope.
Results

*Multiple DNA damage stimuli activate Erk in several cell lines.* To determine whether Erk is activated by DNA damage signals, early passage MEF cells were treated with ETOP, which induces double strand DNA breaks by interfering with the function of DNA topoisomerase II [34]. As expected, ETOP induced stabilization of p53 and up-regulation of p21CIP1 (data not shown). ETOP induced Erk activation (as measured by phosphorylation) with two phases (Fig. 1A). The first phase of Erk activation starts at 1hr and ends at 8hr of ETOP exposure, while the second takes place after 15hr in response to ETOP (Fig. 1A). Increasing ETOP concentrations resulted in increasing levels of Erk activation with maximal Erk activation observed at or higher than 25µM (data not shown), indicating a correlation between intensity of DNA damage and level of Erk activation. To determine whether Erk activation is an unique property of ETOP or MEF cells, we used multiple DNA damage stimuli to initiate Erk activation. ETOP, ADR and UV led to Erk activation in primary (MEF), immortalized (NIH3T3), and transformed (MCF7) cells (Fig. 1B). Additionally, IR and HU also activated Erk in MEF and IMR90 (data not shown), confirming that DNA damage indeed leads to Erk activation.

Since Erk activation is known to require dual phosphorylation on T183 and Y185 by
MEKs, we sought to determine whether DNA damage-induced Erk activation also requires MEK activity. Addition of the MEK1 inhibitors, PD98059 (Fig 3B) and U0126 (data not shown), prevented ETOP induced Erk activation. Complete abrogation of Erk activation was also observed when cells incubated with PD98059 or U0126 were exposed to ADR, UV or IR (data not shown), confirming the role of MEK1 in mediating DNA damage-induced Erk activation. Taken together, these results show that Erk activation is a component of cellular DNA damage responses initiated by diverse stimuli.

Erk activation in response to ETOP requires ATM. Since ATM plays a central role in the relay of DNA damage signals and ATM has been shown to play a role in the DNA damage-induced activation of JNK and p38, we sought to determine if ATM played a role in DNA damage-induced Erk activation. As a member of the PI-3 kinase family, the kinase activity of ATM is inhibited by the PI-3 kinase inhibitor, wortmannin. Addition of wortmannin dose-dependently inhibited ETOP but not UV induced Erk activation (data not shown), consistent with the fact that wortmannin inhibits the kinase activity of ATM but not ATR and that UV induced DNA damage responses are generally ATR dependent. To confirm ATM dependence of ETOP-induced Erk activation, both ATM-/- (GM05823) and IMR90 (wild type) human fibroblasts were used. Expression of ATM in IMR90 but not in GM05823 was confirmed by western blot using an anti-ATM (Ab-3) antibody (data not shown). Addition of ADR or ETOP to IMR90 cells led to p53 stabilization, associated with phosphorylation on S15 and up-regulation of p21CIP1 (Fig. 2). S15 phosphorylation-associated p53 stabilization was significantly reduced and p21CIP1 induction was abolished in GM05823 cells in response to
ADR or ETOP (Fig. 2), further confirming the lack of ATM function in the ATM-/- fibroblasts. Importantly, ETOP induced Erk activation was absent in GM05823 but present in IMR90 (Fig. 2), confirming a role of ATM in ETOP induced Erk activation. However, ADR induced Erk activation was not attenuated in the ATM-/- fibroblast line (Fig 2), suggesting that ATR might also play an important role in this setting. Caffeine (2mM), which inhibits both ATM and ATR did prevent ADR induced Erk activation in this context, supporting the supposition that ATR plays a role in the transmission of ADR mediated DNA damage to Erk (data not shown).

**DNA damage induced Erk activation is independent of p53.** Since p53 is the major effector protein downstream of ATM mediating the DNA damage response, we sought to investigate if Erk activation played a role in the p53 pathway, as has been suggested by others. Kinetically, p53 stabilization by ETOP (Fig 3A), ADR and IR (data not shown) preceded Erk activation in all cell lines studied. Fig 3A shows MCF-7 cells. MEK1 inhibition with PD98059 inhibited Erk activation but was without effect on p53 stabilization in response to DNA damage in MEF, NIH3T3 and MCF-7 cells (Fig. 3B; NIH3T3), suggesting that Erk activation was not upstream of p53. Conversely, pre-treatment of cells with cycloheximide (CHX) for 30 minutes to destroy endogenous p53 prevented ETOP (data not shown) and ADR (Fig.3C; NIH3T3) induced p53 stabilization but not Erk activation, indicating that Erk activation is not downstream of p53. However, given conflicting data suggesting that Erk activation may be either upstream or downstream of p53 after DNA damage, we wished to confirm our observations. In support of our results, maximal level of Erk activation was not attenuated in MEFp53-/- compared to wild type MEF (Fig. 3D inset), although the fold increase of Erk
activation is slightly lower in MEFp53-/- due to higher basal level of Erk activity in these p53 negative cells (Fig 3D). ADR and UV also induced Erk activation in MEFp53-/- (data not shown). Taken together, these data indicate quite conclusively that under physiologic conditions, DNA damage- induced Erk activation is independent of p53.

**Erk activation maximizes p21CIP1 induction upon DNA damage.** The two paradigmatic cellular responses to DNA damage are cell cycle arrest (allowing the damage to be repaired) or apoptosis in response to low or high intensities of DNA damage respectively. The Cdk inhibitor, p21CIP1 is up-regulated in this process, and plays a major role in arresting cells in either G1 or at G2/M in response to DNA damage 42;43. We have observed that wortmannin affected Erk activation and p21CIP1 induction in a more clearly dose dependent manner than p53 stabilization after DNA damage (data not shown), suggesting that Erk activation might up-regulate p21CIP1. This is consistent with the observations that enforced Erk activation by over-expression of a constitutively activated Raf1 led to p21CIP1 induction 44;45.

Consequently, we examined the effect of inhibition of Erk activation on p21CIP1 induction after DNA damage. Inhibition of Erk activation by PD98059 reduced p21CIP1 induction by half (Fig. 3B and 4) but had no effect on p53 stabilization by ETOP (Fig. 3B). PD98059 also attenuated ADR induced p21CIP1 induction (Fig. 4). As expected, over-expression of a constitutively activated MEK1/Q56P 23 strongly activated Erk in NIH3T3 and MEF cells (data not shown) and led to p21CIP1 induction that was also inhibited by PD98059
Further, expression of MEK1/Q56P in MEFP53/- cells also resulted in p21CIP1 induction (data not shown), indicating that Erk activation is capable of inducing p21CIP1 expression, consistent with prior observations with forcibly expressed Raf1. Our data, though, is the first to demonstrate p21CIP1 induction by Erk under physiologic conditions.

**Erk activation is required for cell cycle arrest upon DNA damage.** As Erk activation partially contributes to p21CIP1 induction (Fig. 3B and 4), and p21CIP1 plays an important role in both induction of G1 arrest and maintenance of arrest at the G2/M checkpoint, we sought to determine whether the magnitude of p21CIP1 induction induced by Erk activation played a functional role in cell cycle arrest in response to DNA damage. Whilst PD98059 100µM alone results a degree of G1 arrest (Fig 5B), consistent with the notion that ERK activity facilitates G1 progression, addition of PD98059 to ETOP 0.5µM (Fig 5D) led to a marked release from the G2/M arrest seen with etoposide 0.5µM alone (Fig 5C). We confirmed that G2/M release was indeed occurring by mitotic labeling with nocodazole trapping and showed an increase of mitotic cells from 5 to 11% at 24h when PD98059 is added to ETOP 0.5µM (data not shown). To further confirm a role of Erk in maintaining G2/M arrest in response to ETOP, a more specific MEK1 inhibitor, U0126, was also used. While 50µM U0126 alone has a minimal effect on G1 phase (Fig 5E), 10µM and 50µM U0126 again dramatically released ETOP (0.5µM) induced G2/M arrest (Fig. 5E). Similar results were observed with ADR (data not shown), although the effect of PD98059 on cell cycle arrest induced by ADR is less prominent than that
induced by ETOP, consistent with the observation that inhibition of Erk activation was more effective in preventing ETOP- than ADR- mediated p21CIP1 induction (Fig. 4).

**Erk activation facilitates DNA damage induced apoptosis.** Given our data for a pro-cell cycle arrest function of Erk in response to DNA damage, we wished to determine if DNA damage- induced Erk activation also mediated apoptotic responses. Addition of ETOP 10µM for 24 hours led to DNA fragmentation in NIH3T3 cells, which was essentially prevented by ERK inhibition (Fig 6A). Overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-xL also prevented apoptosis in response to ETOP, indicating that the mitochondrial cellular execution pathway plays a role in DNA damage- induced apoptosis (Fig 6A). Quantification of apoptosis using terminal deoxyuridine end-nick labeling (TUNEL) revealed that 65% of cells were apoptotic when exposed to 10µM ETOP for 24 hours, and that ERK inhibition with PD 98059 dose- dependently reduced this to less than 10%, suggesting a major role for ERK in DNA damage induced apoptosis in response to ETOP (Fig 6B). Similarly, Bcl-2 or Bcl-xL overexpression also reduced apoptosis to approximately 10%, indicating again the importance of the mitochondrial execution pathway (Fig 6B). Identical results were also obtained when U0126 was used. While U0126 alone at 50µM induced no apoptosis, addition of 10µM and 50µM strongly inhibited 10µM ETOP induced apoptosis (Fig 7). When TUNEL was used to quantify apoptotic cells in Fig 7, ETOP alone resulted in 70% apoptosis and U0126 at 10 or 50µM reduced this to 12 and 4% respectively (data not shown), confirming the importance of Erk activity in ETOP induced apoptosis.

We reasoned that if ERK is central in induction of apoptosis in response to DNA damage,
then overexpression of ERK should further sensitize cells to undergo programmed death when exposed to ETOP. Indeed, TUNEL revealed marked (15%) apoptosis in NIH3T3 cells infected with retrovirus for MEK1/Q56P, a constitutively active MEK23, in response to 5μM ETOP for 18 hours, a condition usually associated with <5% apoptosis in NIH3T3 cells infected with control retrovirus (Fig 8). Inhibition of MEK-1 with PD98059 again essentially prevented this (Fig 8).

Since our data indicated that Erk was activated by a number of DNA damage stimuli (Fig. 1B), we sought to ensure that pro-apoptotic signaling through ERK was a general response to DNA damage. Consequently, the effect of PD98059 on UV and ADR induced cell death were also examined. Inhibition of Erk activation by PD98059 increased cell survival in response to both UV and ADR treatments (Fig. 9A and B). Taken together, these results show that Erk activation facilitates DNA damage induced apoptosis.


**Discussion**

DNA damage surveillance systems play an important role in maintaining genomic integrity. Inactivations in or losses of components of these pathways results in genetic instability, and may lead to the accumulation of multiple genetic alterations and development of neoplasia [47-49]. All human tumors display genetic instability [47], and functional inactivation of p53, a major player in DNA damage checkpoint pathways, occurs in more than 50% of cancers [50]. Lack of a functional ATM kinase, the best characterized transducer of DNA damage, is also characterized by chromosomal instability in response to DNA damage and the development of cancer [51;52].

In this paper, we add novel information to an already very complex DNA damage response. We initially observed that DNA damage agents were able to initiate Erk activation as measured by Erk phosphorylation in several cell lines (Fig. 1), and the degree of Erk activation correlated with the intensity of DNA damage. This is consistent with recent reports that treatment of cells with UV, mitomycin-C, and cisplatin led to Erk activation [25;26;29]. Our new observations result from detailed characterization of the mechanisms and consequences of Erk activation after ETOP-induced DNA damage. We have mapped Erk activation to be downstream of ATM in response to ETOP, but, importantly, independent of p53 (Figs 2 and 3). Together with p53, such Erk activation contributed to DNA damage-induced cell cycle arrest and apoptosis.

DNA damage activates the kinase activity of ATM, which subsequently modifies a number of downstream targets [4], including the phosphorylation of S15 at the N-terminus of p53.
This modification prevents ubiquitin-dependent p53 degradation by interfering with the interaction between p53 and Mdm2. We report here that ETOP is unable to activate Erk in ATM-/- fibroblasts but does so in wild type IMR90 fibroblasts, indicating that Erk is a downstream target of ATM in response to DNA damage. Supporting this is our observation that wortmannin, a PI-3 kinase inhibitor, also blocked ETOP- induced Erk activation. Interestingly, it has been reported recently that ATM kinase functions in the insulin signaling pathway to stimulate protein synthesis by phosphorylating 4E-BP1, supporting the hypothesis that the role of ATM in cell signaling may not be restricted to the maintenance of genomic stability. This raises the possibility that Erk might also be a target of ATM normally and therefore lack of a functional ATM kinase might interfere with Erk activation and cell proliferation. This would be consistent with the observations that ATM-/- cells proliferate poorly and require high levels of growth factors. To further support this scenario, we have observed that wortmannin at concentrations of or higher than 100nM also inhibits the basal level of Erk activation (unpublished observations). Since the IC50’s of wortmannin for lipid PI-3 kinase, DNA-PK, and ATM kinase are less than 5nM, 16nM, and approximately 100nM, respectively, these observations indicate a potential role for ATM in broader cell signaling by affecting Erk activation.

Since p53 protein is a major effector in the DNA damage response, it is essential to determine any linkage between p53 and Erk activation after DNA damage. Like other oncogenic signals, Erk activation leads to p14ARF dependent p53 activation in primary but not immortalized MEF. In the case of DNA damage response, however, we have found
that Erk activation occurs independently of p53. This is based on following observations: 1) the MEK1 inhibitor, PD98059, prevented Erk activation but not p53 stabilization in response to DNA damage (Fig. 3B) and 2) pre-incubation with CHX at 10µg/ml for 30 minutes to destroy endogenous p53 completely blocked DNA damage induced p53 stabilization but not Erk activation (Fig. 3C) and 3) the maximal level of Erk activation by UV, ETOP, and ADR was not attenuated in MEFp53-/- (Fig 3D). This is consistent with the observation that enforced activation of Erk leads to cell cycle arrest in a p53 independent manner (our unpublished observations) 44;45.

Our results are different from reports that Erk activation lies either upstream or downstream of p53 in response to various DNA damage agents. Inhibition of Erk activation by PD98059 was found to prevent cisplatin- induced p53 stabilization by reducing phosphorylation of S15 in the N-terminal region of p53 25. Although it was not clearly demonstrated, the authors indicated that by interaction with p53, Erk phosphorylated p53 on S15. It is unclear how Erk, a proline directed kinase, could phosphorylate S15 of p53 since this residue is not followed by a proline, but rather a Q residue. Further, cisplatin induced Erk activation could be inhibited by suramin, a growth factor receptor antagonist, suggesting that Erk activation might not be related to cisplatin- induced DNA damage 29. However, the possibility remains that these observations may be due to the different DNA damage stimuli used. This is supported by data indicating that mitomycin C and actinomycin D- induced Erk activation was abolished in a p53 defective tumor cell line 26, although in this case p53 would be placed upstream of Erk activation.

In support of the notion that p53 is required for some forms of DNA damage- induced
Erk activation, Lee at al. 26 showed that over-expression of wild type p53 itself led to Erk activation. An alternative explanation for this is that over-expression of p53 leads to feedback activation of a DNA damage response pathway, resulting in ATM kinase activation. ATM kinase in turn leads to Erk activation, consistent with our observation that Erk activation depends on ATM after DNA damage. Supporting this inference was the observation that over-expression of wild type p53 led to activation of p53 26. However, it is known that simply increasing the abundance of p53 is insufficient to activate p53 pathway 62.

Although independent of p53, our data shows that Erk activation makes important contributions to functional outcomes- cell cycle arrest and apoptosis- after DNA damage. Inhibition of Erk activation by PD98059 or U0126 (data not shown) diminished p21CIP1 induction, explaining the partial release by these inhibitors from cell cycle arrest in response to low concentrations of either ETOP (Fig 5) or ADR (data not shown), since p21CIP1 is required to maintain G2/M arrest 42. Although enforced activation of Erk is known to lead to p21CIP1 induction in a p53 independent manner 44;45, our demonstration here is the first that Erk activation contributes to the induction of p21CIP1 under physiologic conditions. In response to higher doses of ETOP, ADR, and UV, inhibition of Erk activation by PD98059 and U0126 attenuated apoptosis (Figs.6 and 7). Since DNA damage- induced cell cycle arrest and apoptosis requires p53, our results indicate the existence of a parallel Erk dependent pathway also functional in DNA damage- induced cell cycle arrest and apoptosis.

The data presented here demonstrate that Erk activation after DNA damage plays a
functional role in surveillance and response to DNA damage, independently of p53. Since may malignant cells display high levels of Erk activation, these findings may help explain the heightened sensitivity of some transformed cells to DNA damaging chemotherapeutic agents when compared to primary cells. It will be instructive to examine such lines for amplifications of components of the Erk signaling pathway.
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Figure Legends

Figure 1. **Erk activation is seen in response to various DNA damage stimuli.** (A) MEF cells were exposed to ETOP 100µM for the indicated times. Erk activation was assessed by western blot using an anti-phospho-Erk antibody. Total Erk protein was assessed by western blotting of the same membranes with an anti-Erk antibody. (B) 2 x 10⁶ primary (MEF), immortalized (NIH3T3), and transformed (MCF7) were seeded for 24 hours in 60mm plates. Cells were treated with ETOP at 100µM for 2 hours, ADR at 1.6µM for 4 hours or UV at 100mJ/cm² for 4 hours. Erk activation was assessed by western blot using an anti-phospho-Erk antibody. Densitometric data are shown with standard error bars. All experiments were performed at least three times.

Figure 2. **Erk activation by DNA damage signals requires ATM.** Wild type fibroblast IMR90 and ATM-/- fibroblast cells, GM05823, were seeded and grown to confluency in 60mm plates. Cells were treated with ETOP at 100µM for 2 hours or ADR at 1.6µM for 4 hours as indicated. Western blot was carried out to determine p53 stabilization with an anti-p53 antibody, phosphorylation on the S15 of p53 with anti-phospho-p53(S15), up-regulation of P21CIP1 with anti-p21CIP1, and Erk activation with an anti-phospho-Erk antibody. Blots were routinely re-probed with an anti-actin antibody to control for loading. All experiments were performed at least in triplicate, and representative radiographs shown.

Figure 3. **Erk activation by DNA damage occurs independently of p53.** (A) MCF-7 cells were seeded for 24 hours in 60mm plates and then treated with ETOP at 100µM for the indicated
times. Stabilization of p53 with an anti-p53 antibody and Erk activation with an anti-phospho-
Erk antibody were determined. **(B)** NIH3T3 cells were pre-treated with PD98059 (100µM) for
30 minutes before addition of 100µM ETOP for 2 hours. Erk activation, p53 stabilization, and
p21CIP1 up-regulation were then examined by western blot with their respective antibodies.
Blots were routinely re-probed with anti-actin to ensure equivalence of loading (data not
shown). **(C)** NIH3T3 cells were pre-treated with cycloheximide (10µg/ml) for 30 minutes before
addition of 1.6µM ADR for 4 hours. Erk activation and p53 stabilization were determined by
western blotting with their respective antibodies. Blots were routinely re-probed with anti-actin
to ensure equivalence of loading (data not shown). **(D)** Wild type and p53−/− MEFs were
treated with ETOP at 100µM for 2 hours, ADR at 1.6µM for 4 hours or UV at 100mJ/cm² for 4
hours. Erk activation was determined by western blot using anti-phospho-Erk antibody (inset
gel) and quantified. Densitometric results from three independent experiments are shown with
error bars.

**Figure 4. Erk activation contributes to DNA damage- induced p21CIP1 up-regulation.** NIH3T3
cells were seeded for 24 hours in 60mm plates and then treated with ETOP at 100µM for 2 hours
or ADR at 1.6µM for 4 hours with or without PD98059 (100µM) as indicated. Similarly, cells
infected with retrovirus expressing MEK1/Q56P were grown for 24 hours and then treated with
PD98059 (100µM) as indicated. The abundance of p21CIP1 protein was determined by western
blot and quantified. Densitometric results from three independent experiments are shown with
error bars.

**Figure 5. Erk activation functions in DNA damage- induced cell cycle arrest.** Experiments
shown are after 24 hours exposure to the indicated compounds and were performed in at least triplicate. The percentages of cells in each phase were determined by propidium iodide staining followed by fluorescent automated cell sorting. (A) NIH3T3 cells exposed to vehicle (DMSO). (B) NIH3T3 cells were exposed to PD98059 (100µM). (C) NIH3T3 cells were exposed to ETOP (0.5µM). (D) PD98059 (100µM) was added to ETOP (0.5µM). (E) U0126 was used in the indicated concentrations instead of PD 98059 with or without ETOP as indicated. The percentage of cells in each phase is shown graphically. Error bars are based on the results of four separate experiments.

Figure 6. Erk activation mediates DNA damage-induced apoptosis. NIH3T3 cells were infected with either a control retrovirus, pBabe, or retrovirus expressing Bcl-2 or Bcl-xL and seeded for 24 hours in 60mm plates. (A) Cells were then treated with ETOP (10µM) with or without PD98059 (100µM) and DNA fragmentation determined. (B) Cells were treated with ETOP (10µM) with or without PD98059 at the indicated concentrations and apoptosis determined by TUNEL. Results from three independent experiments are shown with error bars.

Figure 7. Erk activation mediates DNA damage-induced apoptosis. NIH3T3 cells were treated with DMSO (1), 50µM U0126 (2), 10µM ETOP (3), 10µM U0126 plus 10µM ETOP (4), or 50µM U0126 plus 10µM ETOP (5) for 24 hours. Cells were then stained with propidium iodide, followed by detection of apoptotic cells using FACS. Cells with sub-G1 DNA content are apoptotic and the percentage of total cells in this area is given. Results shown are representative of four separate experiments.

Figure 8. Enforced Erk activation sensitizes to DNA damage-induced apoptosis. NIH3T3 cells were infected with either a control retrovirus, pBabe, or retrovirus expressing MEK1/Q56P and...
seeded for 24 hours in 60mm plates. Cells were treated with ETOP at the indicated concentrations with or without PD98059 (100µM) and apoptosis determined by TUNEL staining. Results were from three independent experiments and are shown with error bars.

**Figure 9. Erk activation contributes to UV and ADR- induced cell death.** NIH3T3 cells were seeded for 24 hours in 60mm plates. (**A**) Cells were treated with UV at 100mJ/cm² with or without PD89059 (50µM) for the indicated time periods and viability determined as described in Materials and Methods. Results were from three independent experiments and are shown with error bars. (**B**) Cells were treated with ADR for 19 hours at the indicated concentrations with or without PD89059 (50µM) and viability determined. Results were from three independent experiments and are shown with error bars.
Reference List


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   *J.Biol.Chem.* **276**, 2686-2692


Figure 2
A
ETOP (hr)
0  1  2  4  6  8
-- p53
-- Erk-P
-- Actin

B
PD98059
Etoposide
DMSO
<table>
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<tr>
<th></th>
<th>+</th>
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Fold Increase p21
1  18.4  10.8

C
ADR
CHX
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-- Erk-P
-- p53

D
MEF-wt
MEF-P53-/-

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Erk activity (fold increase)
ETOP  UV  AD

DNA damages
Figure 4
Figure 5E
Figure 6
Figure 9
ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53
Damu Tang, Dongcheng Wu, Atsushi Hirao, Jill M. Lahti, Lieqi Liu, Brie Mazza, Vincent J. Kidd, Tak W. Mak and Alistair J. Ingram

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