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TAO kinases mediate activation of p38 in response to DNA damage

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Thousand and one amino acid (TAO) kinases are Ste20p-related MAP kinase kinase kinases (MAP3Ks) that activate p38 MAPK. Here we show that the TAO kinases mediate the activation of p38 in response to various genotoxic stimuli. TAO kinases are activated acutely by ionizing radiation, ultraviolet radiation, and hydroxyurea. Full-length and truncated fragments of dominant negative TAOs inhibit the activation of p38 by DNA damage. Inhibition of TAO expression by siRNA also decreases p38 activation by these agents. Cells in which TAO kinases have been knocked down are less capable of engaging the DNA damage-induced G2/M checkpoint and display increased sensitivity to IR. The DNA damage kinase ataxia telangiectasia mutated (ATM) phosphorylates TAOs *in vitro*; radiation induces phosphorylation of TAO on a consensus site for phosphorylation by the ATM protein kinase in cells; and TAO and p38 activation is compromised in cells from a patient with ataxia telangiectasia that lack ATM. These findings indicate that TAO kinases are regulators of p38-mediated responses to DNA damage and are intermediates in the activation of p38 by ATM.

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Introduction

Evolutionarily conserved mechanisms exist in cells to sense and respond to aberrations in genomic integrity. Without such mechanisms, deleterious mutations would be passed on to subsequent generations and diminish organism viability. The DNA damage response is executed by a network of proteins that include sensors that detect the damage, proteins that halt the cell cycle and recruit repair machinery, and proteins that activate apoptosis if repair is insufficient to alleviate the insult (Sancar *et al*, 2004). It is remarkable that only a handful of evolutionarily conserved proteins take part in this process, given that DNA damage can be caused by many agents that induce different types of damage. Key players in this pathway include the ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) Ser/Thr protein kinases, which orchestrate multiple aspects of the DNA damage response via phosphorylation of effectors on well-characterized SQ/TQ motifs (Matsuoka *et al*, 2000; Sapkota *et al*, 2002; Zhou and Bartek, 2004). Two such substrates are Chk1 (phosphorylated on Ser317 and Ser345 by ATR) and Chk2 (phosphorylated on Thr68 by ATM), protein kinases that engage the G1/S, intra-S phase and G2/M cell cycle checkpoints in response to DNA damage.

Many cellular stressors like heat shock, osmotic stress, and microtubule depolymerization induce cell cycle arrest via robust activation of p38 MAP kinase (MAPK). p38 also regulates cell cycle checkpoints in response to DNA damage, including the G1/S and the early G2/M checkpoints induced by UV radiation. This occurs in part through its ability to activate MAPKAP Kinase-2 (MK2). MK2, like Chk1 and Chk2, phosphorylates Ser323 in Cdc25B and Ser216 in Cdc25C, both in 14-3-3-binding sites (Bulavin *et al*, 2001; Manke *et al*, 2005). Phosphorylation of Cdc25 phosphatases inactivates them, preventing cell cycle progression. The importance of p38 in cell cycle checkpoints induced by ionizing radiation (IR) is less well understood. MEK6 and p38γ have been implicated in the IR-induced G2/M checkpoint (Wang *et al*, 2000). The same study also proposed that IR activated p38 via ATM, because p38 activation was diminished in ATM$^{-/-}$ fibroblasts. How p38 is activated by DNA damage is not known. Neither direct phosphorylation of p38 by ATM/ATR nor direct interactions between p38 and damaged DNA have been reported. The compromised p38 activation in ataxia telangiectasia (AT) cells suggests that cell cycle checkpoints are also activated via indirect ATM/ATR-mediated regulation of p38. Identification of components of the signaling cascade that impinges on p38 is crucial to understanding the mechanism by which multivariate stressors converge to detect and repair DNA damage.

TAOs are MAP3Ks in the p38 MAPK cascade that were originally identified based on their kinase domain similarity to the yeast Ste20p MAP4K. TAOs regulate p38 via their ability to phosphorylate and activate the MAPK kinases MEK3 and 6 (Hutchison *et al*, 1998; Chen *et al*, 2003). TAOs have been implicated in regulation of cytoskeleton stability, G protein-coupled receptor signaling to p38, and cell survival (Hutchison *et al*, 1998; Aza-Blanc *et al*, 2003; Chen *et al*, 2003; Mitsopoulos *et al*, 2003; MacKeigan *et al*, 2005). Here, we identify TAOs as key intermediates in the activation of p38 by DNA damage.

Results

TAO kinases are activated by DNA-damaging agents

p38 is a required component of the DNA damage response to genotoxic stress. Robust activation of p38 ($\sim$40-fold) was

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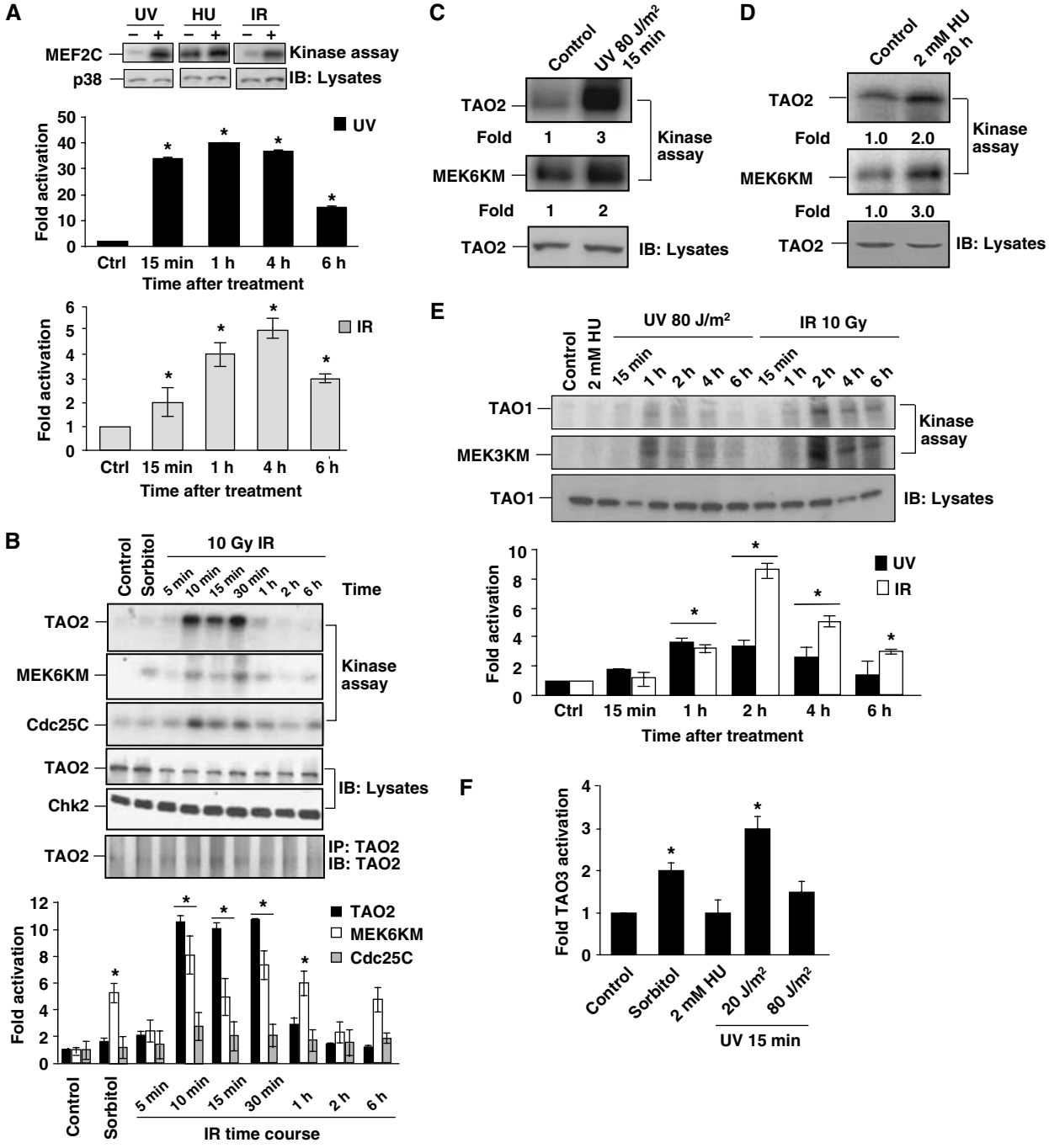

Figure 1 DNA-damaging agents activate TAO kinases. (**A**) HeLa cells were untreated (Ctrl) or treated with 2 mM HU overnight, 80 J/m^2 UV, or 10 Gy IR. p38 activity was assayed with MEF2C as substrate. (**B**) HeLa cells were untreated or treated with 0.5 M sorbitol for 15 min or with 10 Gy IR. TAO2 and Chk2 activities were assayed using MEK6 KM and Cdc25C (200–254) as substrates, respectively. (**C**) HeLa cells were untreated or treated with 80 J/m^2 UV for 15 min. TAO2 activity was assayed. (**D**) HeLa cells were untreated or treated with 2 mM HU. TAO2 activity was assayed. (**E**) HeLa cells were treated as in (A). TAO1 activity was assayed by autophosphorylation and phosphorylation of MEK3 KM. (**F**) HeLa cells were treated as above. TAO3 activity was assayed with MBP as substrate. Values are mean ± s.e.m. ($n = 3$). *$P \leqslant 0.05$.

seen in UV-treated HeLa cells within 15 min and sustained for many hours (Figure 1A). Both IR and hydroxyurea (HU) also activated p38 approximately fivefold, with greater IR-mediated activation observed at later times (1–2 h post radiation) (She *et al*, 2002). p38 is activated robustly by UV because multiple signaling pathways are triggered when this agent stresses the cell. IR specifically activates the DNA damage response by causing double-strand DNA breaks, an event that activates ATM and DNA-PK (Stiff *et al*, 2004; Chen BP *et al*, 2005). Hence, the comparatively modest activation of p38 by IR is likely due to the limited number of upstream pathways activated by this stress.

To determine if TAOs are activated by DNA damage, TAOs were immunoprecipitated from stressed cells and assayed. TAO2 was activated up to 10-fold by IR within 30 min of treatment (Figure 1B). The time-course of activation was similar to that of the known checkpoint kinase Chk2 as determined by monitoring phosphorylation of Cdc25C.

Activation of TAO2 by UV and HU was a modest twofold (Figure 1B–D). Although neither TAO1 nor TAO2 was activated by HU, both were activated several-fold by UV and IR (Figure 1A and E). Thus, IR significantly activated TAO1 and TAO2 with different kinetics; UV modestly activated all three TAOs; HU activated TAO2 but to a lesser extent than IR or UV. IR activates TAOs more dramatically than other known TAO regulators, suggesting TAOs as potential mediators of IR-induced p38 activation. These findings suggest a common function for TAOs in activation of p38 by DNA damage.

Dominant negative TAOs block p38 activation by genotoxic stress

To determine if the activation of p38 by DNA damage requires TAOs, dominant-negative TAO mutants were expressed in cells that were subsequently treated with HU, UV, or IR to invoke the DNA damage response. While basal p38 activity was unaltered (data not shown), inactive full-length TAO1, TAO2 (1–451), or TAO3 (1–442) inhibited HU-, UV-, or IR-induced p38 activation by 50% or more (Figure 2A–D).

The relative contributions of MEK3 and 6 in p38 activation by DNA damage are poorly defined (Wang *et al*, 2000). We found that both MEK3 and 6 were required to varying extents for p38 activation. DNA damage-mediated activation of p38 appeared to be primarily through MEK6, as inactive MEK6 inhibited p38 activity by 70% or more relative to control (Figure 2E). Inactive MEK3 inhibited p38 activation 50% or less (Figure 2E). Inactive MEK1 and MEK5, coupled to the ERK1/2 and ERK5 MAPK pathways, respectively, had little effect on p38 activity (data not shown). These experiments demonstrate a requirement for TAOs in p38 activation by genotoxic stress.

Knockdown of TAO kinases inhibits activation of p38 by DNA damage

To obtain additional evidence that TAOs are required for p38 activation by DNA damage, we used siRNA to examine p38 activation by UV, HU and IR in cells with reduced TAO expression. Knockdown of TAOs 1 and 3 was efficient (70–80%); however, knockdown of TAO2 was less reliable (50% or less). Knockdown of one TAO did not affect amounts of the others (Figure 3A). TAO3 knockdown had pronounced effects on cell viability, resulting in cell death by 48 h (Aza-Blanc *et al*, 2003; MacKeigan *et al*, 2005). The surviving cells expressed reduced amounts of TAO3. Knockdown of TAO1/2 had no effect on cell viability over a two-week period. Consistent with the extent of TAO knockdown, p38 activation by UV, HU, and IR was diminished up to 50% in TAO knockdown cells (Figure 3B–D).

Reduced TAO activation in cells lacking ATM

ATM may play a role in the activation of p38 by IR (Wang *et al*, 2000). To assess the requirement for ATM in the activation of p38 and TAOs by DNA damage, we tested if TAOs or p38 were activated in cells lacking ATM. Canonical ATM and ATR substrates are phosphorylated on Ser/Thr followed by Gln (pSQ/pTQ). All three TAOs have SQ/TQ sites in their C-terminal domains (TAO1: 6, TAO2: 7 and TAO3: 9, Figure 4A). Protein kinase activities of p38 and TAO2 were assayed from wild-type human skin fibroblasts (1BR3) and skin fibroblasts from a patient with AT that do not express detectable ATM. Activation of p38 and TAO2 was intact in wild-type fibroblasts treated with IR. However, in AT cells, the activation of these protein kinases was diminished, implying that ATM is required for their activation (Figure 4B and C).

Inhibition of TAOs impairs activation of the G2/M checkpoint

Given the evidence that TAO kinases appear to be important mediators of the DNA damage response in the activation of p38, we reasoned that siRNA of TAOs would result in the abrogation of the G2/M checkpoint induced by either UV or IR. To investigate this, we used a fluorescence-activated cell sorting-based assay to quantify the percentage of cells in mitosis. TAO expression was knocked down by siRNA and the cells were treated with UV for 2 h. The number of cells in mitosis was determined by dual staining with propidium iodide (DNA content) and phosphoSer10 Histone H3 (pSer10-H3), a marker for mitotic cells. In control siRNA-transfected cells, UV treatment arrested cells in G2, and negligible staining was seen with pSer10-H3, indicating that the G2/M checkpoint was efficiently engaged (Figure 5A and B). Knockdown of TAO1 and 2 had no effect on the cell cycle in the absence of DNA damage (Figure 5A and data not shown). In contrast to control cells which arrested in G2, a higher proportion of UV-treated cells depleted of TAO1 or TAO3 entered mitosis, indicating a role for these kinases in damage-induced arrest (Figure 5A and B). Knockdown of TAO1 alone partially inhibited the IR-induced G2/M checkpoint.

G2/M arrest is caused by inhibition of mitotic cyclin/Cdc2. Cyclin/Cdc2 is tightly regulated by multiple phosphorylations. Cdc2 is inhibited by Myt1/Wee1 phosphorylation on Tyr15 (Lundgren *et al*, 1991; Parker and Piwnica-Worms, 1992). The key step in its activation is dephosphorylation of Tyr15 by Cdc25C. G2/M arrest by DNA damage causes the accumulation of inactive pTyr15-Cdc2. In cells transfected with control oligonucleotide, HU or UV treatment caused accumulation of pTyr15-Cdc2 (Figure 5C). However, knockdown of either TAO1 or TAO3 decreased the amount of pY15-Cdc2 substantially, indicating that Cdc2 was activated despite the presence of DNA damage. These data further support the idea that TAO-mediated regulation of p38 in response to DNA damage is critical for regulating entry into mitosis. Decreasing the expression of TAOs was sufficient to bypass inhibition of mitotic cyclin/Cdc2 in response to damaged DNA.

We examined effects of depleting TAO1 and TAO2 on cell survival following γ-radiation. Depletion of TAO3 resulted in marked cell death within 2 days of irradiation, preventing its study in this assay. After 7 days, surviving colonies were stained with crystal violet and counted. Depletion of TAO1 and 2 sensitized cells to γ-radiation (Figure 5D). These data further suggest that TAO kinases are MAP3Ks regulating the p38 arm of the G2/M DNA damage checkpoint.

ATM is upstream of TAOs in the DNA damage response

To determine if TAOs can be phosphorylated by ATM *in vitro*, we carried out ATM kinase assays using inactive TAOs as substrates. Cells were transfected individually with either ATM or inactive TAO1 or TAO3. ATM-transfected cells were irradiated with 10 Gy IR to activate ATM. Cells were harvested and the proteins were immunoprecipitated via their epitope

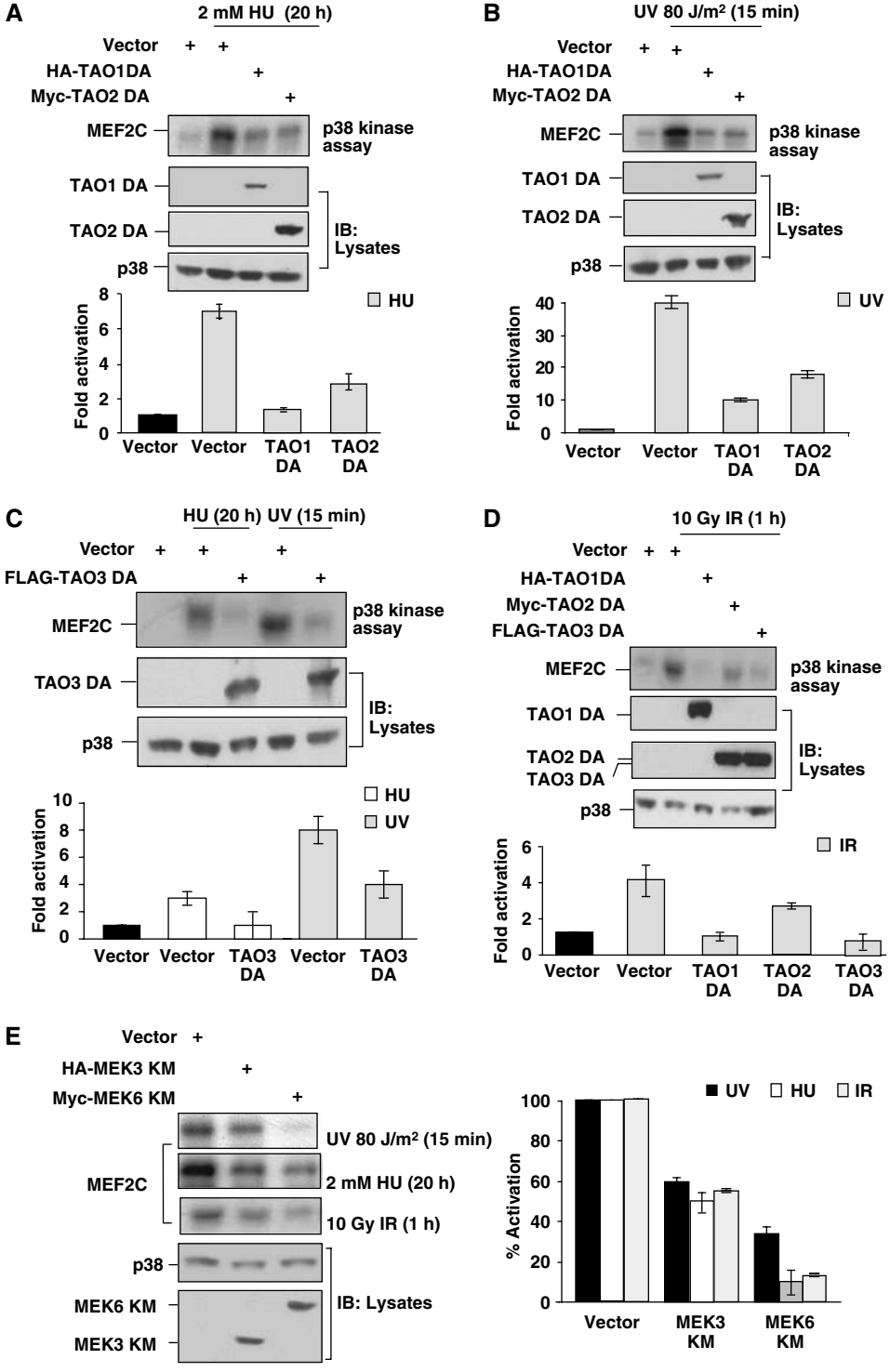

Figure 2 Activation of p38 by DNA damage is inhibited by dominant negative TAOs. (**A**) Cells transfected with vector alone and HA-TAO1 D169A or Myc-TAO2 (1–451) D169A were untreated or treated with 2 mM HU. p38 activity was assayed as in Figure 1. (**B**) Cells transfected as in (A) were untreated or treated with 80 J/m^2 UV, and p38 activity was assayed. (**C**) Cells were transfected with vector alone or FLAG-TAO3 (1–442) D165A and untreated or treated with 2 mM HU or 80 J/m^2 UV. p38 activity was assayed. (**D**) Cells were transfected with the indicated constructs and untreated or irradiated with 10 Gy IR. p38 activity was assayed. (**E**) Cells were transfected with vector alone or HA-MEK3 K64M, and Myc-MEK6 K82M and treated with either 2 mM HU, 80 J/m^2 UV, or 10 Gy IR. p38 activity was assayed. Values are mean $\pm$ s.e.m. ($n = 3$). All experimental values were significantly different from control values at the $P \leqslant 0.05$ level.

tags. *In vitro* kinase assays were carried out by mixing the immunoprecipitates containing ATM and kinase-dead TAOs. In these assays, ATM phosphorylated both kinase-dead TAO1 and TAO3 (Figure 6A), but not TAO1 T643A, T785A, or S990A (Figure 6B). We observed no phosphorylation of MEK6 KM or WNK1 DA, despite the presence of numerous SQ/TQ sites in these proteins (Figure 6A, data not shown). Overexpression of any of a number of SQ/TQ mutants of TAO1 resulted in up

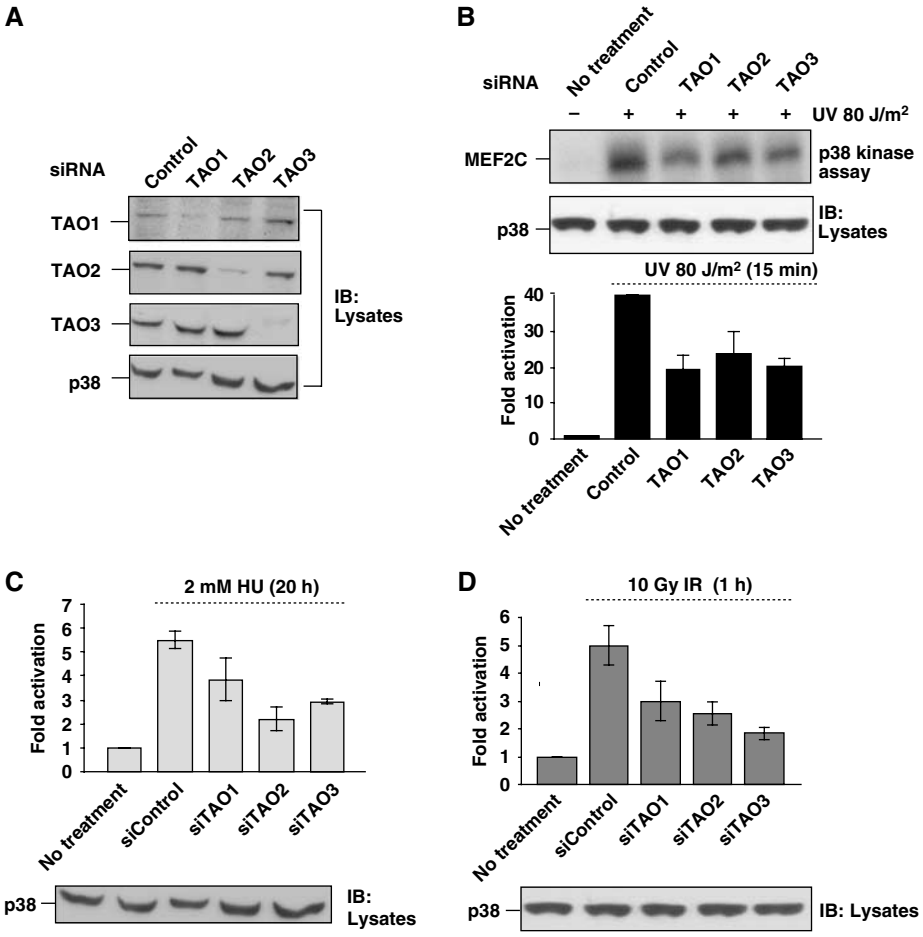

Figure 3 TAO kinases are required for DNA damage-induced p38 activation. (**A**) HeLa cells were transfected with siRNA oligonucleotides to rat TAO2 (Control) or TAO1–3. Lysates were probed with anti-TAO antibodies. (**B**) HeLa cells were transfected with siRNA oligonucleotides as above. Cells were treated with 80 J/m^2 UV for 15 min and p38 activity was assayed. (**C**) HeLa cells were untreated or transfected with siRNA oligonucleotides as above and untreated or treated with 2 mM HU. p38 activity was assayed. (**D**) HeLa cells were untreated or transfected with oligonucleotides as above and treated with10 Gy IR for 1 h. p38 activity was assayed. Values are mean ± s.e.m. ($n = 3$). All experimental values were significantly different from control values at the $P \leqslant 0.05$ level.

to 50% inhibition of p38 activation by IR, suggesting that these sites on TAO1 are important in p38 activation (Figure 6C). In addition, these mutants also inhibited the IR-induced G2/M checkpoint (Figure 6D). However, a mutant in which a serine not followed by glutamine (S375) was replaced with alanine did not impair the G2/M checkpoint in response to IR (Figure 6D).

To determine if TAOs may be direct ATM substrates, cells were transfected with kinase-dead Myc-TAO3 or HA-TAO1 and the impact of exposure to IR on their phosphorylation was assessed. Cells were labeled with ^{32}P for 1 h prior to a 1-h irradiation. As shown in Figures 7A and B, irradiation caused a substantial increase in ^{32}P incorporation into both TAO proteins. The IR-induced phosphorylation did not occur in cells lacking ATM (Figure 7C). Immunoprecipitated TAOs from a second set of untreated or irradiated cells were analyzed by mass spectrometry. With or without radiation, a phosphopeptide corresponding to a putative autophosphorylation site was found (Supplementary Figure S1). In TAO3 from IR-treated cells, a second phosphopeptide corresponding to pS324Q was also identified (Figure 7D). This phosphopeptide was found in both wild-type and kinase-dead TAO3 from both IR-treated 293 and Hela cells (Figure 7D), but was absent from untreated cells (Supplementary Figure S1). We tested the functional effects of the mutant lacking TAO3 S324 in both p38 protein kinase assays as well as in eliciting the G2/M checkpoint (Figure 7E). TAO3 S324A inhibited p38 activation as well the G2/M checkpoint in response to both UV and IR. These findings further support the idea that TAOs are direct substrates of ATM and implicate ATM in the activation of TAOs and p38 by genotoxic stress (Figure 8).

Discussion

DNA damage occurs both as a result of normal metabolism as well as by exogenous inputs such as UV and IR at a rapid rate in every cell. To counterbalance the deleterious effects of damage occurring in essential genes, cells rapidly detect damaged DNA and attempt to redress the damage. One of the hallmarks of the DNA damage response is the ability to halt the cell cycle to allow for damaged DNA to be repaired. Cell cycle checkpoints occur at the G1/S boundary, within S phase, and at the G2/M boundary. The G2/M checkpoint is thought to be the final 'net' that catches cells that have escaped the earlier checkpoints, protecting cells from undergoing mitosis until the damage has been repaired (Bakkenist

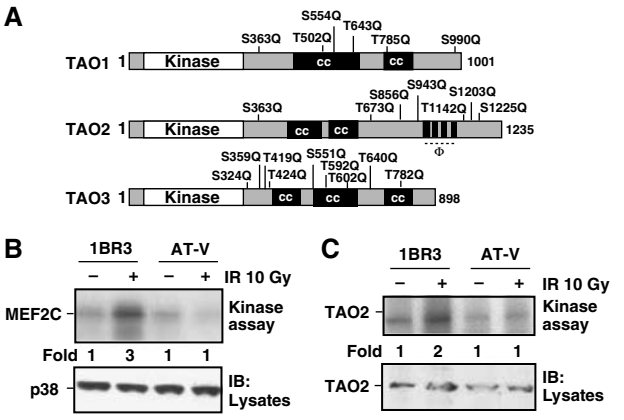

Figure 4 Reduced TAO activation in cells lacking ATM. (**A**) Positions of SQ/TQ sites in TAOs. CC: coiled coils, ϕ: hydrophobic domain. (**B**) Human skin fibroblasts derived from a normal (1BR3) and an AT individual were treated with 10 Gy IR. p38 activity was assayed. (**C**) 1BR3 and ATV cells were treated with 10 Gy IR and TAO2 activity was determined.

and Kastan, 2004). Failure to engage these checkpoints will result in the propagation of potentially lethal mutations to daughter cells. Chk1 and Chk2 are critical regulators of cell cycle checkpoints and can engage checkpoints at every stage of the cell cycle depending on the type of genotoxic stress (Bartek and Lukas, 2003). p38 also participates in the DNA damage response, for example, in double-strand breaks that occur during V(D)J recombination (Bulavin *et al*, 2001; Pedraza-Alva *et al*, 2006).

Multiple lines of evidence indicate that TAOs are important regulators of the response to genotoxic stress. The activation of these kinases is a critical determinant of the DNA damage response. Damage-induced p38 activation is blocked by catalytically deficient TAOs or TAO RNAi. RNAi of TAOs diminishes not only p38 activation but also impairs the DNA damage-activated G2/M cell cycle checkpoint. The inability to engage the damage-induced G2/M checkpoint in cells with reduced TAO expression parallels the persistent activation of mitotic cyclin/Cdc2. Importantly, reducing TAO expression enhanced sensitivity to γ-radiation in colony

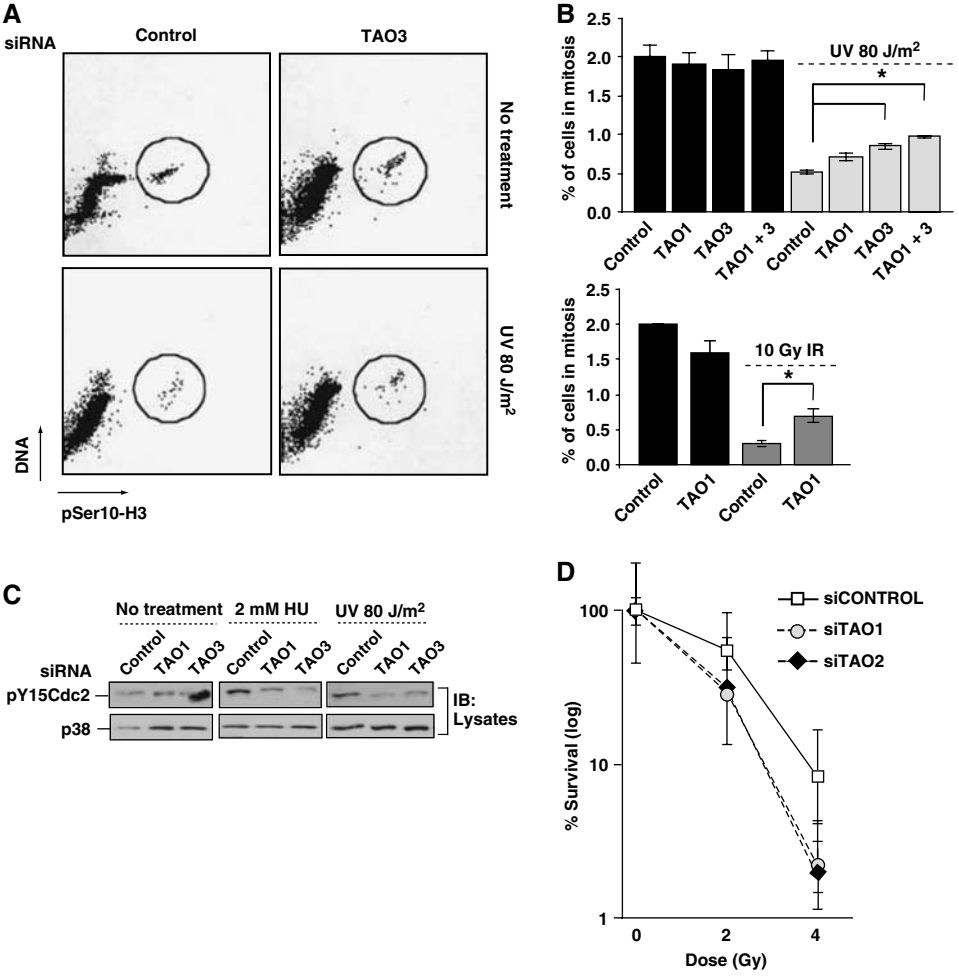

Figure 5 TAO kinases are required for the DNA damage response. (**A**) HeLa cells were transfected with siRNA oligonucleotides to rat TAO2 (Control) or TAO1 and TAO3 either singly or in combination. Cells were treated with 80 J/m^2 UV or 10 Gy IR. After 2 h, the percentage of mitotic cells was determined by dual staining with propidium iodide (DNA) and anti-pSer10-H3. Representative FACS of TAO3 shown. (**B**) Quantitation of mitotic cells in TAO1 and TAO3 knockdown cells (upper panel, UV; lower panel, IR). (**C**) HeLa cells were transfected as in (A) and untreated or treated with 2 mM HU or 80 J/m^2. Lysate proteins were probed with anti-pY15-Cdc2 and anti-p38. In the upper left panel, the dark band at an angle across one of the lanes is an artifact. (**D**) HeLa cells were transfected with siRNA oligonucleotides as in (A). Surviving colonies were stained with crystal violet and survival curves are shown. Values shown are means ± s.e.m. ($n = 3$) $P \leqslant 0.05$ (*).

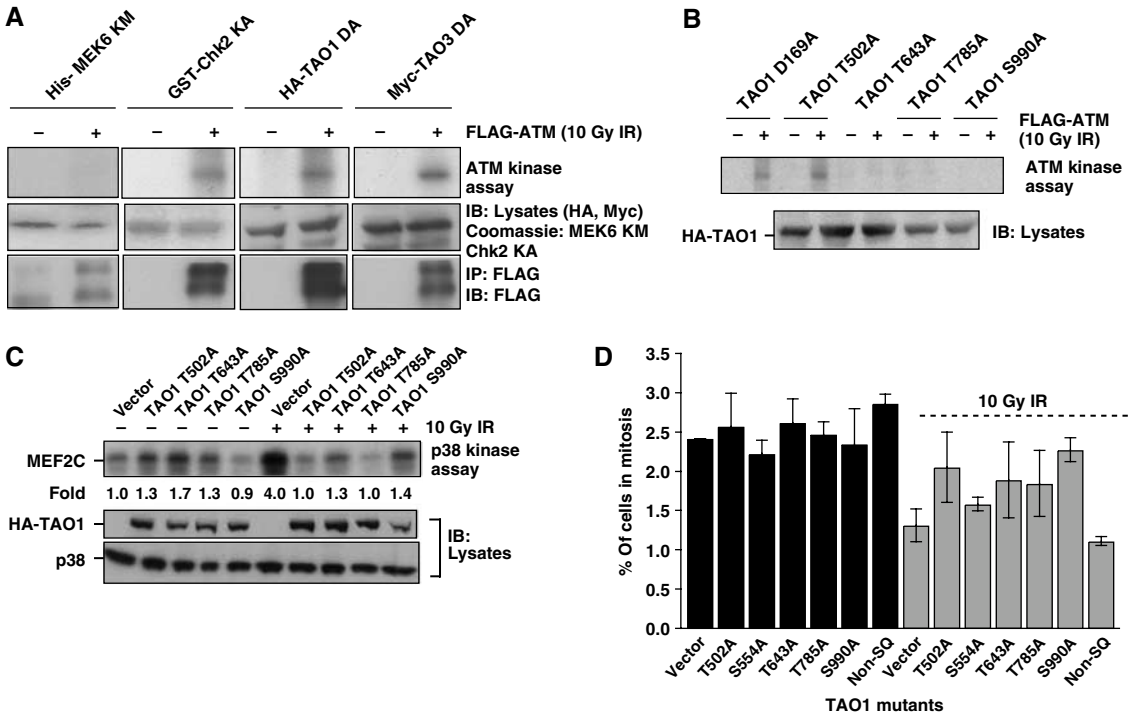

Figure 6 ATM is upstream of TAOs. (**A**) HEK293 cells were transfected with vector alone, or kinase-dead HA-TAO1 or Myc-TAO3 with or without FLAG-ATM. Top panel shows phosphorylation of Chk2 (positive control) and TAOs 1 and 3 by ATM. MEK6KM was used as a negative control. (**B**) Cells were transfected with the indicated HA-TAO1 SQ/TQ point mutants and assayed as in (A). (C) Cells were transfected with the indicated HA-TAO1 SQ/TQ mutants and were untreated or treated with 10 Gy IR for 2 h. Half the cells were lysed and p38 activity was assayed. (**D**) The other half of the cells in (C) were analyzed by FACS for their ability to elicit the G2/M checkpoint following irradiation (non-SQ = S375A). Values shown are means ± s.e.m. ($n = 3$).

survival assays. In aggregate, these findings provide strong evidence that the TAOs are required for activation of p38 and the G2/M checkpoint upon DNA damage.

Further support comes from experiments that implicate TAO kinases as key intermediates in the activation of p38 by ATM/ATR. Significantly, TAO activation by DNA damage is diminished in AT cells, as is activation of p38. TAOs appear to be direct ATM substrates. They contain canonical ATM/ATR phosphorylation sites, are *in vitro* substrates of ATM, and at least one of the TAOs is phosphorylated on an SQ site in response to DNA damage. Mutation of this site on TAO3 interferes with the IR- and UV-induced checkpoint. Furthermore, the IR-induced phosphorylation of TAO3 is dependent on ATM. Similar experiments with candidate phosphorylation sites on TAO1 reduced p38 activation by IR and UV (data not shown) and interfered with engagement of the checkpoint, suggesting that these sites are required for activation of TAO1 by ATM/ATR and its function in regulating p38 in response to DNA damage.

Why is p38 required for checkpoint activation when protein kinases such as Chk1 and Chk2 engage these checkpoints in response to DNA damage? DNA is damaged not only by radiation but also as a consequence of cell stress by other agents. For example, osmotic stress also induces double-strand DNA breaks (Kultz and Chakravarty, 2001; Dmitrieva *et al*, 2003) but does not induce Chk1 phosphorylation. Hence, pathways alternative to Chk1/2 must exist that sense DNA damaged by other events. p38 is ideal for this purpose because of its stress sensitivity. An array of MAP3Ks are linked to p38, providing the wiring to engage p38 as a consequence of many cellular insults. Specific MAP3Ks are responsive to overlapping stresses. TAOs are activated more strongly by DNA-damaging agents than by other cellular stresses. This suggests that among their primary functions is p38 activation in response to DNA damage, either due to activation of ATM/ATR or through other mechanisms.

In summary, we have identified the TAO family of protein kinases as a primary component linking the DNA damage response induced by genotoxic insults through ATM/ATR to p38. This is the first report of a family of MAP3Ks regulating p38 activation in response to DNA stressors.

Materials and methods

Cell culture and treatments

HEK293 and HeLa cells were from ATCC. Human skin fibroblasts from a normal individual (1BR3.3GN2) and a patient with AT (AT5.BIVA) were gifts from Benjamin Chen (Department of Radiation Oncology). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. 1BR3 and AT5 cells were grown in α-minimal essential medium (MEM) with 10% FBS. For γ-radiation, cells were exposed to a ^{137}Cs source at the indicated doses. UV was administered with 254 nm (UV-C) using a Stratalinker 2400 (Stratagene) at 20–80 J/m^2. Times indicated in experiments with IR/UV refer to time of incubation following radiation treatment. Cells were treated with 2 mM HU (Sigma) overnight.

Cloning and siRNA oligonucleotides

TAO3 was amplified by PCR from HEK293 cDNA and ligated into pCMV5 and p3XFLAG-CMV7.1 (Sigma). TAO1 mutants were generated using Quikchange according to the manufacturer's

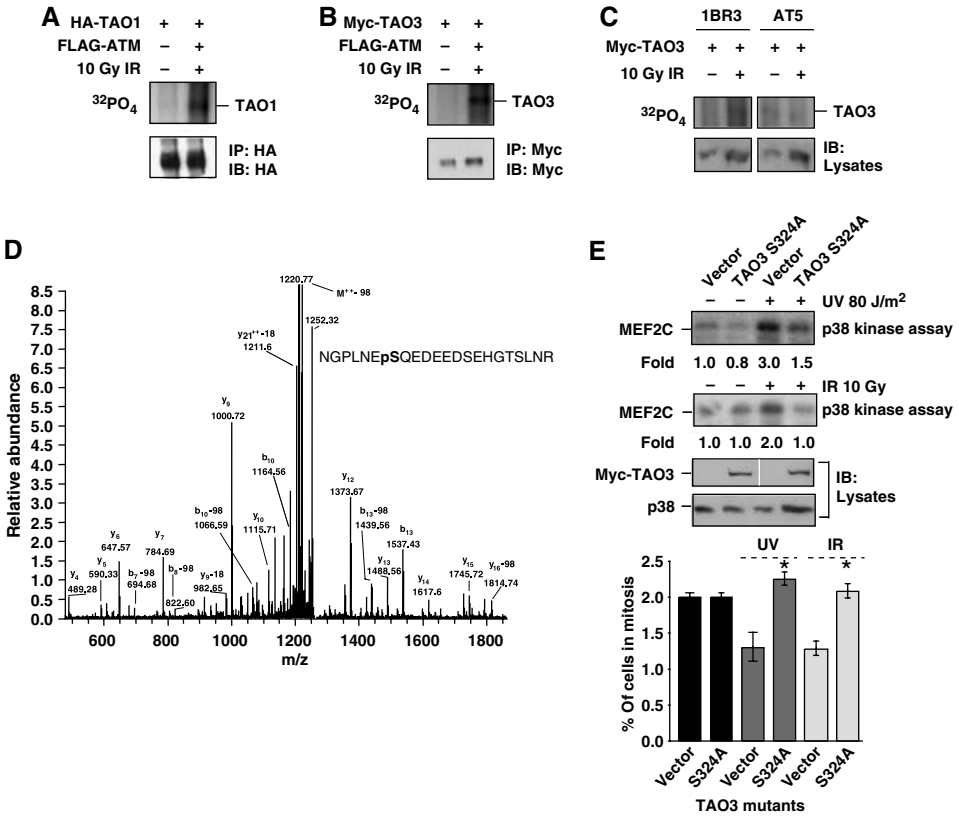

Figure 7 TAOs are phosphorylated *in vivo* following irradiation. HeLa cells were transfected with FLAG-ATM and kinase-dead HA-TAO1 (**A**) or Myc-TAO3 (**B**). Cells were labeled with ^{32}P for 1 h and then irradiated for 1 h. TAOs were immunoprecipitated and ^{32}P incorporation was assessed by autoradiography. (**C**) Human skin fibroblasts from normal (1BR3) or ATM-deficient (AT5) cells were transfected with kinase-dead Myc-TAO3. Cells were labeled with ^{32}P for 1 h and irradiated for a further 1 h. Myc-TAO3 was immunoprecipitated and ^{32}P incorporation was detected by autoradiography. (**D**) HeLa cells were transfected with kinase-dead Myc-TAO3 and not treated or treated with 10 Gy IR for 1 h. TAO3 was immunoprecipitated and subjected to mass spectrometry. Mass spectroscopic data of the phosphopeptide (pS324) in the irradiated samples is shown. (**E**) Cells were transfected with vector alone or Myc-TAO3 S324A and were untreated or treated with 80 J/m^2 UV or 10 Gy IR for 2 h. p38 activity was assayed. The graph shows the G2/M checkpoint activation by FACS. Values shown are means ± s.e.m. ($n = 3$) $P \leqslant 0.05$ (*).

instructions (Stratagene). Dominant negative mutants of TAOs and MEKs are described elsewhere (Hutchison *et al*, 1998; Chen and Cobb, 2001). TAO3 (1–442) wild type and D165A were amplified by PCR from full-length constructs and ligated into p3XFLAG-CMV7.1. Chk2 and Cdc25C were gifts from Helen Piwnica-Worms (HHMI, Washington University). Cdc25C (200–259) was amplified by PCR and ligated into pGEX-KG. The pcDNA3-FLAG ATM plasmid was a gift from Michael Kastan (St Jude's Children's Hospital, Tennessee). All constructs were verified by sequencing. Small interfering oligonucleotides (siRNAs) corresponding to the TAO1 sense 5′-CCAGGCCAGGUGAAACUUGdTdT-3′ and antisense strands 5′-CAAGUUUCACCUGGUCUGGdTdT-3′, and the TAO3 sense 5′-GGACAGUAUGAUGGGAAAGdTdT-3′ and antisense strands 5′-CUUUCCCAUCAUACUGUCCdTdT-3′ were designed as in Elbashir *et al* (2001) and purchased from Ambion. SMARTpool TAO2 siRNA oligonucleotides were purchased from Dharmacon (MAH.061305.1). Oligonucleotides were used at a final concentration of 100 nM. Oligonucleotides were transfected into trypsinized cells with oligofectamine (Invitrogen) using the manufacturer's protocol. A second round of siRNA transfections was performed after 48 h and cells were harvested following indicated treatments after another 24 h.

Transfection and cell treatment

Cells were trypsinized and transfected with the indicated plasmid combinations using Fugene (Roche) as per the manufacturer's instructions. Transfection efficiency ranged from 70 to 80% based on expression of LacZ. Fragments of TAO2 and 3 were used in these experiments as full-length proteins express poorly. Cells were treated either with HU after 24 h for a further 20 h or after 48 h with UV/IR. Cells were washed in 1 × phosphate-buffered saline (PBS) and then lysed with buffer A (50 mM HEPES (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 10 mM sodium fluoride, 2 mM Na_3VO_4, 10 μg/ml aprotonin, 2 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF).

Antibodies and immunoblotting

The antibody to pTyr15-Cdc2 was from Cell Signaling Technologies. Goat-TAO3 antibody was from Serotec and mouse anti-TAO1 was from BD Biosciences. TAO2 and p38 antibodies are described (Chen *et al*, 1999). Mouse anti-FLAG antibody was from Sigma. Proteins in sample buffer were fractionated by SDS–PAGE and transferred onto nitrocellulose for immunoblotting (Chen *et al*, 1999).

Expression of recombinant proteins and kinase assays

Methods for expression of GST-MEF2C (204–321) and GST Cdc25C (200–259) were as described (Chen *et al*, 2003). Kinase assays were in 30 μl reactions containing 10 mM $MgCl_2$, 5 μM ATP, 15 cpm/fmol [γ-^{32}P] ATP, 1 mM DTT, and 10 mM HEPES (pH 7.5), and ~10 pmol recombinant substrates or 5 μg of myelin basic protein (MBP). Reactions were incubated at 30°C for 30 min and terminated with 5 × SDS sample buffer. Samples were boiled and resolved by SDS–PAGE, and processed for autoradiography.

ATM transfection and kinase assays

HEK293 cells in 100-mm dishes were transfected with empty vector or 10 μg pcDNA-Flag-ATM, HA-TAO1 D169A, or Myc-TAO3 D165A. After 48 h, cells transfected with the empty vector or ATM plasmid

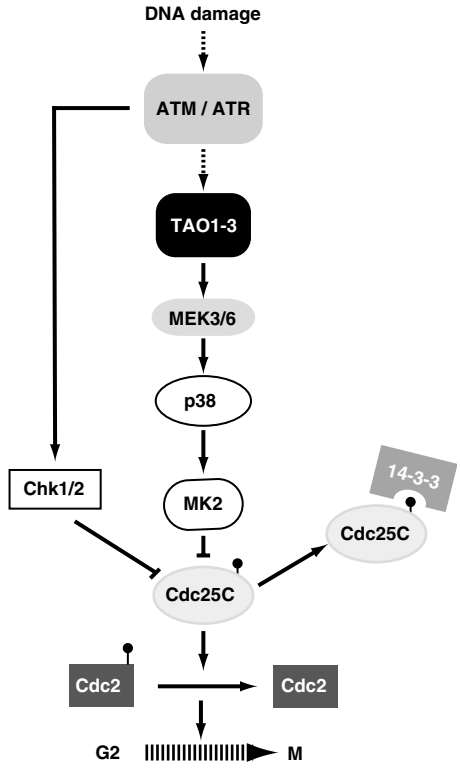

Figure 8 Model for p38 activation by DNA damage. TAO kinases are required for the activation of p38 by DNA damage.

were irradiated with 10 Gy IR to activate ATM. At 1 h after irradiation, cells were lysed in 0.5 ml of buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.2% Tween-20, 20 mM NaF, 2 mM Na_3VO_4, 1 mM DTT, 10 μg/ml aprotonin, 2 μg/ml leupeptin, and 10 μg/ml pepstatin). Lysates were rocked at 4°C for 30 min and clarified by centrifugation at 13 000 r.p.m. for 20 min. Immunoprecipitation was overnight at 4°C with anti-FLAG, anti-Myc or anti-HA, and 30 μl 1:1 slurry of protein A-Sepharose (Amersham Biosciences). Beads were washed twice with lysis buffer and with kinase buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 μg/ml aprotonin, and 2 μg/ml leupeptin). ATM-bound and TAO-bound beads were mixed in 20 μl kinase buffer with 5 μM ATP (15 cpm/fmol [γ-^{32}P] ATP). Reactions were incubated for 30 min at 30°C and terminated with 5 × SDS sample buffer. Samples were processed as above.

FACS

Cells were untreated or irradiated with 80 J/m^2 UV or 10 Gy IR for 2 h and then trypsinized and fixed in 70% ethanol overnight at 4°C, and permeabilized with 0.25% Triton X-100/PBS for 10 min on ice. Cells were stained with 10 μg/ml of the mitotic marker pSer10-H3 for 3 h at room temperature. After washing, cells were incubated for 30 min at room temperature in the dark with FITC-conjugated goat-anti-rabbit secondary antibody. Samples were washed and incubated with 0.25 μg/ml propidium iodide, 0.1 mg/ml RNase A in PBS for 30 min at 37°C and processed for FACS. Data were collected with a Becton Dickinson FACS machine and analyzed using Cell Quest software.

Colony survival assays

HeLa cells were transfected twice 2 days apart with siRNA oligonucleotides. After an additional 24 h, cells were trypsinized and counted. In all, 3000 cells were plated in 60-mm dishes and allowed to adhere for 3–4 h. Cells were either untreated or irradiated at the indicated doses. After 7 days, surviving colonies were stained with crystal violet stain (0.05% crystal violet, 1% formalin in PBS); colonies containing more than 50 cells were counted. Samples were analyzed in triplicate and percentage survival was calculated by comparison to matched nonirradiated plates.

^{32}P labeling

HeLa, 1BR3, or AT5 cells were transfected with kinase-dead HA-TAO1 or Myc-TAO3 in the presence or absence of FLAG-ATM. After 48 h, cells were incubated in phosphate-free medium containing 1 mCi/ml [^{32}P]orthophosphate (MP Biomedicals) for 1 h. Cells were untreated or irradiated for 1 h. Cell lysates were prepared as described above and TAOs were immunoprecipitated via antibodies to their epitope tags. Proteins were resolved by electrophoresis and ^{32}P incorporation was visualized by autoradiography.

Mapping phosphorylation sites in TAO3 by mass spectrometric analysis of tryptic peptides

In-gel digestion. The protein of interest was cut out from the gel. Protein in-gel digestion, peptide extraction, and peptide cleaning using a μ-C18 Ziptip were carried out as previously reported (Zhao *et al*, 2004).

HPLC/MS/MS analysis

Tryptic digests of the protein were analyzed by nano-HPLC/LTQ mass spectrometry (ThermoFinigan, San Jose, CA) coupled with Agilent 1100 nano-flow HPLC system (Agilent, Palo Alto, CA) for identification of phosphopeptide and precise location of phosphorylation site. Briefly, each sample was dissolved in 4 μl of HPLC buffer A (1% acetic acid in water (v/v)) and 2 μl were injected into the HPLC. Peptides were separated on a homemade capillary HPLC column (100 mm length × 75 μm ID, 5 μm particle size, 100 Å pore diameter) with Luna C18 resin (Phenomenex, St Torrance, CA) and directly electrosprayed into the mass spectrometer using nano-spray source. The peptides were eluted from the column with a linear gradient of 0–80% buffer B (90% acetonitrile/9.95% water/0.05% acetic acid (v/v/v) in buffer A (2% acetonitrile/97.95% water/0.05% acetic acid (v/v/v)) over 50 min. The MS/MS spectra were acquired in a data-dependent mode. The 10 strongest ions in each MS spectrum were automatically selected for fragmentation.

Protein sequence database search and manual verification

All MS/MS spectra were searched against NCBI-nr protein sequence database with the specification of the possible phosphorylation modification at serine, threonine, and tyrosine residues using MASCOT database search engine. Phosphorylated peptides identified with MASCOT score >20.0 were manually examined with the rules previously described (Chen Y *et al*, 2005b) and all phosphorylation sites have to be identified by consecutive b- or y ions so that the possibilities that phosphorylation (+98 Da) occurs on adjacent residues were eliminated.

Statistics

Error bars in all figures represent s.e.m. Student's two-tailed *t*-test was used to determine the significance of experimental values relative to control values.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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