The role of RelA (p65) threonine 505 phosphorylation in the regulation of cell growth, survival, and migration

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ABSTRACT The NF-κB family of transcription factors is a well-established regulator of the immune and inflammatory responses and also plays a key role in other cellular processes, including cell death, proliferation, and migration. Conserved residues in the *trans*-activation domain of RelA, which can be posttranslationally modified, regulate divergent NF-κB functions in response to different cellular stimuli. Using *rela*−/− mouse embryonic fibroblasts reconstituted with RelA, we find that mutation of the threonine 505 (T505) phospho site to alanine has wide-ranging effects on NF-κB function. These include previously described effects on chemotherapeutic drug-induced apoptosis, as well as new roles for this modification in autophagy, cell proliferation, and migration. This last effect was associated with alterations in the actin cytoskeleton and expression of cellular migration–associated genes such as WAVE3 and α-actinin 4. We also define a new component of cisplatin-induced, RelA T505–dependent apoptosis, involving induction of NOXA gene expression, an effect explained at least in part through induction of the p53 homologue, p73. Therefore, in contrast to other RelA phosphorylation events, which positively regulate NF-κB function, we identified RelA T505 phosphorylation as a negative regulator of its ability to induce diverse cellular processes such as apoptosis, autophagy, proliferation, and migration.

INTRODUCTION

The NF-κB family of transcription factors is an important regulator of the cellular response to stress and infection, functioning as controllers of the immune and inflammatory responses, as well as key cellular processes such as apoptosis, proliferation, and adhesion

(Hayden and Ghosh, 2008). Aberrantly active NF-κB subunits are associated with a wide range of inflammatory diseases (Kumar *et al.*, 2004). Moreover, NF-κB can contribute to the growth, survival, and malignancy of numerous cancer cell types while also significantly affecting the response of tumors to many types of chemotherapy and ionizing radiation (Kim *et al.*, 2006).

NF-κB complexes in mammalian cells consist of homodimers and heterodimers formed from five subunits: RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (Hayden and Ghosh, 2004). In the absence of an inducing stimulus, NF-κB complexes are typically held in an inactive form, bound to one of a family of IκB inhibitory proteins (Hayden and Ghosh, 2004). A number of pathways can lead to activation of NF-κB, and, of these, the classical (or canonical) pathway, which induces expression of RelA-containing complexes, is the best defined (Hayden and Ghosh, 2004; Perkins, 2007). The classic pathway is typically activated by inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin β, as well as bacterial products such as bacterial lipopolysaccharide (Hayden and Ghosh, 2004). However, in response to other stimuli,

RelA-containing complexes can become activated through different routes. For example, under many circumstances, DNA damage results in nuclear localization of NF-κB essential modulator (NEMO) and phosphorylation by ataxia telangiectasia mutated (ATM), with subsequent relocation back to the cytoplasm, where it activates the IκB kinase (IKK) complex (Wu and Miyamoto, 2007). IKK-independent, atypical pathways of activation have also been described, such as the p38 mitogen-activated protein kinase/CK2–mediated induction of NF-κB in response to UV light (Kato et al., 2003).

Posttranslational modifications can have a major effect on the function of NF-κB subunits (Perkins, 2006). The IKK subunits themselves can phosphorylate NF-κB proteins and modulate their transcriptional activity (Perkins, 2006, 2007). However, parallel signaling pathways can also modify NF-κB subunits, thus providing a mechanism to integrate NF-κB/IKK activity with other cellular processes (Perkins, 2006, 2007). Modification of NF-κB subunits can have diverse effects, including induction of protein degradation, release from IκB, and enhancement of DNA binding, as well as transcriptional effects, such as stimulating or inhibiting coactivator/corepressor binding (Perkins, 2006; O'Shea and Perkins, 2008). This can have dramatic effects on the consequences of NF-κB induction. For example, RelA has a strong antiapoptotic effect following TNFα stimulation but can stimulate cell death following treatment with some inducers of DNA damage (Campbell et al., 2004, 2006; Perkins and Gilmore, 2006; Strozyk et al., 2006; Wu and Miyamoto, 2008; Thayaparasingham et al., 2009). We and others have demonstrated that NF-κB subunits can both induce and repress the expression of the same gene targets, depending on the context of their activation (Campbell et al., 2004, 2006; Thayaparasingham et al., 2009) or cell cycle stage (Barre and Perkins, 2007), and the gene induction or repression correlates with changes in RelA phosphorylation.

The C-terminal RelA transactivation domain (TAD) is responsible for mediating most of its transcriptional effects in cells. Within this domain are a number of highly conserved known and putative phosphorylation sites (Campbell and Perkins, 2004; O'Shea and Perkins, 2008). It was suggested that different patterns of phosphorylation within the RelA TAD provide an explanation for the altered functions of RelA (Perkins, 2006, 2007; Moreno et al., 2010; O'Shea and Perkins, 2010). In particular, we previously defined the threonine 505 (T505) residue as having an important regulatory role under some conditions: T505 can become inducibly phosphorylated by the checkpoint kinase Chk1 in response to cisplatin and induction of the tumor suppressor p14^{ARF} (Rocha et al., 2003, 2005; Campbell et al., 2006). Moreover, T505 is phosphorylated by Chk1 in S phase as a consequence of activation of the DNA-replication checkpoint (Barre and Perkins, 2007). T505 phosphorylation is stimulus specific and is not seen after TNFα treatment or with other inducers of DNA damage such as UV or daunorubicin. Our previous data suggested that T505 phosphorylation induced a proapoptotic form of RelA that could facilitate cell death through transcriptional repression of antiapoptotic target genes such as Bcl-xL. Here we reconstituted rela$^{-/-}$ mouse fibroblasts with wild-type and T505 mutant forms of RelA. We confirm the important role that T505 plays in mediating a proapoptotic effect of RelA in response to cisplatin and extend this analysis to reveal roles in the regulation of autophagy, cellular proliferation, and migration.

## RESULTS
Reconstitution of rela$^{-/-}$ mouse fibroblasts
Previously we observed inducible phosphorylation of RelA at T505 following cisplatin treatment of U-2 OS human osteosarcoma cells (Campbell et al., 2006). Concurrently, we observed repression of the NF-κB target gene Bcl-xL, consistent with a proapoptotic role for RelA under these conditions. Moreover, mutation of RelA at T505 abolished cisplatin-mediated repression of the RelA TAD in luciferase reporter assays. Together, these results suggested that phosphorylation of RelA at T505 following cisplatin treatment mediates a proapoptotic effect and contributes to cisplatin-induced cytotoxicity. To obtain more definitive data concerning the role of T505 phosphorylation after cisplatin treatment, we reconstituted immortalized rela$^{-/-}$ mouse fibroblasts by retroviral transduction with wild-type and T505 mutated forms of human RelA (Figure 1A and Supplemental Figure S1A).

Consistent with our previous results, where we showed T505 phosphorylation during S phase of the cell cycle (Barre and Perkins, 2007), in the untreated asynchronous reconstituted rela$^{-/-}$ cells, RelA displayed a basal level of T505 phosphorylation. This was enhanced almost twofold following 12 h of cisplatin treatment (Figure 1A). Mutation of the threonine 505 residue to either alanine (T505A) or aspartic acid (T505D) reduced the phospho-antibody signal, although some background binding to RelA was still detectable. We also confirmed phosphorylation of endogenous RelA at T505 in untreated asynchronous wild-type Rela MEF cells that was further increased following cisplatin treatment (Supplemental Figure S1B). To demonstrate that the RelA was functional in the reconstituted rela$^{-/-}$ cells, we examined the response of RelA to TNFα. We observed reconstitution of the NF-κB complex by electrophoretic mobility shift assay (EMSA) following TNFα treatment (Supplemental Figure S1C) and resistance to TNFα-induced apoptosis (Figure 1B). Mutation of T505 had no effect on the antiapoptotic NF-κB response to TNF in these cells.

A feature of some isolates of immortalized rela$^{-/-}$ mouse fibroblasts is that they display a mild transformed phenotype and can form colonies in soft agar (Gapuzan et al., 2005). This is also a feature of the isolate of rela$^{-/-}$ cells used here (Figure 1C), which also contain mutant p53, as judged by the high levels of p53 protein seen by Western blot. Consistent with previous data from the Gilmore laboratory (Gapuzan et al., 2005), reconstitution of these cells with RelA suppressed soft agar colony formation, and this process was also not affected by mutation of T505 (Figure 1C).

We next investigated the response to cisplatin treatment. As predicted by our previous observations, we found that reintroduction of RelA enhanced cisplatin-induced cell death (Campbell et al., 2006). Of importance, the T505A mutation abolished the RelA-dependent enhancement of death, whereas the T505D mutation did not (Figure 1D and Supplemental Figure S2A). There was no difference in viability in the untreated control cells between the groups (Figure 1D). Similarly, T505A mutation was also protective against cell death caused by camptothecin and rimcazole but did not protect as well against cell death following treatment with methylmethanesulfonate (MMS; Supplemental Figure S2B). To confirm that these results did not occur due to variability in the individual clones, we examined the effect of cisplatin treatment on cell survival in wild-type Rela MEF cells transiently transfected with an empty RSV vector or with RSV containing human RelA, T505A, or T505D. To reduce background from endogenous Rela, these cells were treated with an small interfering RNA (siRNA) targeting mouse Rela 48 h prior to transient transfection (Supplemental Figure S2C). As in the reconstituted rela$^{-/-}$ cells, transient transfection with RelA enhanced cisplatin-mediated cell death, whereas transfection with a T505A-containing plasmid abolished the RelA-dependent enhancement of cell death (Supplemental Figure S2D). These data confirm the importance of T505 phosphorylation in mediating the response of RelA to specific DNA-damaging agents and, in particular, cisplatin.
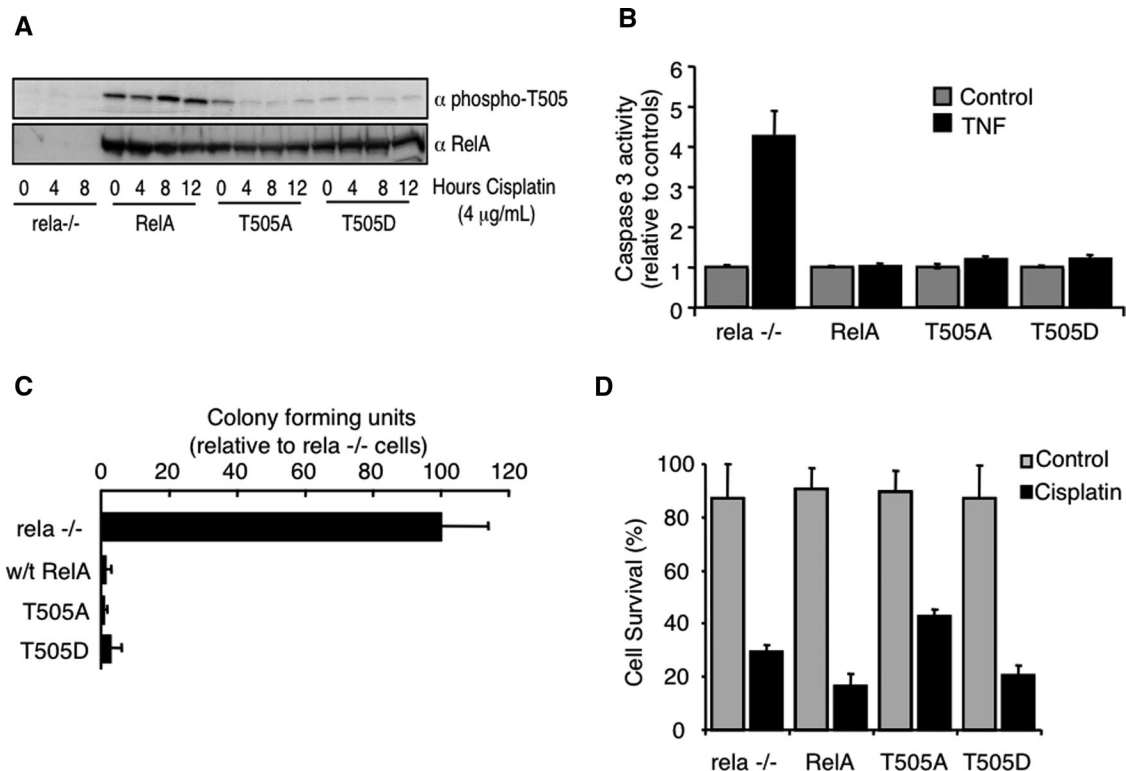
FIGURE 1: Reconstitution of *rela⁻/⁻* mouse fibroblast cells with RelA restores responsiveness to TNFα treatment. (A) Cisplatin stimulates RelA T505 phosphorylation in RelA reconstituted *rela⁻/⁻* MEF cells. Cells from individual MEF clones were treated with cisplatin (4 µg/ml) for 4, 8, or 12 h, and phosphorylation of RelA T505 was determined in whole-cell extracts (20 µg) by Western blotting. (B) Mutation of RelA at T505 protects *rela⁻/⁻* MEFs from TNFα-induced apoptosis. Cells were treated for 6 h with 50 ng/ml TNFα, and caspase 3 activity was detected in 25 µg of cell lysates using the Promega CaspasACE Kit. Data are shown as mean ± SD and expressed as caspase activity relative to the untreated control sample. (C) RelA T505 mutations still reverse the neoplastic phenotype of *rela⁻/⁻* MEFS. A total of 1 × 10⁵ cells were plated in 0.3% agar and incubated for 21 d. Colonies in the agar were counted at 2.5× magnification using a Leica microscope. Data are expressed as colonies formed relative to the *rela⁻/⁻* MEF cells. (D) T505A mutation protects cells from cisplatin-induced cell death. Cells were treated with 4 µg/ml cisplatin for 48 h, and the percentage of living cells was determined by trypan blue exclusion assay. Data are presented as mean ± SD and expressed as percentage survival. All data are representative of a minimum of three independent experiments.

Mutation of T505 abolishes RelA-dependent induction of apoptosis

Our previous data suggested that T505 phosphorylation regulates the apoptotic response (Campbell *et al.*, 2006). Confirming a proapoptotic effect of RelA in the reconstituted *rela⁻/⁻* cells following cisplatin treatment, we observed enhanced caspase 3 activation in the presence of wild-type RelA (Figure 2, A and B). The T505A mutation abolished this RelA-dependent induction of caspase 3. Similar data were seen with wild-type Rela MEFs transfected with RelA, T505A, or T505D (Supplemental Figure S2E). As seen in the *rela⁻/⁻* MEF cells, annexin staining was also reduced in the T505A MEF cells following cisplatin treatment when compared with wild-type RelA cells (Supplemental Figure S2F). In the reconstituted *rela⁻/⁻* MEFs, caspase 3 activation in response to cisplatin treatment in the T505D cells was reduced in comparison to the wild-type RelA cells but showed similar annexin staining (Figure 2B and Supplemental Figure S2F). Annexin staining detects phosphatidylserine exposure in both caspase-dependent and caspase-independent apoptosis (Ferraro-Peyret *et al.*, 2002). Given that these cells displayed the same level of enhanced cell death as seen with wild-type RelA (Figure 1D and Supplemental Figure S2A), the T505D cells may be dying through a caspase-independent pathway. We therefore investigated potential alternative mechanisms through which these cells might be dying.

Recently it has become apparent that autophagy, which can have both prosurvival and prodeath effects, depending on the cellular context, can contribute to cell killing (Mathew *et al.*, 2007; Levine and Kroemer, 2008). We therefore investigated whether autophagy might be involved in cisplatin-induced cell death in RelA reconstituted *rela⁻/⁻* cells. Yeast Atg8 and its mammalian homologue LC3 are ubiquitin-like modifiers that are localized on isolated membranes and play crucial roles in the formation of autophagosomes. Conversion of soluble LC3-I to lipid-bound LC3-II is associated with the formation of autophagosomes. We therefore analyzed the effects of RelA and cisplatin treatment on LC3 conversion in these cells. By Western blot, we observed, in both wild-type RelA and T505D reconstituted cells, an enhanced basal level of the LC3-II form that was further increased following cisplatin treatment (Figure 2A). By contrast, the control *rela⁻/⁻* cells and T505A reconstituted cells displayed a lower level of the LC3-II form, which, although still inducible by cisplatin treatment, never reached the levels seen in the wild-type RelA or T505D cells. This effect was analyzed more quantitatively by acridine orange staining, which undergoes a color conversion in autophagic vesicles that can be detected by fluorescence-activated cell sorting (FACS; Paglin *et al.*, 2001; Kanzawa *et al.*, 2003). In agreement with the Western blot data, both higher basal level and cisplatin-induced levels of autophagy were observed
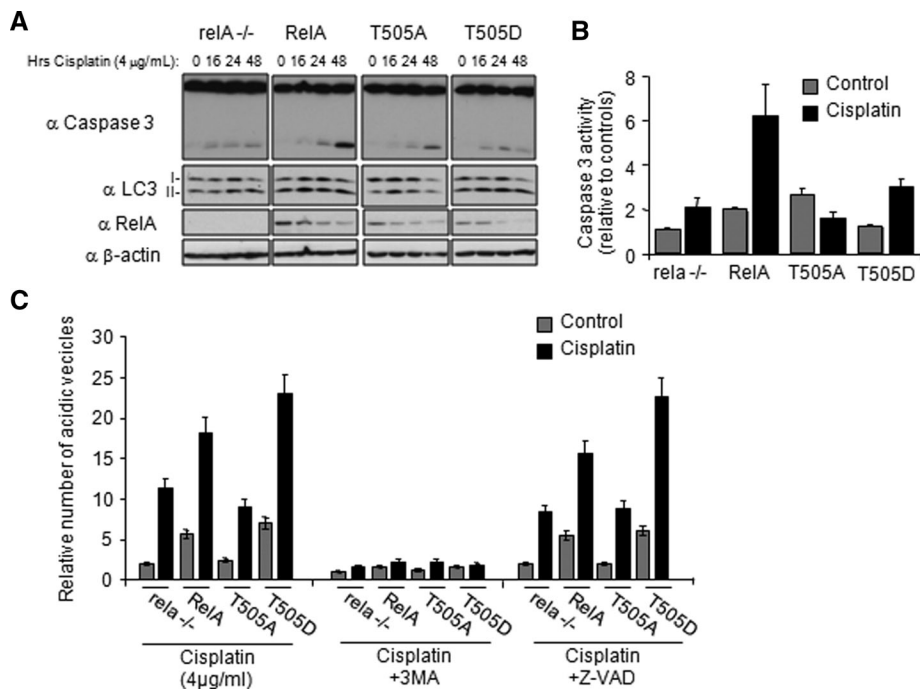
FIGURE 2: Mutation of T505 abolishes RelA-dependent induction of apoptosis. (A) Reconstituted *rela*−/− MEF cells were treated with 4 μg/ml cisplatin for 16, 24, or 48 h, and 20 μg of a whole-cell extract was analyzed by Western blot for levels of total and cleaved caspase 3, LC3-I and LC3-II isoforms, RelA. and β-actin. (B) RelA T505A mutation protects against cisplatin-induced apoptosis. Cells were treated for 24 h with 4 μg/ml cisplatin, and caspase 3 activity was detected in 25 μg of cell lysates using the Promega CaspasACE kit. Data are presented as mean ± SD and expressed as caspase activity relative to the dimethyl sulfoxide (DMSO) control sample. (C) The development of acidic vesicular organelles, indicative of autophagy, was examined following treatment with 4 μg/ml cisplatin, with or without 1-h pretreatment with the inhibitor 3-MA (1 mM) or Z-VAD (20 μM) for 24 h. Cells were stained with acridine orange, and the intensity of the red fluorescence was measured using FACS. Data are expressed as mean ± SD. All data represent a minimum of three individual experiments.

in the wild-type RelA and T505D reconstituted cells (Figure 2C). Confirming that the signal generated by acridine orange staining resulted from autophagy, this was inhibited by treatment with the autophagy inhibitor 3-methyladenine (3-MA) but not by the caspase inhibitor Z-VAD. However, 3-MA treatment did not totally protect against cell death in these cells, indicating that this is not an explanation for the enhanced level of cell death seen in wild-type RelA and RelA T505D reconstituted cells (Supplemental Figure S2A). This is consistent with our previous observation on the role of autophagy in response to cisplatin treatment (Barre and Perkins, 2010). A preapoptotic, protective lag phase in response to cisplatin treatment of renal cells has been shown to result from autophagy (Kaushal *et al.*, 2008; Periyasamy-Thandavan *et al.*, 2008). Moreover, autophagy has also been shown to have a protective effect against apoptosis induced in tumor cell lines (Harhaji-Trajkovic *et al.*, 2009; Barre and Perkins, 2010).
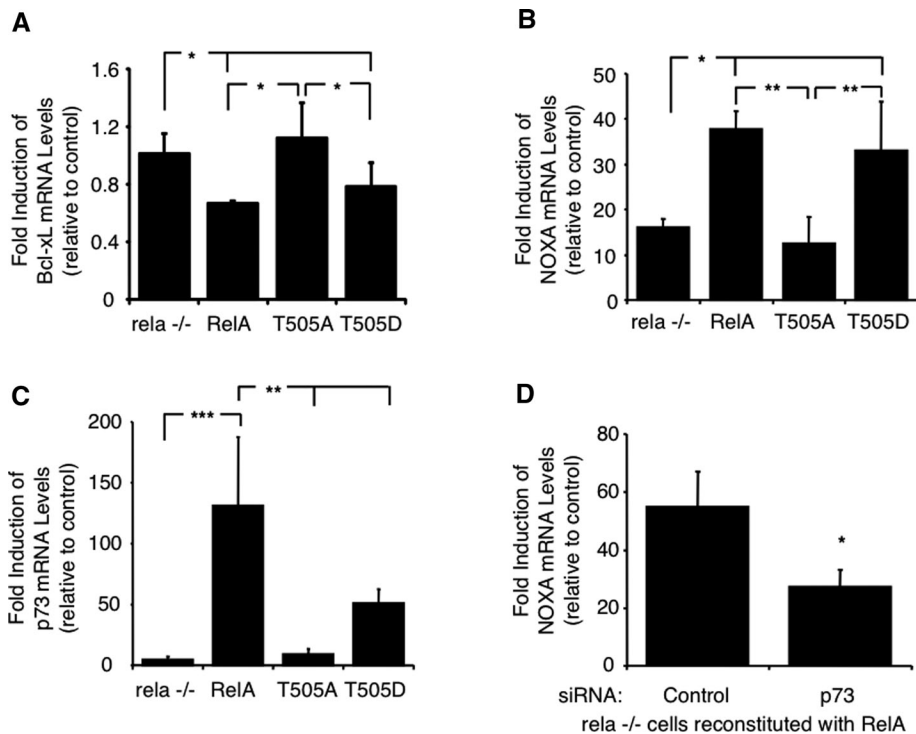
Regulation of NOXA gene expression by RelA and T505 dependence

Previously we observed that RelA can repress the expression of the antiapoptotic gene Bcl-xL in response to cytotoxic drugs, including cisplatin (Campbell *et al.*, 2004, 2006). In agreement with our previous data, in the reconstituted *rela*−/− MEFs we also observed significant RelA-dependent repression of Bcl-xL mRNA and protein levels, which was impaired in the T505A cells following cisplatin treatment (Figure 3A and Supplemental Figure S3A). Analysis of the

expression of other genes with a role in apoptosis indicated that NOXA (also known as PMA-induced protein 1, PMAIP1) was induced in these cells in a RelA-dependent manner upon cisplatin treatment (Figure 3B and Supplemental Figure S3B). Moreover, consistent with the effects on caspase 3 activation, T505A mutation inhibited RelA-dependent induction of this gene in both reconstituted *rela*−/− and wild-type MEF cells (Figure 3B and Supplemental Figure S3B). We previously showed that the RelA-dependent repression of the Bcl-xL gene results from RelA binding and recruitment of histone deacetylase (HDAC) to the Bcl-xL promoter (Campbell *et al.*, 2006). The NOXA promoter region contains a number of putative NF-κB–binding sites (Supplemental Figure S3C), and to determine whether RelA directly regulates NOXA gene expression we examined RelA binding to the NOXA promoter using chromatin immunoprecipitation. After 16 h of cisplatin treatment there was a moderate increase in RelA binding to the NOXA promoter in the wild-type RelA and T505A reconstituted *rela*−/− cells (Supplemental Figure S3D). We did not see any changes in HDAC1 binding at this time; however, this does not exclude the possibility that other classes of deacetylases are involved in this response. Recently it was shown that NOXA expression is regulated by p73 and that NF-κB is required for this induction (Martin *et al.*, 2009; O'Prey *et al.*, 2010). p73 is a member of the p53 tumor suppressor family and shares considerable homology and overlapping functions with p53. It is believed to act as a tumor suppressor and can bind to p53 gene targets and regulate apoptosis, although it has also been implicated in development and immunity (Davis and Dowdy, 2001). Of interest, NF-κB has also been implicated in regulation of p73 stability in response to TNFα treatment (Kikuchi *et al.*, 2006) and may represent another way in which NF-κB can mediate both antiapoptotic and proapoptotic actions. To determine whether mutation of RelA can alter p73 levels and thus also influence NOXA levels, we examined the level of p73 mRNA in the reconstituted *rela*−/− and wild-type MEF cells. As with NOXA, there was a significant increase in p73 mRNA levels in both the RelA and T505D MEF cells that was impaired in the T505A and *rela*−/− cells (Figure 3C and Supplemental Figure S3E), which suggests that RelA may regulate NOXA expression primarily through regulation of p73 levels.

To test this hypothesis, we examined the effect of p73 knockdown on cisplatin-induced NOXA mRNA levels in wild-type Rela MEF cells and *rela*−/− MEF cells reconstituted with human RelA. We observed a basal induction of NOXA following cisplatin treatment that was RelA independent, as seen in the *rela*−/− cells, that was further induced in the presence of RelA (Figure 3B and Supplemental Figure S3B). Of importance, knockdown of p73 in both the reconstituted *rela*−/− and wild-type MEF cells (Supplemental Figure S3, F and H) significantly inhibited the RelA-dependent, cisplatin-induced increase in NOXA levels, reducing them to levels similar to that seen in the *rela*−/− or T505A cells, but did not effect the RelA-independent

FIGURE 3: RelA regulation of NOXA gene expression. (A, B) RelA T505A mutation induces antiapoptotic and reduces proapoptotic gene expression following cisplatin treatment. Reconstituted $rela^{-/-}$ MEF cells were treated with cisplatin (4 μg/ml) or DMSO as a control for 4 h (Bcl-xL) or 16 h (NOXA). Following cisplatin treatment, RelA T505A induced, whereas RelA and T505D repressed, expression of the antiapoptotic gene Bcl-xL (A; $p < 0.05$, n = 5/group). Conversely, RelA T505A repressed, whereas RelA and T505D induced, expression of the proapoptotic gene NOXA (B; $p < 0.01$, n = 4/group). RNA was extracted from individual clones (A) and from pooled MEF cells (B). mRNA data are normalized relative to 18S mRNA and expressed as fold induction compared with the DMSO control. (C) mRNA was prepared from individual MEF clones following treatment with 4 μg/ml cisplatin for 16 h. The induction in p73 mRNA levels in the cisplatin-treated samples relative to the untreated controls was determined by real-time PCR. (D) Knockdown of p73 mRNA impairs the induction of NOXA following cisplatin treatment. In $rela^{-/-}$ MEF cells reconstituted with wild-type RelA, p73 was knocked down by siRNA, and NOXA mRNA levels were examined by real-time PCR following 16 h of cisplatin treatment. All PCR data are normalized relative to 18S mRNA and expressed relative to the DMSO control sample. Data are presented as mean ± SD and represent a minimum of three individual experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
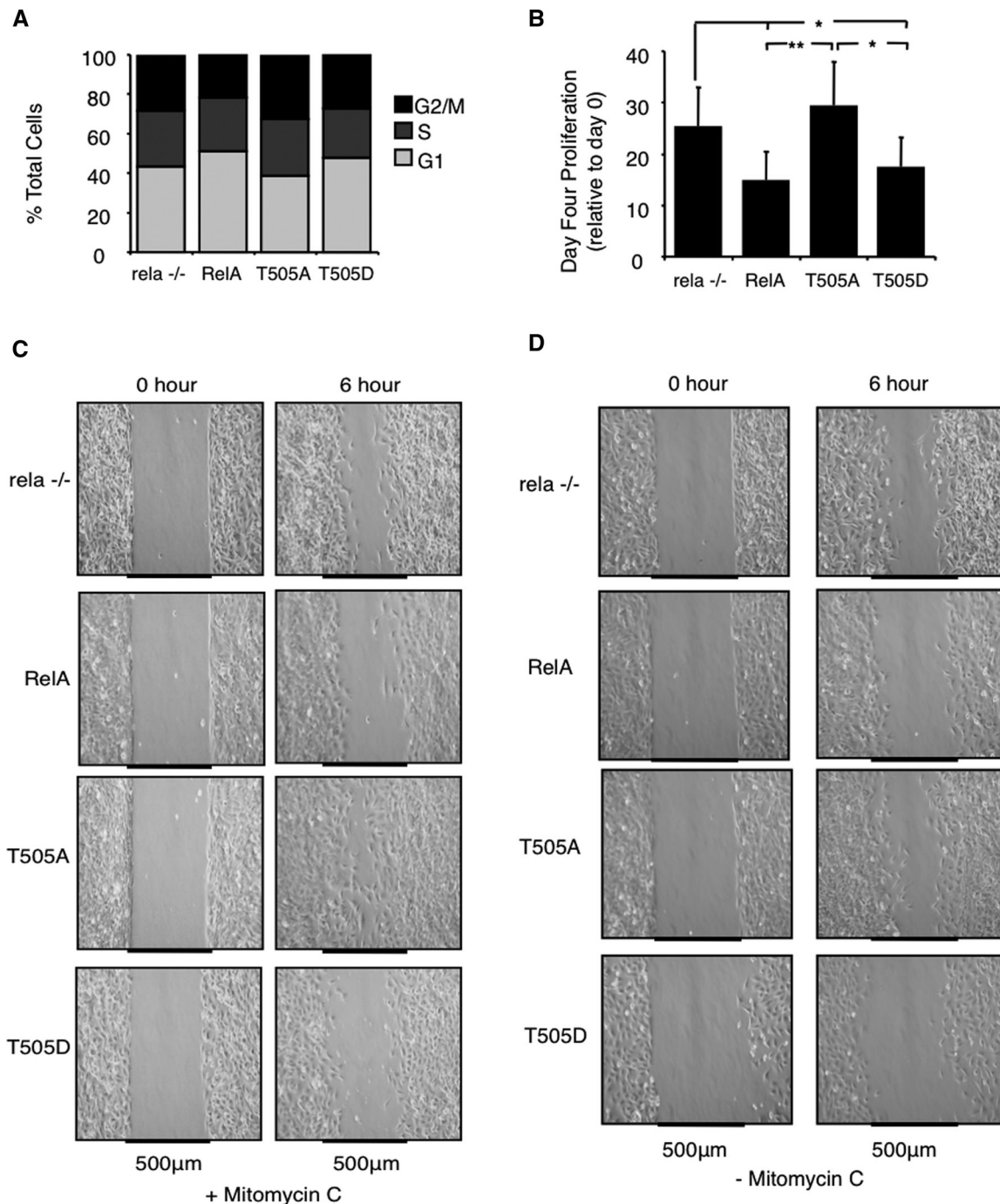
and filopodia (reviewed in Vignjevic and Montagnac, 2008). Analysis of cell proliferation by FACS showed that RelA T505A mutation decreased the proportion of G1-phase cells and increased G2/M-phase cells when compared with the wild-type RelA reconstituted $rela^{-/-}$ MEF cells (Figure 4A). Similarly, use of an MTS assay showed a significant increase in proliferation in the T505A cells when compared with the wild-type RelA and T505D cells (Figure 4B). Furthermore, analysis of cell migration in a wound-healing assay showed that migration is increased in the T505A MEF cells (Figure 4, C and D). This was independent of cell proliferation, as inhibition of proliferation with mitomycin C did not effect the rate of migration when compared with untreated cells (Figure 4, C and D). This effect could also be seen when plasmids containing wild-type or RelA T505 mutations were transfected into both wild-type Rela MEF and human U-2 OS osteosarcoma cells, where the inhibition of migration seen upon RelA expression was lost with the T505A mutation (Supplemental Figure S4, A and B). The increase in migration in T505A cells was also accompanied by an increase in the presence of filamentous actin fibers in these cells (Figure 5).

To determine how this effect of T505A mutation on cell migration is regulated, we examined the expression of a number of genes required for cellular migration. There was a significant increase in the mRNA levels of WAVE3, α-actinin 4, cortactin, N-Wasp, and integrin-α5 in the T505A cells compared with wild-type RelA cells (Figure 6A and Supplemental Figure S5A). However, there was no effect on other migration-associated genes, such as ARP2 (Supplemental Figure S5A). Similar data were observed in wild-type Rela MEFs transfected with RelA, T505A, or T505D plasmids (Figure 6B and Supplemental Figure S5B). To determine whether these gene changes were due directly to RelA, we examined binding of RelA to NF-κB sites in the promoters of WAVE3, α-actinin 4, N-Wasp, and integrin-α5, using chromatin immunoprecipitation (Supplemental Figure S6A). There was significantly increased binding of RelA to all of the promoters in RelA reconstituted $rela^{-/-}$ MEFs when compared with the T505A mutant cells (Figure 6C and Supplemental Figure S6B). This suggests that these genes are RelA targets and that binding of RelA may be involved in the repression of these genes. Overall our data indicate that RelA can alter cellular migration through regulation of specific migration genes and that phosphorylation of Thr505 is important in this regulation.

DISCUSSION

It is now understood that the role of NF-κB in regulation of cell survival and death is more complex than originally thought, with NF-κB capable of acting to both promote and inhibit apoptosis, depending on the stimulus and cell type (Perkins and Gilmore, 2006). Our data are consistent with previous reports of NF-κB and

induction of NOXA (Figure 3D and Supplemental Figure S3G). Our data suggest two mechanisms for the induction of NOXA following cisplatin treatment: a basal induction that is both RelA and p73 independent and a RelA/p73 dependent effect that can further enhance NOXA induction. We propose that the RelA/p73-dependent increase in NOXA expression seen will, together with repression of Bcl-xL, contribute to cisplatin-induced cell death.

Mutation of T505 alters cellular proliferation and migration

Our data demonstrated a role for RelA T505 phosphorylation in regulation of cell death following treatment with DNA-damaging agents. To determine whether this residue may contribute to other processes associated with tumorigenesis, we also examined the effect of mutation of T505 on cell proliferation and migration. The ability of cancer cells to evade proliferative blocks and to invade and migrate into surrounding tissues is recognized as a key hallmark of tumorigenesis and is a crucial step in the formation of cancer metastases (Hanahan and Weinberg, 2000). Cellular migration involves reorganization of the actin cytoskeleton, which provides the force for cell mobility and is initialized by the specialized structures of the lamellipodia

FIGURE 4: RelA T505 mutation affects cell proliferation and migration in MEF cells. (A, B) T505A mutation of RelA restores proliferation to levels matching the rela−/− MEF cells. Cells were seeded into six-well (FACS) or 96-well (MTS) plates, and growing MEF cells were analyzed for cell cycle stage using propidium iodide FACS (A) and for proliferation after 4 d by MTS assay (B). Data are presented as mean ± SD relative to the day-zero control. (C, D) Migration is increased in RelA T505A MEFs. Individual clones of the reconstituted rela−/− MEF cells were seeded in each side of an Ibidi culture insert and incubated for 24 h. Cells were pretreated with mitomycin C (C; 30 μM) or with culture media alone (D) for 30 min before removal of the insert. Cells were photographed at the time of insert removal and 6 h after insert removal and examined for cell migration. Scale bar, 500 μm. All experiments are representative of a minimum of three independent experiments. *p < 0.05, **p < 0.01.

RelA stimulating apoptosis following specific forms of genotoxic stress (Campbell et al., 2004; Wu and Miyamoto, 2008; Martin et al., 2009; O'Prey et al., 2010). Although we previously identified Bcl-xL repression as a component of this process, here we also demonstrate RelA-dependent induction of the proapoptotic gene NOXA. Of importance, T505A mutation reduced the level of

NOXA mRNA following cisplatin treatment (Figure 3B and Supplemental Figure S3B). Although NOXA was previously shown to be a RelA target gene in cortical neurons, with RelA regulating NOXA promoter activity and NOXA mRNA levels in response to cerebral ischemia (Inta et al., 2006), in this study we reported only moderate RelA binding to the NOXA promoter in wild type RelA and
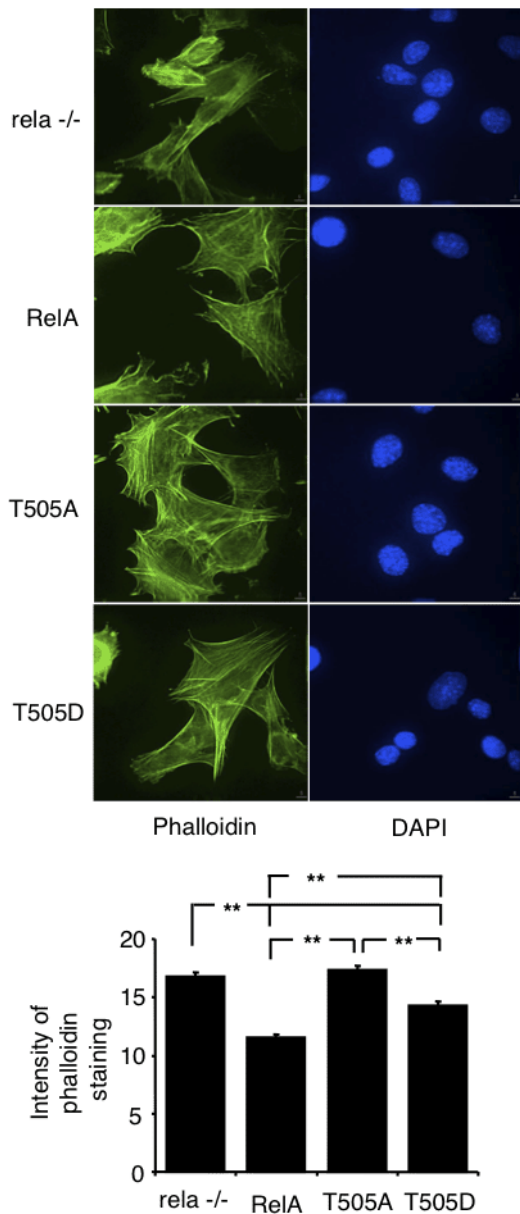
Phalloidin   DAPI

FIGURE 5: RelA T505 mutation alters the actin cytoskeleton. RelA mutation increases filamentous actin fibers in the reconstituted $rela^{-/-}$ MEF cells. Cells were stained with Alexa Flour 488 phalloidin and DAPI, and F-actin was examined using microscopy. The intensity of phalloidin staining was quantified by densitometry using ImageJ. All experiments are representative of a minimum of three independent experiments. **$p < 0.01$.

T505A reconstituted $rela^{-/-}$ cells (Supplemental Figure S3D), suggesting additional mechanisms. Indeed, consistent with a previous report of RelA-dependent NOXA expression by p73 (O'Prey et al., 2010), we found a significant RelA T505–dependent increase in p73 mRNA. Furthermore, knockdown of p73 could significantly inhibit only the cisplatin-induced, RelA-dependent and not the basal RelA-independent induction of NOXA (Figure 3D and Supplemental Figure S3G). Our data suggest that in response to cisplatin treatment, NOXA mRNA levels are induced by two mechanisms: a basal induction that is independent of RelA and a RelA/p73 mechanism that further enhances NOXA induction. Overall our data suggest that by coordinating patterns of gene induction and repression, RelA phosphorylation at Thr505

can determine specific cell death decisions in response to different stimuli.

We also observed RelA T505–dependent regulation of cellular proliferation and migration. Previous data from our group showed that multiple NF-κB subunits with differential posttranslational modifications can control cell cycle progression through regulation of proteins such as cyclin D1, c-Myc, and p21[WAF1] (Schumm et al., 2006; Barre and Perkins, 2007). Phosphorylation of RelA at T505 occurred primarily during S phase and was associated with decreased cyclin D1, c-myc, and skp2 expression (Barre and Perkins, 2007). In this study we showed that the RelA T505A mutation increased cell proliferation compared with wild-type RelA and T505D mutant cells (Figure 4, A and B). Furthermore, we also demonstrated RelA T505–dependent regulation of cell migration in both reconstituted $rela^{-/-}$ and wild-type Rela MEFs, as well as in U-2 OS cells. Here wild-type RelA suppressed cell migration, whereas mutation of T505 to alanine abolished this effect, with enhanced levels of migration being seen (Figure 4, C and D, and Supplemental Figure S4, A and B). This is not the first report to implicate NF-κB in cell migration. However, it is the first study to demonstrate the direct role of an individual NF-κB subunit, RelA, and the role of a specific phosphorylatable residue. Previously, loss of IKKβ was shown to increase invasion and migration (Chen et al., 2006). Conversely, loss of IKKα reduced cell migration, adhesion, and invasiveness, possibly through an NF-κB–independent direct effect of IKKα on target genes such as maspin (Merkhofer et al., 2010; Mahato et al., 2011). We also found RelA T505–dependent regulation of the mRNA expression of a number of migration-associated genes (Figure 6, A and B, and Supplemental Figure S5, A and B). Using chromatin immunoprecipitation (ChIP) analysis, we observed RelA binding to NF-κB elements in the promoters of these genes (Figure 6C and Supplemental Figure S6B), implying that the mRNA changes result at least in part through a direct effect of RelA on these genes.

This study demonstrated the importance of T505 phosphorylation as a negative regulator of RelA-dependent cellular processes. Overall, the effect of the T505A mutation is to create a form of RelA exhibiting enhanced oncogenic characteristics, leading to enhanced cellular proliferation and migration, together with enhanced resistance to chemotherapeutic treatments. We propose that pathways such as RelA T505 phosphorylation normally act to suppress NF-κB activity and oncogenicity. However, loss of these pathways during tumorigenesis will lead to forms of NF-κB with enhanced oncogenic abilities capable of promoting the development of malignant, metastatic cancer cells.

MATERIALS AND METHODS

Cells and DNA/siRNA transfection
Cell lines were grown in 10% fetal calf serum (Life Technologies, Carlsbad, CA)/DMEM (Lonza, Basel, Switzerland) for no more than 30 passages. DNA transfections were performed using polyethylenimine (Polysciences, Warrington, PA), and siRNA duplex oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and transfected using Interferin (Polyplus-transfection, Illkirch, France) as per manufacturer's instructions.

Reconstitution of $rela^{-/-}$ cells
Immortalized $rela^{-/-}$ MEFs were obtained from Ron Hay (University of Dundee, Dundee, United Kingdom). RelA cDNAs were inserted into the PIRESpuro-deNotI, viral vector plasmid, which, together with the VSV-G, viral envelope protein expressing plasmid, and CMVR8.91, the viral packaging plasmid, were transfected into HEK 293 cells to generate lentiviral particles essentially as described (Zufferey et al., 1998).
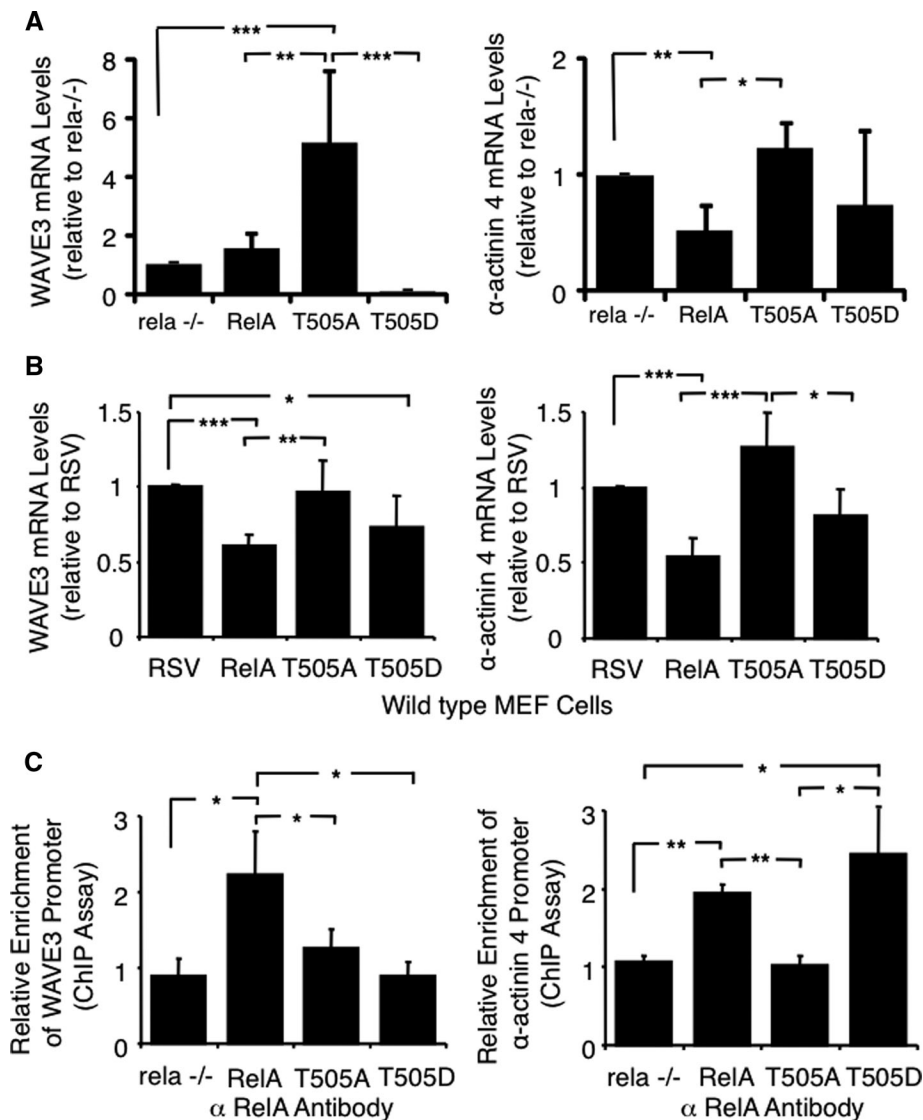
FIGURE 6: RelA T505 mutation alters migration-associated genes in MEF cells. (A, B) RelA T505A mutation alters expression of migration-associated genes. mRNA was extracted from individual reconstituted *rela*−/− MEF clones (A) and wild-type Rela MEFs transfected with a RelA, T505A, or T505D plasmid (B), and mRNA levels of WAVE3 and α-actinin 4 were examined by quantitative PCR. PCR data are normalized relative to 18S mRNA and expressed relative to the *rela*−/− (A) or RSV (B) control samples. (C) RelA binds to the promoter of WAVE3 and α-actinin 4. RelA binding to the promoters of WAVE3 and α-actinin 4 was examined in reconstituted *rela*−/− MEF cells, using chromatin immunoprecipitation. ChIP data were normalized to the individual input samples and are expressed relative to a Gal4 control. Data are presented as mean ± SD. All experiments are representative of a minimum of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

HEK 293 cell–generated viral supernatant was mixed with 4 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) and was used to infect 5 × 10⁵ *rela*−/− MEFs in a 10-cm dish containing 5 ml of media. Selection for reconstituted *rela*−/− MEFs was performed with 4 µg/ml puromycin (Sigma-Aldrich). Experiments were performed as indicated on pooled populations of stably transduced cells or on isolated clones.

Caspase and annexin V assays

To determine whether the cells were dying by apoptosis, both adherent and floating cells were harvested by centrifugation, and cell pellets were washed once in ice-cold phosphate-buffered saline (PBS). Caspase activity was analyzed with the CaspasACE Kit (Promega, Madison, WI), following the manufacturer's instructions, using 25 µg of protein extract. Annexin V staining was analyzed with the Annexin V FITC Apoptosis Detection Kit (Calbiochem, La Jolla, CA) using 0.5 × 10⁶ cells, following the manufacturer's instructions.

Crystal violet stain

Cells were counted, and equal numbers of cells were grown in six-well plates for 48 h before treatment with chemotherapeutic drugs for 48 h. Surviving cells were washed once with PBS and fixed with 100% methanol for 10 min at room temperature before staining with 0.05% (wt/vol) crystal violet in 25% methanol for 5 min. Plates were repeatedly washed with distilled water, allowed to air dry, and scanned for analysis.

Acridine orange assay for autophagy

To quantify the development of acidic vesicular organelles in cells following DNA damage, cells were stained with acridine orange and the intensity of the red fluorescence measured as described previously (Paglin *et al.*, 2001; Kanzawa *et al.*, 2003). Green (510–530 nm) and red (>650 nm) fluorescence emission from cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (BD Biosciences, San Diego, CA), using CellQuest software.

Soft agar assays to assess colony formation

Cells were counted, and 1 × 10⁵ cells were mixed with molten agar (0.3% wt/vol final concentration) in DMEM medium and poured onto a layer of 0.3% (wt/vol) set agar in a six-well dish. The agar was allowed to set at room temperature before incubation at 37°C for 21 d. Colonies were photographed, stained with neutral red (Sigma-Aldrich) for 1 h at 37°C, and counted.

Migration/wound-healing assays

Cells were seeded at a density of 3 × 10⁴ cells on each side of an Ibidi culture insert for live cell analysis (Ibidi, Munich, Germany), with a 500 µM separation between each side of the well, and allowed to grow for 24 h. Cells were pretreated with or without 30 µM mitomycin C for 30 min before removal of the insert, and following removal of the insert cells were incubated in DMEM also containing media alone or media with 30 µM mitomycin C. Cells were photographed using a Powershot A640 camera (Canon, Lake Success, NY) connected to an Axiovert 40C microscope using the 10× objective (Zeiss, Thornwood, NY) at insert removal (0 h) and following 6 and 24 h of incubation.

Phalloidin staining and fluorescence microscopy for F-actin

Cells were seeded on coverslips for 48 h, washed with PBS, and fixed with 3.7% formaldehyde. After a further wash with PBS the

cells were permeabilized with 0.1% Triton X-100, washed twice with PBS, and stained with Alexa Fluor 488 phalloidin (1:200; Invitrogen, Carlsbad, CA) for 20 min at room temperature. Coverslips were washed twice with PBS and mounted with 4′,6-diamidino-2-phenylindole mounting media (Vectashield, Vector Laboratories, Burlingame, CA). Images were taken on a DeltaVision Spectris wide-field deconvolution microscope, using softWoRx software (Applied Precision, Seattle, WA).

Other assays

Transient transfections, siRNA knockdown, EMSAs, trypan blue, and MTS assays of cell viability, RNA extraction, real-time quantitative reverse transcription–PCR, protein extracts, Western blots, FACS, and ChIP were all performed as described previously (Rocha et al., 2003; Rocha et al., 2005; Campbell et al., 2006; Schumm et al., 2006; Barre and Perkins, 2007). Information on oligonucleotides used is shown in Supplementary Methods.

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