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Identification of a novel pro-apopotic function of NF-κB in the DNA damage response

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Abstract

NF-κB is activated by DNA-damaging anticancer drugs as part of the cellular stress response. However, the consequences of drug-induced NF-κB activation are still only partly understood. To investigate the impact of NF-κB on the cell's response to DNA damage, we engineered glioblastoma cells that stably express mutant IκBα superrepressor (IκBα-SR) to block NF-κB activation. Here, we identify a novel pro-apoptotic function of NF-κB in the DNA damage response in glioblastoma cells. Chemotherapeutic drugs that intercalate into DNA and inhibit topoisomerase II such as Doxorubicin, Daunorubicin and Mitoxantrone stimulate NF-κB DNA binding and transcriptional activity prior to induction of cell death. Importantly, specific inhibition of drug-induced NF-κB activation by IκBα-SR or RNA interference against p65 significantly reduces apoptosis upon treatment with Doxorubicin, Daunorubicin or Mitoxantrone. NF-κB exerts this pro-apoptotic function especially after pulse drug exposure as compared to continuous treatment indicating that the contribution of NF-κB becomes relevant during the recovery phase following the initial DNA damage. Mechanistic studies show that NF-κB inhibition does not alter Doxorubicin uptake and efflux or cell cycle alterations. Genetic silencing of p53 by RNA interference reveals that NF-κB promotes drug-induced apoptosis in a p53-independent manner. Intriguingly, drug-mediated NF-κB activation results in a significant increase in DNA damage prior to the induction of apoptosis. By demonstrating that NF-κB promotes DNA damage formation and apoptosis upon pulse treatment with DNA intercalators, our findings provide novel insights into the control of the DNA damage response by NF-κB in glioblastoma.

Keywords: NF-κB • apoptosis • glioblastoma • DNA damage

Introduction

Glioblastoma is the most common malignant brain tumour, which bears a very poor prognosis [1]. Characteristic features of this tumour include uncontrolled proliferation, resistance to apoptosis, robust angiogenesis, diffuse infiltration and genomic instability, pointing to aberrant regulation of multiple signalling pathways [2].

Upon chemotherapy, the damage to DNA is a common initial event [3, 4]. DNA double strand breaks are considered to be one of the key lesions that initiate activation of the DNA damage response and are produced directly or indirectly by many anti-cancer drugs, including DNA intercalating, alkylating or cross-linking agents, topoisomerase inhibitors and nucleotide analogues [3]. Upon DNA double strand breaks, ataxia telangiectasia mutated (ATM) is recruited by the MRE-11-Rad50-NBS1 (MRN) complex to sites of broken DNA and phosphorylates downstream substrates such as checkpoint kinase 2 (Chk2) [4, 5]. Damage to DNA engages DNA repair processes to ensure the cell's survival in case of sublethal damage [3]. Alternatively – if the damage is too severe to be repaired – the DNA-damaging insult is transmitted by the cellular stress response to the activation of effector systems to mediate cell death [3]. In the latter case, various stress-inducible molecules, including NF-κB, p53, JNK or MAPK/ERK, have been implicated in propagating and modulating the cell death signal [6, 7].

The transcription factor NF-κB is composed of hetero- or homodimers of the NF-κB/Rel family of proteins [8]. In most cell types, NF-κB proteins are sequestered in the cytoplasm by their

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interaction with inhibitor of κB (IκB) proteins, predominantly IκB-α [8–10]. NF-κB activity is induced in response to a variety of stimuli, including DNA-damaging anticancer agents [8]. In the context of DNA double strand breaks, NF-κB is activated *via* ATM, which transmits the signal to the cytoplasmic IKK complex through phosphorylation of NEMO [11, 12]. NF-κB can exert pleiotropic functions in the course of the DNA damage response [6]. For example, NF-κB has been reported to transcriptionally activate anti-apoptotic proteins [13], which may promote evasion of apoptosis in case of sublethal damage. *Vice versa*, NF-κB has also been described to actively repress anti-apoptotic target genes upon treatment with certain chemotherapeutic agents, resulting in increased apoptosis [14–16].

We previously reported that inhibition of NF-κB does not translate into enhanced spontaneous or cytotoxic drug-induced apoptosis in glioblastoma cells [17], indicating that NF-κB does not simply orchestrate an anti-apoptotic program in glioblastoma as observed in a variety of other solid cancers or haematological malignancies [8]. To gain further insights into the function of NF-κB in glioblastoma, we investigated in the present study the role of drug-induced NF-κB activity in the regulation of the DNA damage response.

Results

Generation of glioblastoma cell lines with stable inhibition of NF-κB

To investigate the role of NF-κB in the regulation of the DNA damage response in glioblastoma, we generated glioblastoma cell lines, which stably express the dominant-negative superrepressor mutant IκBα-S(32, 36)A (IκBα-SR) or empty vector control. IκBα-SR is resistant to proteasomal degradation, because it cannot be phosphorylated at the two phosphorylation sites serine 32/36, and thus blocks NF-κB activation. For this purpose, we selected two prototypical glioblastoma cell lines, *i.e.* U87MG and T98G, which harbour p53 wild-type and p53 mutant, respectively. Retroviral transduction resulted in strong ectopic expression of IκBα-SR (Fig. 1A). To control functionality of mutant IκBα-SR protein, we assessed NF-κB DNA binding activity by electrophoretic mobility shift assay (EMSA) and apoptosis induction in response to the pro-inflammatory cytokine tumour necrosis factor (TNF)α, a prototypical model of apoptosis inhibition by NF-κB [8]. Ectopic expression of IκBα-SR substantially reduced basal as well as TNFα- or Doxorubicin-stimulated NF-κB DNA binding activity (Fig. 1B). Further, overexpression of IκBα-SR blocked TNFα-triggered NF-κB transcriptional activity, which in turn significantly increased TNFα-induced apoptosis (Fig. 1C and D). This demonstrates that stable overexpression of IκBα-SR results in potent blockade of the NF-κB pathway in a prototype model of the anti-apoptotic function of NF-κB in both U87MG and T98G glioblastoma cells.

DNA intercalators trigger NF-κB DNA-binding activity and transcriptional activation

Initially, we screened a panel of DNA-damaging drugs with different modes of action for their potential to trigger NF-κB activation in glioblastoma cells. To assess NF-κB activation, we analysed NF-κB DNA binding activity after drug treatment for 6 hrs, because we observed a delayed kinetic of NF-κB activation upon treatment with anticancer agents compared to the rapid kinetic of NF-κB activation by the prototypical NF-κB stimulus TNFα (Fig. 1B and [17]), which is in line with previous reports [6, 15, 18]. Interestingly, we found that in particular DNA intercalators, which also inhibit topoisomerase II such as Doxorubicin, Daunorubicin and Mitoxantrone, potently triggered NF-κB DNA binding in a dose-dependent manner in glioblastoma cells (Fig. 2, Table 1). Control experiments using a mutated oligo (competition experiments) confirmed the specificity of NF-κB DNA binding (Fig. S1A). Of note, anticancer drug-induced NF-κB DNA binding was completely prevented by overexpression of IκBα-SR (Fig. 2). Supershift analysis showed that Doxorubicin-induced NF-κB complexes consisted of p50 and p65 NF-κB subunits (Fig. S1B and [17]). By comparison, Etoposide, a topoisomerase II inhibitor that does not intercalate into the DNA, did not trigger NF-κB DNA binding in glioblastoma cells (Table 1). The DNA-alkylating agent Temozolomide caused NF-κB DNA binding in glioblastoma cells, yet less potent and with a delayed kinetic compared to Doxorubicin, Daunorubicin or Mitoxantrone (Table 1). To further explore the role of inducible NF-κB in the control of DNA damage and repair, we selected Doxorubicin, Daunorubicin and Mitoxantrone, because they potently activated NF-κB in the investigated glioblastoma cell lines.

NF-κB promotes DNA intercalator-induced apoptosis

We then assessed the effect of drug-induced NF-κB activation on chemosensitivity of glioblastoma cells. Interestingly, we found that inhibition of NF-κB significantly reduced apoptosis upon treatment with Doxorubicin, Daunorubicin or Mitoxantrone in p53 wild-type U87MG cells, especially after pulse exposure and subsequent removal of the drugs (Fig. 3A). This pro-apoptotic function of NF-κB was less prominent when U87MG cells were continuously incubated with cytotoxic drugs and was not observed in p53 mutant T98G cells (Fig. 3A and B). Also when a wide dose range was assessed, NF-κB inhibition did not significantly alter Doxorubicin-, Daunorubicin- or Mitoxantrone-induced cytotoxicity in both cell lines (Fig. S2).

To further investigate the contribution of NF-κB, we used a second, independent approach to inhibit NF-κB, *i.e.* knockdown of p65 by RNA interference. Western blot analysis confirmed that p65 protein expression was substantially reduced by siRNA against p65 (Fig. 3C). Importantly, silencing of p65 significantly reduced apoptosis following pulse treatment with Doxorubicin

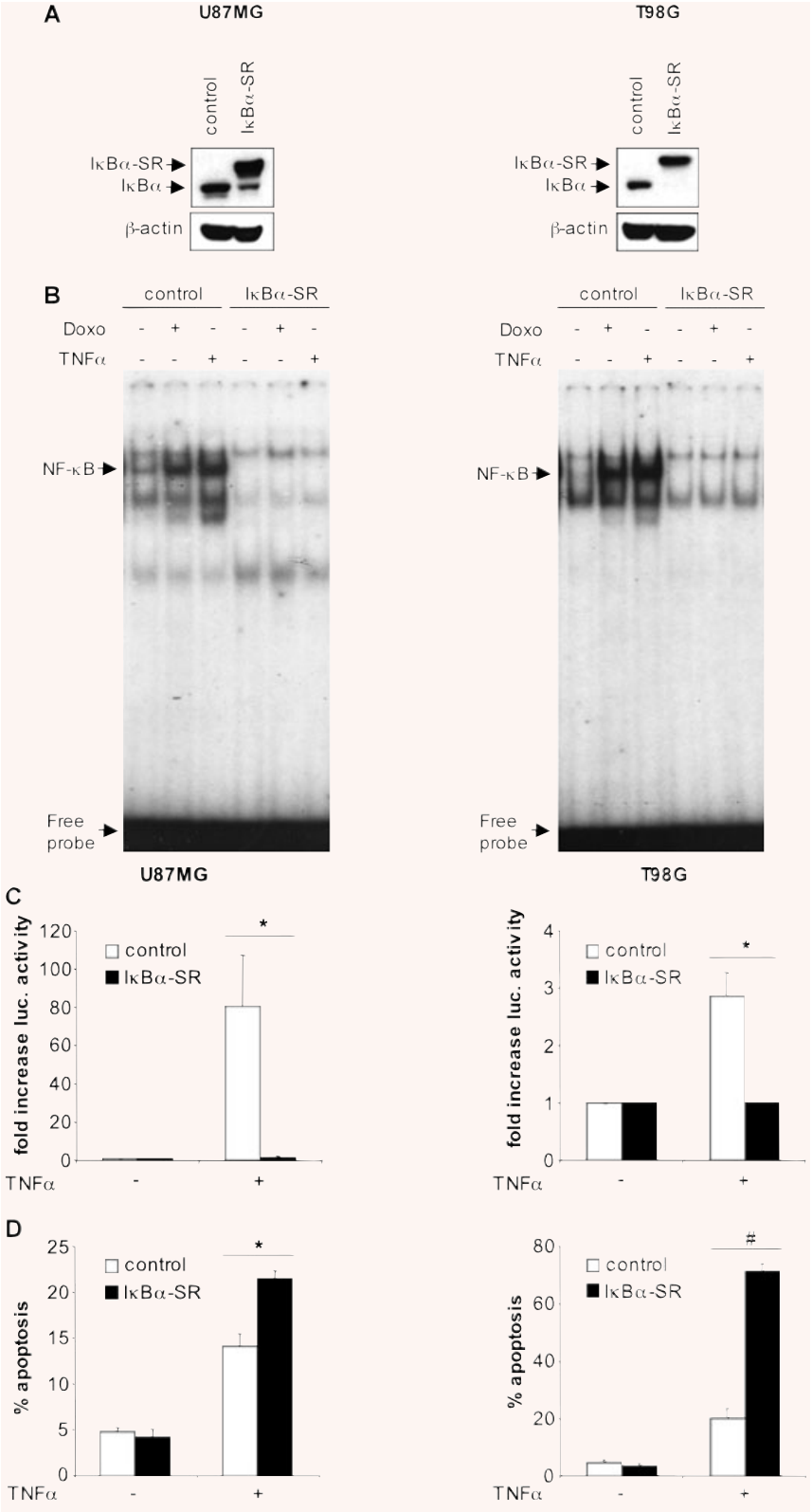

Fig. 1 Generation of glioblastoma cell lines with stable NF-κB inhibition. (**A**) Ectopic expression of IκBα-SR. U87MG and T98G glioblastoma cells were transduced with a control vector or a vector containing IκBα-SR. Protein expression of wild-type IκBα and mutant IκBα-SR was determined by Western blot analysis. β-actin served as loading control. (**B**) Inhibition of NF-κB DNA binding by IκBα-SR. NF-κB DNA binding was assessed by EMSA in nuclear extracts of cells transduced with control vector or a vector containing IκBα-SR that were left untreated or were treated with 0.8 μg/ml (U87MG) or 1 μg/ml (T98G) Doxorubicin for 6 hrs or 10 ng/ml TNFα for 1 hr. (**C**) Inhibition of NF-κB transcriptional activity by IκBα-SR. U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were transiently transfected with firefly and renilla luciferase gene constructs, treated for 6 hrs with 10 ng/ml TNFα and analysed by dual luciferase assay for induction of NF-κB transcriptional activity. Fold increase in luciferase activity relative to unstimulated control is shown. (**D**) Enhancement of TNFα-induced apoptosis by NF-κB inhibition. U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were left untreated (–TNFα) or were treated with 50 ng/ml TNFα for 48 hrs (+TNFα). Apoptosis was determined by FACS analysis of DNA-fragmentation of propidium iodide stained nuclei. Mean values of three independent triplicate experiments with S.D. are shown; $*P < 0.05$ and $\#P < 0.001$ comparing IκBα-SR *versus* control.

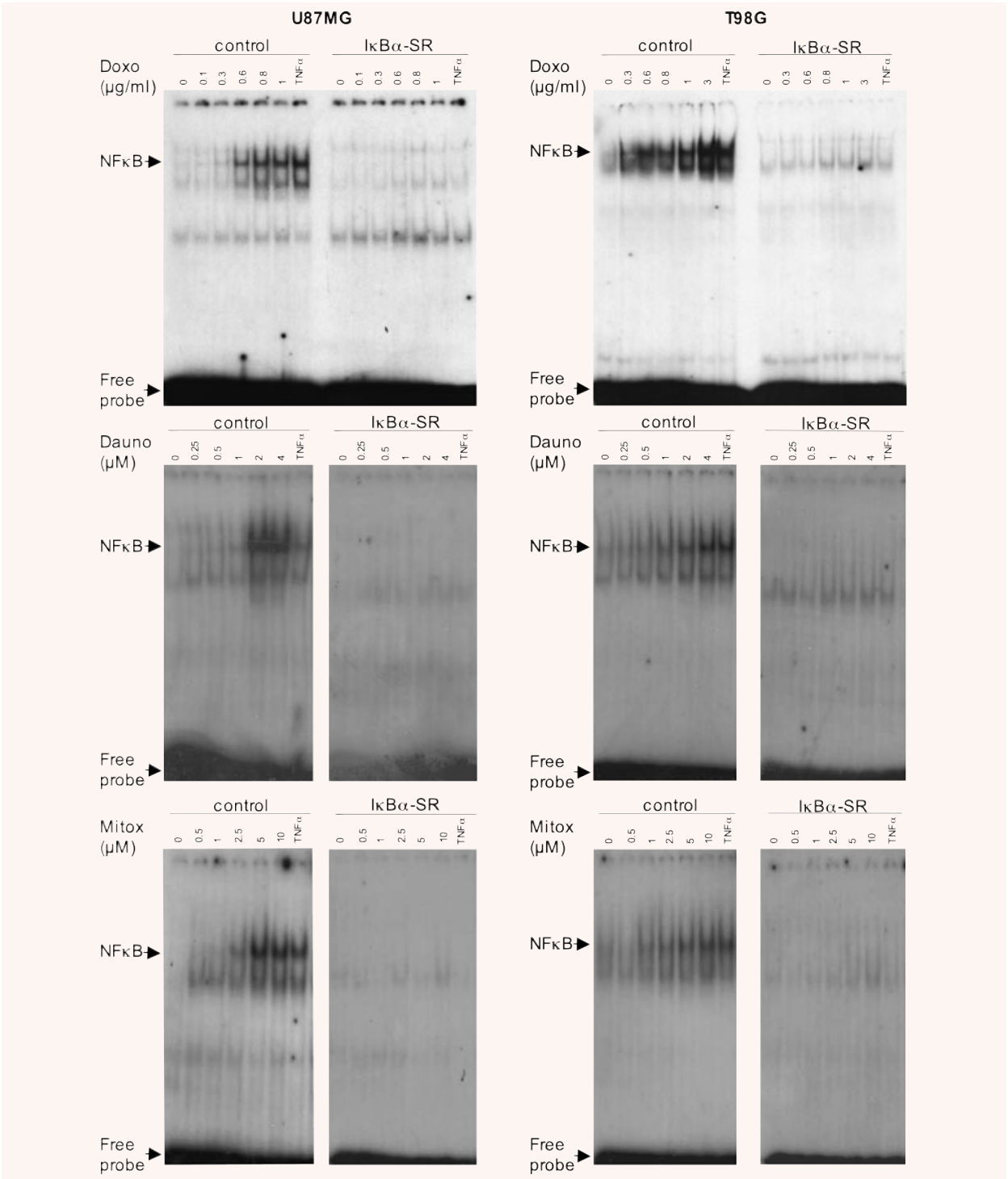

Fig. 2 DNA intercalators trigger NF-κB DNA-binding activity. U87MG (left panels) or T98G (right panels) cells stably transduced with control vector or a vector containing IκBα-SR were treated for 6 hrs with indicated concentrations of Doxorubicin (Doxo), Daunorubicin (Dauno) or Mitoxantrone (Mitox) or for 1 hr with 10 ng/ml TNFα. Nuclear extracts were analysed by EMSA for NF-κB binding to DNA.

Table 1 Activation of NF-κB upon treatment with different DNA-damaging drugs

Drug	Mode of action	Concentrations	U87MG	T98G
Doxorubicin	DNA intercalation	0.3–3 μg/ml	+++	+++
	TopoII inhibition			
	ROS generation			
Daunorubicin	DNA intercalation	0.25–4 μM	+++	+++
	TopoII inhibition			
	ROS generation			
Mitoxantrone	DNA intercalation	0.5–10 μM	+++	+++
	TopoII inhibition			
Bleomycin	Radiomimetic	40–320 μg/ml	–	+
Etoposide	Topo II inhibition	3–30 μg/ml	–	–
Camptothecin	Topo I inhibition	0.01–10 μM	+	+
Topotecan	Topo I inhibition	0.03–0.3 μg/ml	–	–
BCNU	DNA alkylation	30–300 μM	+	+
Temozolomide	DNA alkylation	0.6–2 mM	++	++
Cisplatin	DNA adduct formation	10–60 μg/ml	–	–

U87MG and T98G glioblastoma cells were treated for 6 hrs (or 24 and 48 hrs for Temozolomide) with the respective drugs and NF-κB DNA binding activity was assessed by EMSA. The ability of the different drugs to activate NF-κB is summarized in this table: +++ strong DNA binding; ++ intermediate DNA binding; + weak binding; – no binding.

(Fig. 3D), further supporting the pro-apoptotic function of NF-κB in the context of Doxorubicin-induced apoptosis.

Because anticancer agents have recently been reported to activate a transcriptionally repressive form of NF-κB [15, 16], we determined NF-κB transcriptional activity in response to drug treatment to find out whether NF-κB promotes apoptosis in glioblastoma cells by transcriptional suppression of anti-apoptotic genes. Notably, Doxorubicin, Daunorubicin and Mitoxantrone enhanced NF-κB transcriptional activity in both U87MG and T98G glioblastoma cells as determined by luciferase reporter assay, which was completely blocked in cells that express IκBα-SR (Fig. 3E). Also here, control experiments using a luciferase reporter construct without NF-κB binding sites confirmed specific NF-κB activation (Fig. S3A) and hence transcriptional activity as shown by mRNA expression levels of TNFα, an established NF-κB target gene [8] (Fig. S3B). This indicates that transcriptional repression of anti-apoptotic genes does not account for the pro-apoptotic function of NF-κB in the course of drug-induced apoptosis in glioblastoma cells as shown by Western blot analysis of anti-apoptotic target genes of NF-κB (Fig. 3F).

Effect of NF-κB inhibition on Doxorubicin uptake or efflux and cell cycle

To elucidate the underlying molecular mechanisms for the observed pro-apoptotic function of NF-κB in DNA damage-induced apoptosis, we systematically analysed different steps of the cell's stress response. First, we determined whether NF-κB alters drug uptake and/or efflux, as anthracyclines are known substrates of multidrug-resistant related proteins, which are among the NF-κB target genes [8]. Flow cytometric analysis showed no differences in Doxorubicin uptake or efflux in cells overexpressing IκBα-SR or control vector (Fig. 4A), indicating that differential drug uptake or efflux is unlikely responsible for the observed differences in apoptosis.

Moreover, we assessed cell cycle progression, because NF-κB target genes comprise cell cycle regulatory proteins [4, 8]. Doxorubicin triggered a similar arrest in the S/G2 phase of the cell cycle irrespective of NF-κB activation (Fig. 4B). This indicates that the observed differences in Doxorubicin-induced cell death are not simply the consequence of NF-κB-mediated changes in cell cycle progression.

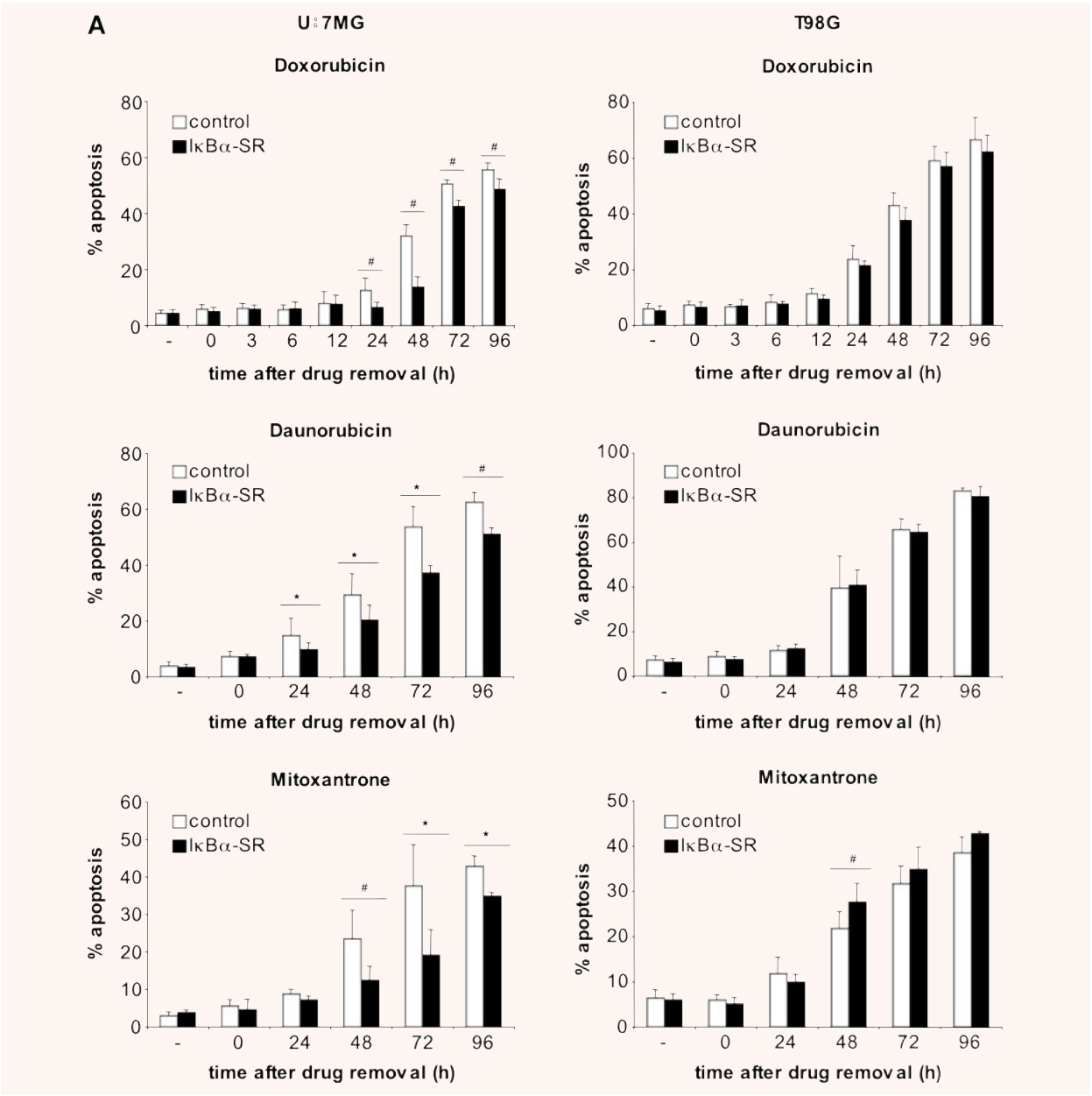

Fig. 3 NF-κB promotes DNA intercalator-induced apoptosis. In (**A**), U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated with 0.8 μg/ml (U87MG) or 1 μg/ml (T98G) Doxorubicin for 18 hrs, 2 μM Daunorubicin for 18 hrs or 2.5 μM Mitoxantrone for 3 hrs (U87MG) or 6 hrs (T98G), followed by a complete exchange of medium. After the indicated time points, apoptosis was determined by FACS analysis of DNA-fragmentation of propidium iodide stained nuclei. In (**B**), U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were continuously treated for indicated times with 0.8 μg/ml (U87MG) or 1 μg/ml (T98G) Doxorubicin, 2 μM Daunorubicin or 2.5 μM Mitoxantrone and apoptosis was assessed by FACS analysis of DNA fragmentation of propidium iodide stained nuclei. In (**C**) and (**D**), U87MG cells were transiently transfected twice consecutively with p65 siRNA or control siRNA. Forty-eight hours after the second transfection cells were reseeded (seed) and the next day treated with 0.8 μg/ml Doxorubicin for 18 hrs (treat), followed by a complete exchange of medium. In (**C**), Protein expression of p65 was analysed by Western blotting the day of seeding, treating and 0 hr, 24 hrs and 48 hrs after drug removal. β-actin served as loading control. In (**D**), U87MG cells were transiently transfected twice with control siRNA (white bars) or p65 siRNA (hatched bars).

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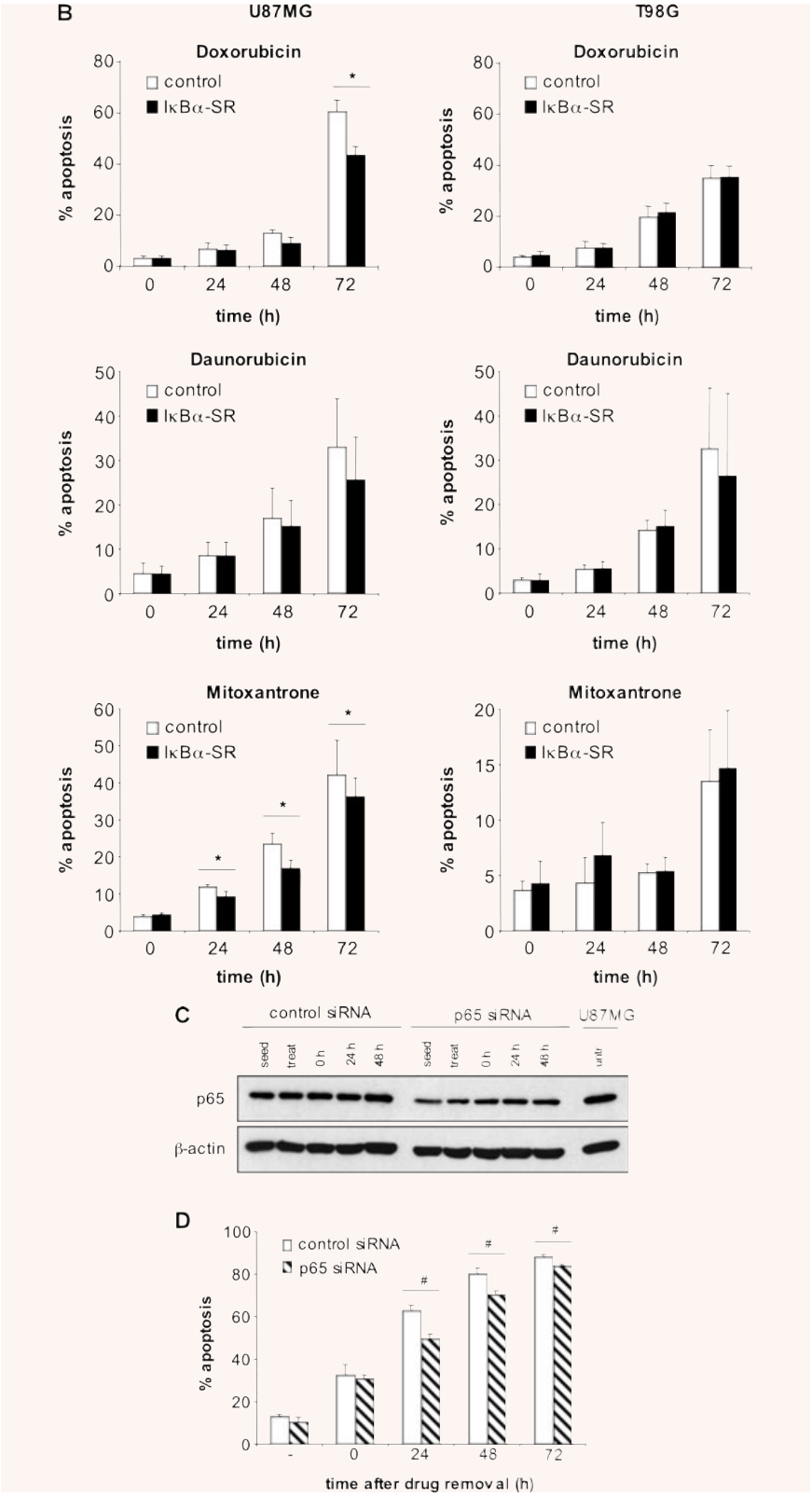

After the indicated time points, apoptosis was determined by FACS analysis of DNA-fragmentation of propidium iodide stained nuclei. In (**E**), U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated for 24 hrs with 0.8 μg/ml (U87MG) or 1 μg/ml (T98G) Doxorubicin, 2 μM Daunorubicin or 2.5 μM Mitoxantrone and analysed by dual luciferase assay for induction of NF-κB transcriptional activity. Fold increase in luciferase activity relative to unstimulated control is shown. In (**F**), U87MG cells stably transduced with control vector or a vector containing IκBα-SR were treated for 12 and 18 hrs with 0.8 μg/ml Doxorubicin and expression levels of anti-apoptotic proteins were determined by Western blotting. EBV-transformed B cells were used as positive control (PC) for cIAP2 expression. Actin served as loading control in (**A**), (**B**), (**D**) and (**E**), mean + S.D. of three independent experiments performed in triplicates is shown. $*P < 0.05$ and $\#P < 0.001$ comparing IκBα-SR *versus* control or p65siRNA *versus* control siRNA.

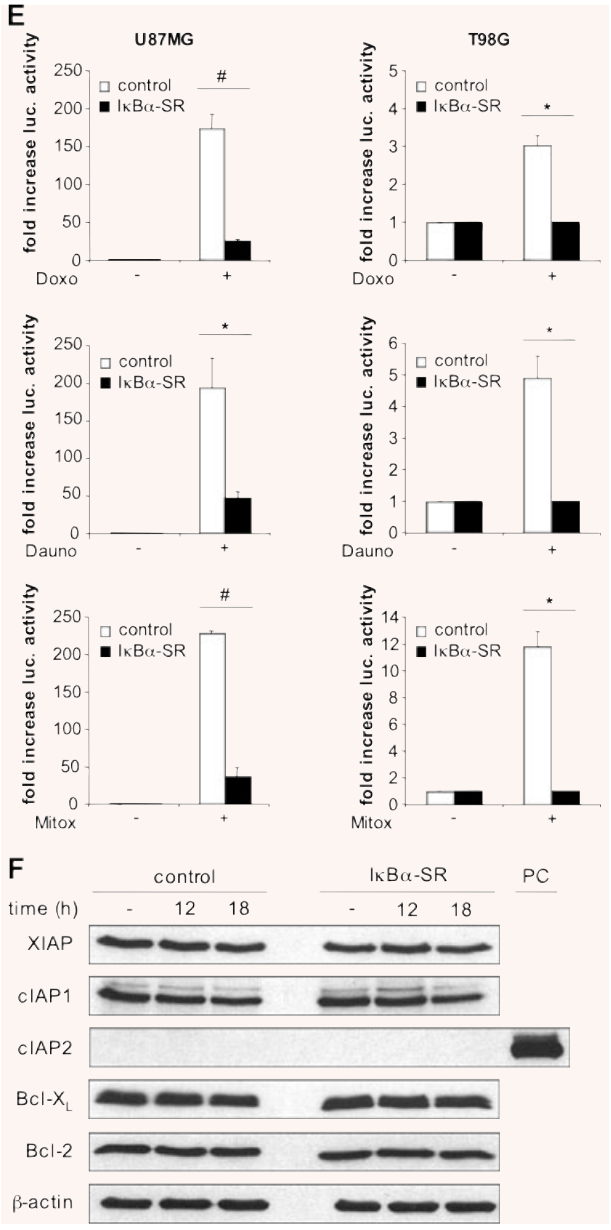

Fig. 3 Continued

NF-κB enhances Doxorubicin-induced apoptosis in a p53-independent manner

Because we observed modulation of apoptosis by NF-κB in p53 wild-type U87MG cells but not in p53 mutant T98G cells, we then asked whether p53 is involved in the regulation of apoptosis by NF-κB. To address this point, we stably knocked down p53 by RNA interference in p53 wild-type U87MG cells. Control experiments confirmed that stable expression of p53 shRNA vectors prevented the accumulation of p53 upon Doxorubicin treatment (Fig. 5A). Of note, knockdown of p53 had no effect on Doxorubicin-induced apoptosis, neither in the presence nor in the absence of NF-κB activity (Fig. 5B). These findings point to p53-independent regulation of Doxorubicin-induced apoptosis by NF-κB.

NF-κB enhances DNA intercalator-induced DNA damage

Finally, we assessed the effect of NF-κB on drug-induced DNA damage and repair. To this end, we used the alkaline Comet assay the read-out of which, referred to as Olive Tail Moment, is considered to correlate directly with the amount of DNA double and single strand breaks [19]. When cells were continuously exposed to Doxorubicin, Daunorubicin or Mitoxantrone, minor effects on drug-induced DNA damage were observed in the presence or absence of NF-κB activity (Fig. 6A), consistent with little effect of NF-κB on the induction of apoptosis under continuous drug treatment (Fig. 3B). Intriguingly, when cells were instead pulse treated with Doxorubicin, Daunorubicin or Mitoxantrone and subsequently monitored for the resolution of DNA damage over a 24-hr time period, inhibition of NF-κB resulted in a significant reduction of DNA damage in U87MG cells (Fig. 6B). This diminished DNA damage in IκBα-SR overexpressing cells corresponded to the significant reduction in drug-induced apoptosis in these cells (Fig. 3A). By comparison, no differences in DNA damage upon pulse treatment with Doxorubicin, Daunorubicin or Mitoxantrone were detected between T98G cells expressing IκBα-SR or control vector (Fig. 6B), in line with our findings that NF-κB had minimal effects on drug-induced apoptosis in these cells (Fig. 3A).

To further elucidate the mechanism leading to the observed differences in DNA strand breaks as detected by Comet assay we used the protein synthesis inhibitor Cycloheximide (CHX) to see whether they were dependent on gene expression. Treatment of U87MG control cells with Doxorubicin and CHX significantly diminished DNA damage (Fig. 6C) as well as apoptosis (Fig. 6D) in comparison to Doxorubicin pulse-treated cells to a level comparable to Doxorubicin-treated IκBα-SR overexpressing cells. This indicates that NF-κB's transcriptional activity likely is required for the observed differences in DNA strand breaks and apoptosis between control and IκBα-SR expressing cells. To test whether this involves DNA repair enzymes we inhibited PARP1 by using the pharmacological inhibitor 3-Aminobenzamide (3-AB) [20]. Treatment of U87MG control cells with Doxorubicin and 3-AB did not alter the amount of DNA damage assessed by Comet assay (Fig. S4), suggesting that PARP1-mediated differences in DNA repair do not account for the differences in DNA strand breaks and apoptosis.

To finally exclude that the observed differences in DNA strand breaks were simply due to caspase-mediated apoptotic DNA

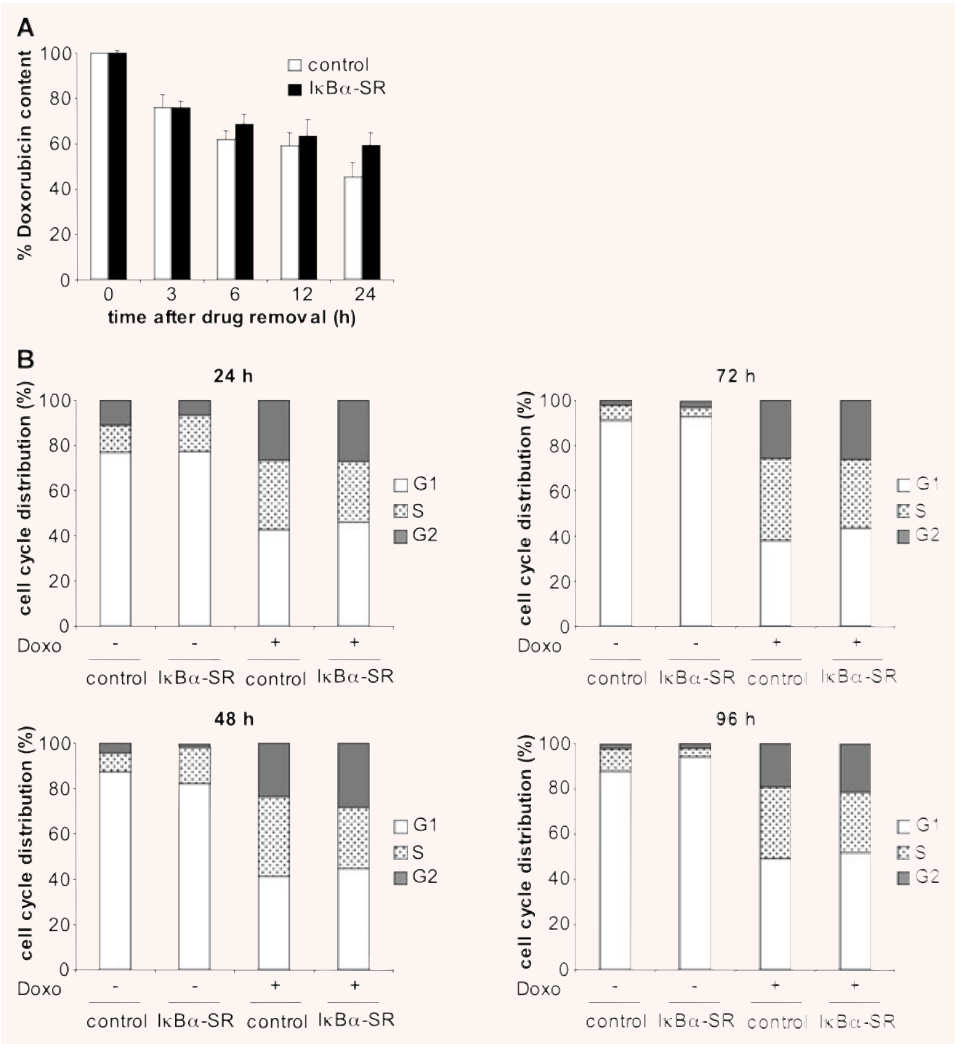

Fig. 4 Effect of NF-κB inhibition on Doxorubicin-induced cellular stress response. (**A**) Effect of NF-κB inhibition on Doxorubicin uptake and efflux. U87MG cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated with 0.8 μg/ml Doxorubicin for 18 hrs, followed by a complete exchange of medium. After the indicated time-points, Doxorubicin content was assessed by flow cytometry. Mean + S.D. of three independent experiments performed in triplicate are shown. (**B**) Effect of NF-κB inhibition on Doxorubicin-induced cell cycle arrest. U87MG cells stably transduced with control vector or a vector containing IκBα-SR were treated with 0.8 μg/ml Doxorubicin for 18 hrs. followed by a complete exchange of medium. After the indicated timepoints, cell cycle progression was assessed by flow cytometry. Mean of three independent experiments performed in triplicate are shown.

fragmentation, we also performed these experiments in the presence of the broad-range caspase inhibitor zVAD.fmk. Notably, zVAD.fmk did not prevent Doxorubicin-induced DNA damage (Fig. 6E), whereas zVAD.fmk completely blocked Doxorubicin-mediated apoptosis (Fig. 6F). This demonstrates that Doxorubicin-induced DNA damage occurs independently of caspase-mediated apoptotic events. Together, this set of experiments indicates that NF-κB inhibition reduces DNA damage and subsequently apoptosis in response to pulse treatment with the DNA intercalators Doxorubicin, Daunorubicin and Mitoxantrone in U87MG cells.

Because so far we found differences in DNA damage and apoptosis in only one of the two glioblastoma cell lines tested, we extended our studies to another glioblastoma cell line, *i.e.* p53 wild-type A172 cells, to rule out that the observed effects seen were cell type specific. Control experiments showed that stable overexpression of IκBα-SR inhibited NF-κB activation (Fig. 7A–C) and enhanced TNFα-induced apoptosis in A172 cells (Fig. 7D). Importantly, NF-κB inhibition significantly reduced DNA damage and apoptosis after pulse treatment with Doxorubicin (Fig. 7E and F). These experiments confirm in an independent cell line that NF-κB enhances DNA intercalator-induced DNA damage and apoptosis.

Discussion

NF-κB is activated upon treatment with DNA-damaging anticancer drugs as part of the cellular stress response [6]. However, the consequences of drug-induced NF-κB activation on downstream

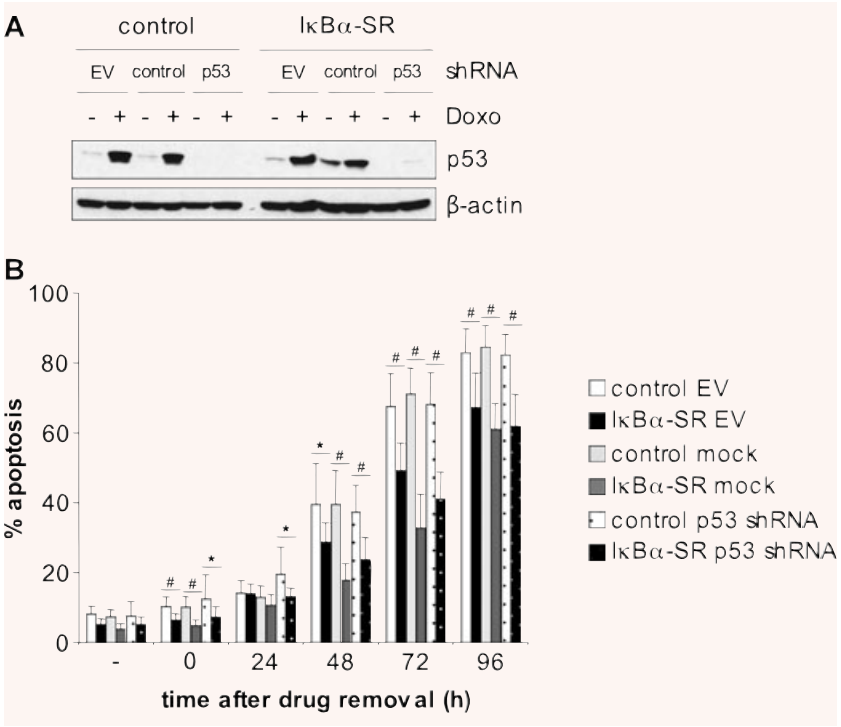

Fig. 5 NF-κB enhances Doxorubicin-induced apoptosis in a p53-independent manner. (**A**) Knockdown of p53. U87MG cells stably transduced with control vector or a vector containing IκBα-SR and stably infected with empty vector (EV), control shRNA or p53 shRNA vector were treated with 0.8 μg/ml Doxorubicin for 18 hrs. Protein expression of p53 was analysed by Western blotting. β-actin served as loading control. (**B**) Effect of p53 knockdown on Doxorubicin-induced apoptosis. U87MG cells stably transduced with control vector (bright bars) or a vector containing IκBα-SR (dark bars) and stably infected with empty vector (EV), control shRNA or p53 shRNA vector were treated with 0.8 μg/ml Doxorubicin for 18 hrs, followed by a complete exchange of medium. After the indicated time-points, apoptosis was assessed by FACS analysis of DNA fragmentation of propidium iodide stained nuclei. Mean + S.D. of three independent experiments are shown. $*P < 0.05$ and $\#P < 0.001$ comparing IκBα-SR *versus* control.

cellular events, for example on induction of cell death, are still only partly understood and likely context dependent.

Pro-apoptotic role of NF-κB after transient DNA damage in glioblastoma

In the present study, we identify a novel pro-apoptotic role of NF-κB in the course of the DNA damage response in glioblastoma cells. This conclusion is supported by our data in two independent glioblastoma cell lines showing that DNA intercalators such as the anthracyclines Doxorubicin and Daunorubicin and the anthracenedione Mitoxantrone stimulate NF-κB DNA binding and NF-κB transcriptional activity prior to the induction of cell death. Further, specific inhibition of DNA damage-induced NF-κB activation by dominant-negative IκBα-SR or p65 siRNA significantly reduces apoptosis upon treatment with DNA intercalators. This pro-apoptotic function of NF-κB in glioblastoma cells becomes especially evident after pulse drug exposure, indicating that NF-κB contributes to drug-induced apoptosis during the recovery phase following the initial DNA damage formation. These findings highlight the schedule dependency of NF-κB in the regulation of drug-induced apoptosis, a novel aspect of the present study that has previously not yet been identified.

NF-κB is known for its anti-apoptotic action [21] and has for example in glioblastoma cells been associated with reduced cytotoxicity of the alkylating agent BCNU, the platin compound carboplatin and the topoisomerase I inhibitor SN-38 [22]. More recently, NF-κB has also been linked to the induction of apoptosis in the context of DNA-damaging anticancer agents [6]. The pro-apoptotic function of NF-κB in response to topoisomerase II inhibitors or UVC irradiation has been attributed to active repression of anti-apoptotic genes by the NF-κB subunit p65 (RelA) because of lack of key post-translational modifications that are required for the role of p65 (RelA) as transcriptional activator [14–16]. However, topoisomerase II poisons have also been reported to stimulate NF-κB transcriptional activity [23, 24]. In glioblastoma cells – as demonstrated in the present study – treatment with topoisomerase II inhibitors that also intercalate into the DNA results in the production of NF-κB complexes that are competent not only for DNA binding but also for transcriptional activation from NF-κB reporter constructs as well as for induction of apoptosis. It is interesting to note that a pro-apoptotic role of NF-κB has also been described in several models of neuronal cell death. For example, neuronal death in response to ischemia, glutamate, or NMDA receptor triggering has been reported to require NF-κB [25–28]. Further, dopamine-induced cell death of pheochromocytoma cells, doxorubicin-, fenretinide- or betulinic acid-induced apoptosis of neuroblastoma cells or cell death triggered by MMP-9 inhibition in medulloblastoma cells have all been shown to depend on NF-κB activation [29–33]. A pro-apoptotic role of NF-κB in neuronal cells is further supported by data showing that anti-inflammatory drugs such as aspirin prevent neuronal cell death *via* inhibition of NF-κB [34]. These reports indicate that

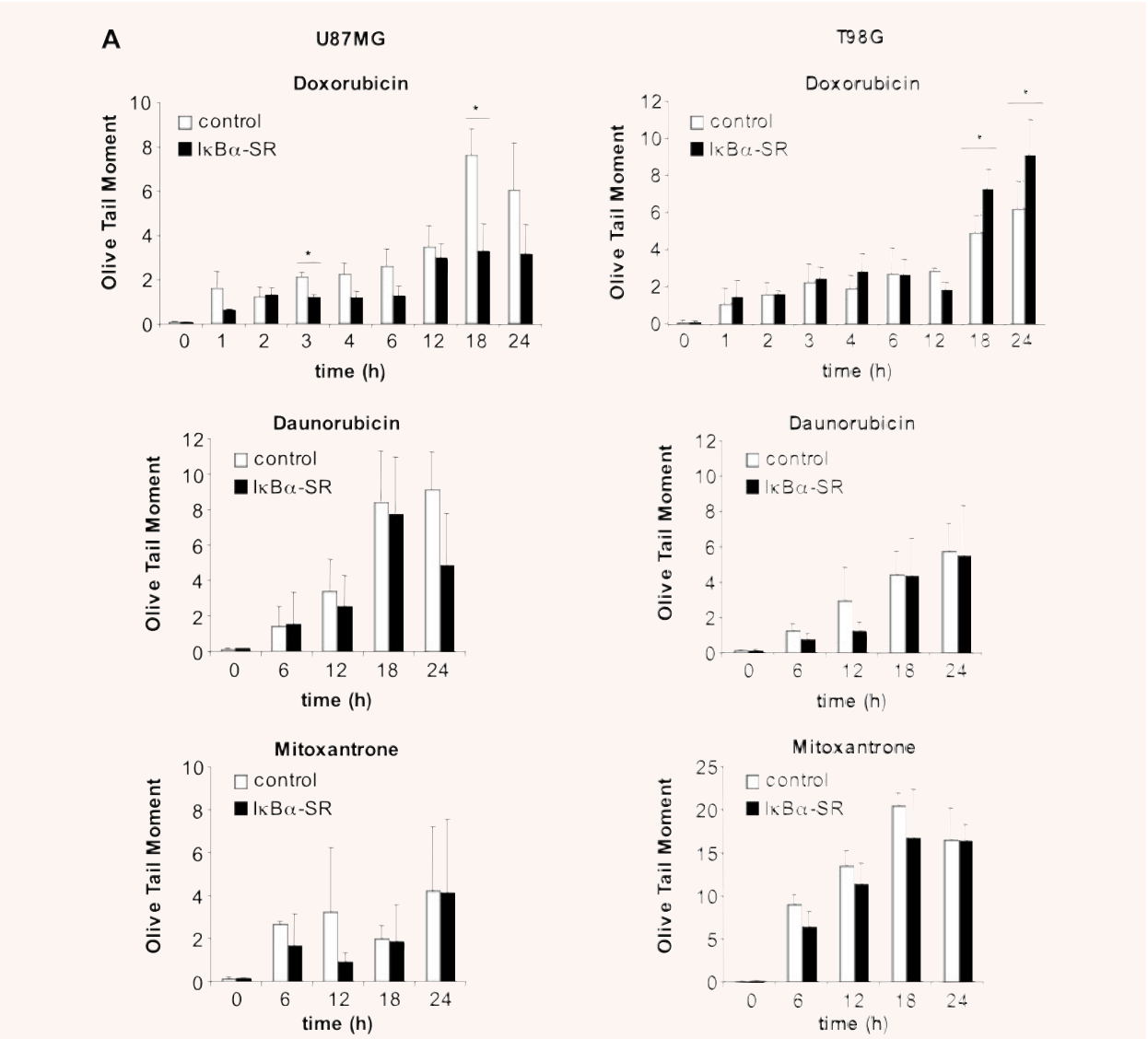

Fig. 6 NF-κB enhances DNA intercalator-induced DNA damage. (**A**) U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were continuously treated for indicated times with 0.8 μg/ml Doxorubicin (U87MG) or 1 μg/ml Doxorubicin (T98G), 2 μM Daunorubicin or 2.5 μM Mitoxantrone and DNA damage was assayed by Comet assay and is displayed as Olive Tail Moment. (**B**) U87MG (left panels) or T98G (right panels) cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated with Doxorubicin (U87MG: 0.8 μg/ml for 18 hrs; T98G: 1 μg/ml for 18 hrs), 2 μM Daunorubicin for 18 hrs or 2.5 μM Mitoxantrone for 3 hrs (U87MG) or 6 hrs (T98G), followed by a complete exchange of medium. After the indicated time points, DNA damage was assayed by Comet assay and is displayed as Olive Tail Moment. In (**C**) and (**D**), U87MG cells stably transduced with control vector were treated with 0.8 μg/ml Doxorubicin in the absence (white bars) or presence (hatched bars) of 10 μg/ml CHX for 18 hrs, followed by a complete exchange of medium and re-addition of CHX. DNA damage was assayed after 6 and 24 hrs by Comet assay and is displayed as Olive Tail Moment (**C**), apoptosis was assessed by FACS analysis of DNA fragmentation of propidium iodide stained nuclei (**D**). In (**E**) and (**F**), U87MG cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated for 18 hrs with 0.8 μg/ml Doxorubicin in the absence or presence of 50 μM zVAD.fmk, followed by a complete exchange of medium and re-addition of zVAD.fmk. DNA damage was assayed after 24 hrs by Comet assay and is displayed as Olive Tail Moment (**E**), apoptosis was assessed by FACS analysis of DNA fragmentation of propidium iodide stained nuclei (**D**). Median (**A–C**, **E**) or mean (**D**, **F**) + S.D. of three independent experiments are shown; $*P < 0.05$ and $\#P < 0.001$ comparing IκBα-SR *versus* control.

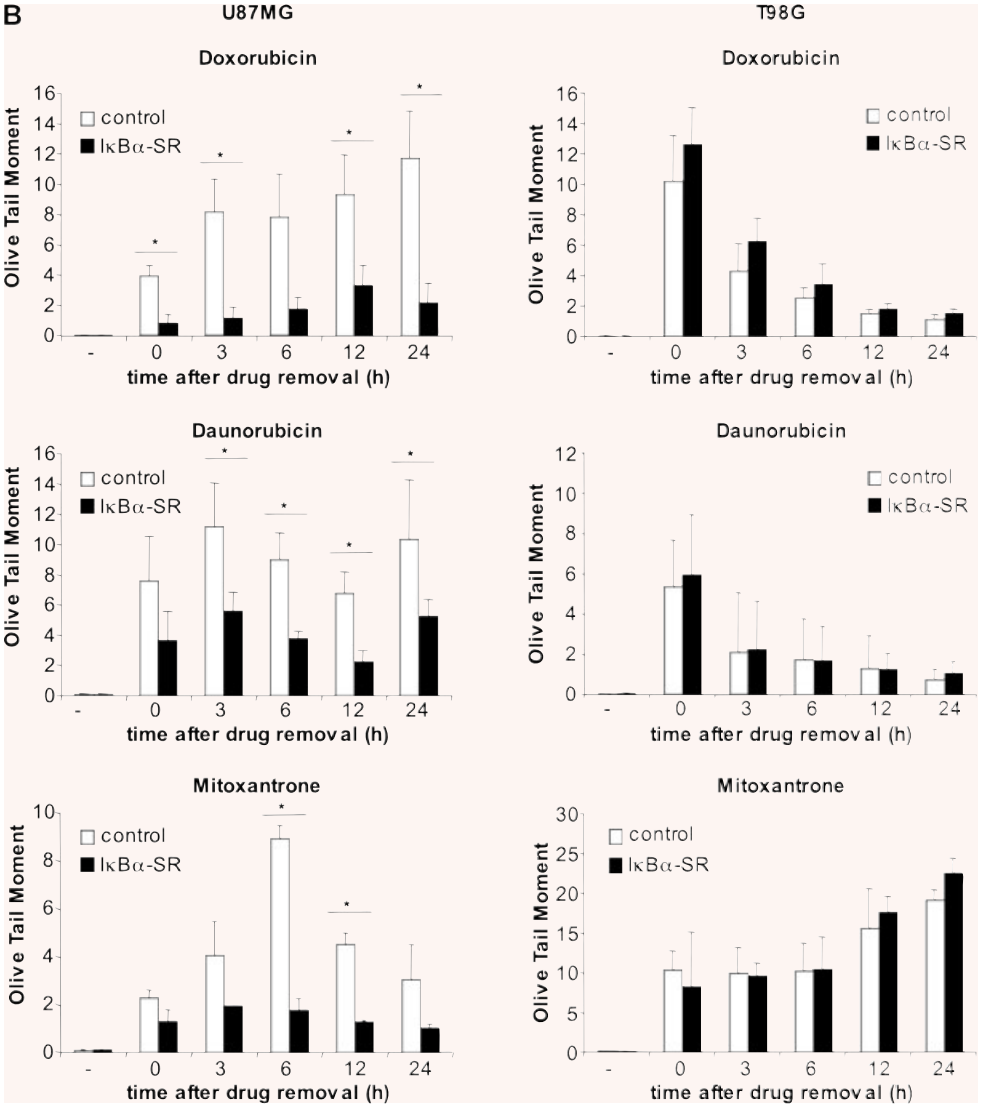

Fig. 6 Continued

it might in particular be the nervous system, where NF-κB controls pro-apoptotic programs. Together, these findings contribute to the growing body of evidence that NF-κB regulates apoptosis in a highly context-dependent manner, *i.e.* cell type-, stimulus- and also schedule specific.

Analysis of the underlying molecular mechanisms how NF-κB controls cell death in glioblastoma cells revealed no impact of NF-κB on drug uptake or efflux, excluding that changes in the multidrug-resistant phenotype are responsible for the NF-κB-mediated regulation of drug-induced apoptosis. Also, no differences in cell cycle progression were found in the presence or absence of NF-κB activity. Of potential clinical relevance is our finding that NF-κB promotes drug-induced apoptosis independently of p53, as p53 signalling is one of the core pathways that has recently been identified in a genomic survey to be altered in the vast majority of glioblastoma [35]. By comparison, p53 has previously been reported to positively and negatively regulate Temozolomide and chloroethylating anticancer drugs-induced cell death, respectively [36, 37], pointing to a stimulus-dependent function of p53 in glioblastoma cells. Furthermore, our study provides for the first time evidence that NF-κB modulates the DNA damage/repair response in glioblastoma cells by demonstrating that drug-induced DNA strand breaks are elevated in cells in which NF-κB was concomitantly activated. This NF-κB-dependent increase in DNA strand breaks precedes the induction of apoptosis, pointing to a link between DNA damage formation and apoptosis. Because the alkaline Comet assay detects both DNA single and double strand breaks [19], a higher score in the Olive Tail Moment may represent enhanced DNA damage or

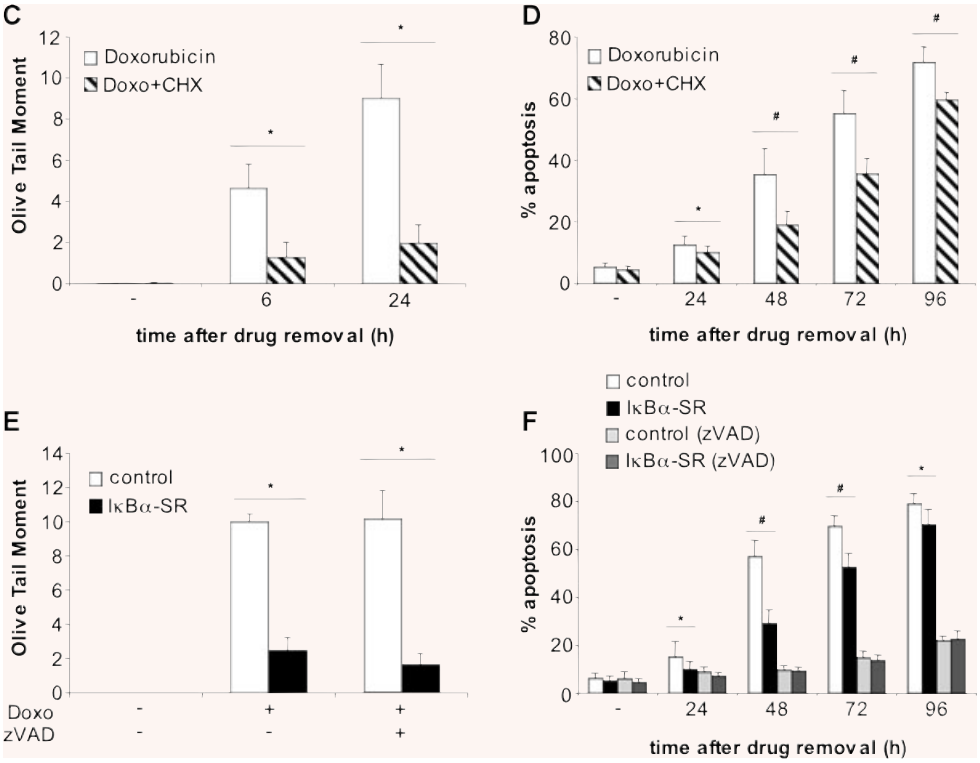

Fig. 6 Continued

alternatively, more DNA single strand breaks as DNA repair intermediates. Because the NF-κB-dependent increase in DNA strand breaks was especially observed after drug removal, NF-κB may modulate in particular the processing of DNA damage and DNA repair processes. As the observed differences in DNA strand breaks and apoptosis depend on protein synthesis, NF-κB transcriptional activity is likely involved. Notably, the conclusion that NF-κB promotes DNA damage and apoptosis is supported by data obtained in two independent glioblastoma cell lines and by two distinct approaches to inhibit NF-κB. How NF-κB regulates DNA damage and repair in glioblastoma cells is currently being explored in a genome-wide approach.

NF-κB: tumour suppressor or promoter in glioblastoma?

While NF-κB has traditionally been viewed as a tumour promoter, there is mounting evidence that it can also act as tumour suppressor under certain circumstances [38]. By demonstrating that NF-κB increases apoptosis in glioblastoma cells, our findings may point to a tumour suppressor rather than a tumour promoter function of NF-κB in glioblastoma. However, NF-κB-mediated increase in DNA damage may also contribute to genetic instability, thereby promoting tumour progression. In clinical specimens from patients with glioblastoma, increased NF-κB activity has been detected [39–44], but so far has only been correlated with higher tumour grade and adverse patients' prognosis in one recent study [44]. Thus, the functional relevance of NF-κB activity in glioblastoma, *e.g.* in distinct stages of tumour progression, awaits further investigations.

In conclusion, by demonstrating that NF-κB promotes DNA damage and apoptosis upon treatment with DNA intercalators, our findings provide novel insights into the role of NF-κB in the control of the DNA damage response in glioblastoma.

Materials and methods

Cell culture and chemicals

Human glioblastoma cell lines U87MG, T98G and A172 were obtained from ATCC and grown in DMEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 1% penicillin/streptomycin, 1 mmol/l L-glutamine (both from Invitrogen), 10% foetal calf serum and 25 mmol/l HEPES (both from Biochrom AG, Berlin, Germany). Recombinant human TNFα was purchased from Biochrom, BCNU, Bleomycin, Camptothecin, Cisplatin, Doxorubicin, Daunorubicin, Etoposide, Mitoxantrone, Topotecan, CHX and hydrogen peroxide (H_2O_2) from Sigma (Sigma-Aldrich, Taufkirchen, Germany), the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD.fmk) from Bachem (Bubendorf, Switzerland) and Temozolomide was provided by the National Cancer Institute (Bethesda, MA, USA).

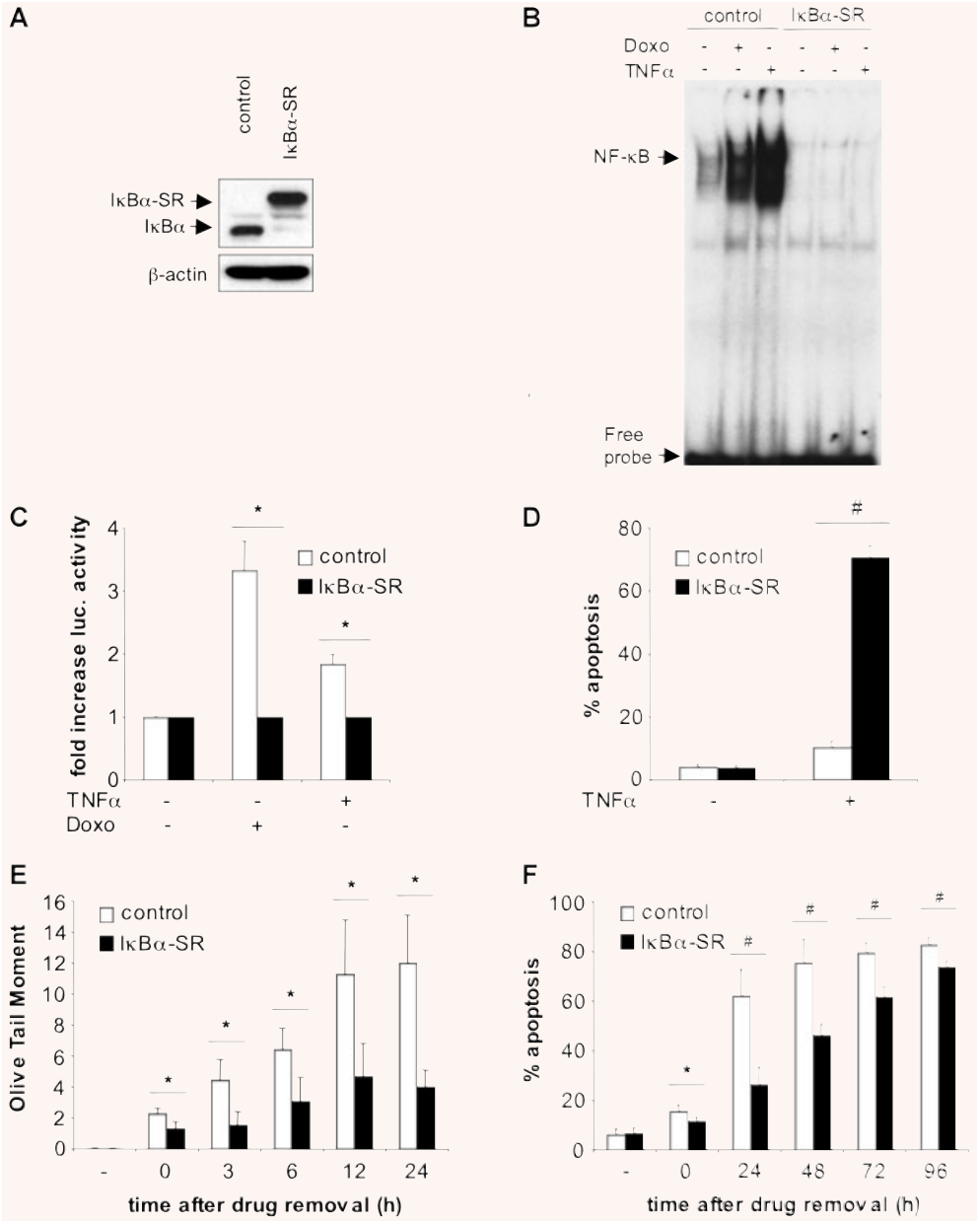

Fig. 7 Doxorubicin-induced NF-κB activation enhances DNA damage and apoptosis in A172 glioblastoma cells. (**A**), Ectopic expression of IκBα-SR. A172 glioblastoma cells were stably transduced with a control vector or a vector containing IκBα-SR. Protein expression of wild-type IκBα and mutant IκBα-SR was determined by Western blot analysis. β-actin served as loading control. (**B**), Inhibition of NF-κB DNA binding by IκBα-SR. NF-κB DNA binding was assessed by EMSA in nuclear extracts of A172 cells transduced with control vector or a vector containing IκBα-SR that were left untreated or were treated with 0.8 μg/ml Doxorubicin for 6 hrs or 10 ng/ml TNFα for 1 hr. (**C**), Inhibition of NF-κB transcriptional activity by IκBα-SR. A172 cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were transiently transfected with firefly and renilla luciferase gene constructs, treated for 6 hrs with 10 ng/ml TNFα or for 24 hrs with 0.8 μg/ml Doxorubicin and analysed by dual luciferase assay for induction of NF-κB transcriptional activity. Fold increase in luciferase activity relative to unstimulated control is shown. (**D**), Enhancement of TNFα-induced apoptosis by NF-κB inhibition. A172 cells transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were left untreated (–TNFα) or were treated with 50 ng/ml TNFα for 48 hrs (+TNFα). Apoptosis was determined by FACS analysis of DNA-fragmentation of propidium iodide stained nuclei. (**E**), NF-κB promotes Doxorubicin-induced DNA damage. A172 cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated with 0.8 μg/ml Doxorubicin for 18 hrs, followed by a complete exchange of medium. After the indicated time-points, DNA damage was assayed by Comet assay and is displayed as Olive Tail Moment. (**F**), NF-κB promotes Doxorubicin-induced apoptosis. A172 cells stably transduced with control vector (white bars) or a vector containing IκBα-SR (black bars) were treated with 0.8 μg/ml Doxorubicin for 18 hrs, followed by a complete exchange of medium. After the indicated time-points, apoptosis was determined by FACS analysis of DNA-fragmentation of propidium-iodide stained nuclei. Median (**E**) or mean (**C**, **D** and **F**) + S.D. of three independent experiments are shown; $*P < 0.05$ and $\#P < 0.001$ comparing IκBα-SR *versus* control.

Nuclear protein extraction and electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described [33]. In brief, after appropriate incubation, cells were washed, scraped and collected by centrifugation at 1000 × *g* for 5 min. at 4°C. Cells were resuspended in low salt buffer, allowed to swell on ice for 12 min., a 10% Igepal CA-630 (Sigma-Aldrich) solution was added and after vortexing the cell suspension was centrifuged again. The pelleted nuclei were resuspended in high salt buffer, incubated on ice and vortexed at times for 20 min. Nuclear supernatants were obtained by centrifugation at 12,500 × *g* at 4°C for 12 min. Protein concentrations were determined using the BCA Protein assay Kit (Pierce, Rockford, IL, USA). For EMSA, the following sequence was used as specific oligomer for NF-κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (sense). Single-stranded oligonucleotides were labelled with γ-[^{32}P]-ATP by T4-polynucleotide kinase (MBI Fermentas GmbH, St. Leon-Rot, Germany), annealed to the complementary oligomer strand and purified on sephadex columns (Micro Bio-Spin P30, Biorad Laboratories, Munich, Germany). Binding reactions containing 5 μg nuclear extract, 1 μg Poly(dI:dC) (Sigma), labelled oligonucleotide (10,000 cpm) and 5× binding buffer were incubated for 30 min. on ice. Binding complexes were resolved by electrophoresis in non-denaturing 6% polyacrylamide gels using 0.3× TBE as running buffer and assessed by autoradiography. Representative EMSAs are shown.

Luciferase assay

Dual-Luciferase® reporter Assay System (Promega, Madison, WI, USA) was used to determine firefly and renilla luciferase activities according to the manufacturer's instructions. 0, 8 × 10^5 cells were seeded in 12-well plates 24 hrs prior to transfection. Cells were transiently transfected with 0.5 μg of a 3× κB-firefly luciferase vector containing 3× κB consensus motif (CCCTGAAAGG) or as negative control a vector without any κB motif and 0.005 μg of a renilla luciferase vector under the control of the ubiquitin promoter [45]. Transfection was done using Fugene (Roche, Mannheim, Germany) in serum-free medium. After 24 hrs, cells were stimulated appropriately and thereafter lysed with passive lysis buffer (Promega). Luciferase activity was determined using a Berthold luminometer (Bad Wildbad, Germany). Values for firefly luciferase activity were normalized to renilla luciferase activity.

Production of retrovirus and retroviral transduction

The pCFG5-IEGZ retroviral vector system, as described by [46], was used to infect U87MG and T98G glioblastoma cells as previously described [17]. In brief, stable PT67 producer cells (Clontech, Palo Alto, CA, USA) were transfected with empty pCFG5-IEGZ vectors or pCFG5-IEGZ vectors containing IκB-α-(S32; 36A) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation and selected with 0.25 mg/ml Zeocin (Invivogen, San Diego, CA, USA). Stable U87MG, A172 and T98G bulk cultures were obtained by retroviral spin transduction and subsequent selection with 0.7 mg/ml (U87MG, A172) or 0.35 mg/ml (T98G) zeozin.

To knockdown p53, shRNA targeting p53 sequence (gactccagtggtaatctac) [47] was cloned into pRETRO-SUPER as previously described [48]. A sequence with no corresponding part in the human genome (gatcatgtagatacgctca) was used as control. Stable bulk cultures were generated by retroviral transduction and selected with puromycin (0.5 μg/ml).

RNA interference

Cells were transfected with siRNA using TransMessenger transfection reagent (Qiagen, Hilden, Germany) as previously described [49], using the following Stealth RNAi constructs from Invitrogen: RELA (RELAHSS109159, RELAHSS109161, RELAHSS184266) for RelA and Stealth Negative Universal Control Medium RNAi (12935–300) for negative control.

Western blot analysis

Western blotting was performed as previously described [33] using the following antibodies: mouse anti-β-actin (Sigma), mouse anti-Bcl2 (BD Pharmingen), mouse anti-Bcl-X_L (BD Biosciences, San Jose, CA, USA), goat anti-cIAP1 (R&D Systems, Inc.), rabbit anti-cIAP2 (Epitomics), rabbit anti-IκBα (Santa Cruz Biotechnology Inc., Heidelberg, Germany), mouse anti-p53 (BD Biosciences) and mouse anti-XIAP (BD Biosciences). Proteins were visualized using either anti-rabbit or anti-mouse IgG-HRP conjugated antibody (Santa Cruz Biotechnology Inc.) and ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Freiburg, Germany), according to manufacturer's instructions. One representative of at least three independent experiments is shown unless otherwise indicated.

Determination of apoptosis, cell cycle progression and drug uptake and efflux

Apoptosis and cell cycle progression were assessed by fluorescence-activated cell-sorting (FACScan, Becton Dickinson, Heidelberg, Germany) analysis of DNA fragmentation or cell cycle progression of propidium iodide-stained nuclei as previously described [50]. Briefly, cells were pelleted and resuspended in a propidium-iodide solution containing 0.1% TritonX-100, 0.1% tri-sodium citrate dehydrate and 50 μg/ml propidium iodide (Sigma) and analysed by flow cytometry. Drug uptake and efflux was determined by flow cytometry.

Single-cell gel electrophoresis (Comet) assay

DNA damage was assayed by the alkaline Comet assay. Cells were seeded in 6 cm dishes and allowed to settle overnight. After drug exposure, cells were washed with PBS, collected by centrifugation and resuspended in PBS. Aliquots of 10 μl were suspended in 120 μl low melting point agarose (0.5%) (Invitrogen) and spread onto microscope slides pre-coated with a thin layer of 1.5% agarose (Roth, Karlsruhe, Germany). Cells were exposed to lysis buffer (2.5 mM NaCl, 100 mM Na_2EDTA, 10 mM Tris) at 4°C overnight. Thereafter, alkaline denaturation was allowed in pre-chilled electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA) for 25 min., followed by alkaline electrophoresis for 25 min. at a pH > 13 (4°C). Slides

were then neutralized (0.4 M Tris), desiccated (absolute alcohol, 5 min.) and stained with ethidium bromide. For each condition, two slides were prepared, and for analysis 50 randomly selected cells of each slide were measured by image analysis (Kinetic Imaging Komet 5.0 Software, Andor Technology Ltd., Berlin, Germany) using an Olympus AX70 'Provis' microscope (Hamburg, Germany). DNA damage is expressed as Olive Tail Moment.

Statistics

Statistical significance was assessed by Student's t-test using Winstat software (R. Fitch Software, Bad Krozingen, Germany).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 DNA damaging drugs induce specific NF-κB DNA binding. (**A**) Competition experiments. U87MG cells stably transduced with control vector or a vector containing IκBα-SR were treated for 6 hrs with 0.8 μg/ml Doxorubicin (Doxo), 2 mM Daunorubicin (Dauno) or 2.5 μM Mitoxantrone (Mitox) or for 1 hr with 10 ng/ml TNFα. Nuclear extracts were analyzed by EMSA using either an oligo containing NF-κB-specific consensus (3×κB) or 5'mutated (5`mut) binding sites. (**B**) Supershift analysis of NF-κB complexes. U87MG cells stably transduced with control vector were treated for 6 hrs with 0.8 μg/ml Doxorubicin or for 1 hr with 10 ng/ml TNFα. Nuclear extracts were subjected to EMSA with or without preincubation with specific antibodies against p50, p65, c-Rel or IgG as control.

Fig. S2 Effect of NF-κB inhibition on drug-induced loss of cell viability. U87MG (left panels) or T98G (right panels) cells stably transduced with a control vector (white bars) or a vector containing IκBα-SR (black bars) were treated with indicated concentrations of Doxorubicin (**A**), Daunorubicin (**B**) or Mitoxantrone (**C**) for 24–72 hrs. Cell viability was assessed using MTT assay and is expressed as percentage of untreated controls. Mean+SD of three independent experiments performed in triplicates is shown.

Fig. S3 DNA damaging drugs induce specific NF-κB transcriptional activation and target gene expression. (**A**) Specificity of NF-κB transcriptional activity. U87MG cells stably transduced with control vector were transiently transfected with a firefly luciferase gene construct containing 3xκB binding sites (3xκB) or not (EV) and renilla luciferase vector, treated for 6 hrs with 10 ng/ml TNFα or for 24 hrs with 0.8 μg/ml Doxorubicin (Doxo), 2 μM Daunorubicin (Dauno) and 2.5 μM Mitoxantrone (Mitox), and analyzed by dual luciferase assay for induction of NF-κB-specific transcriptional activity. Fold increase in luciferase activity relative to unstimulated control is shown. Mean + SD of three independent experiments performed in triplicates is shown; $^{*}P < 0.05$ and $^{\#}P < 0.001$. (**B**) Analysis of Doxorubicin-induced, endogenous NF-kB gene expression. U87MG cells stably transduced with control vector or a vector containing IκBα-SR were treated with 0.8 μg/ml Doxorubicin for indicated times and mRNA expression levels of TNFα were analyzed by RTPCR. GAPDH served as loading control. C, PCR water control; M, marker.

Fig. S4 Effect of PARP1 inhibition on Doxorubicin-induced DNA damage. In (**A**) U87MG stably transduced with control vector were treated with 0.8 μg/ml Doxorubicin in the absence (white bars) or presence (hatched bars) of 4 mM of the PARP1 inhibitor 3- Aminobenzamide (3-AB) for 18 hrs, followed by a complete exchange of medium and readdition of 3-AB. DNA damage was assayed after 6 hrs and 24 hrs by Comet assay and is displayed as olive tail moment. Median+SD of three independent experiments is shown. In (**B**) activity of 3-AB was controlled by intracellular staining of Poly ADP-Ribose (PAR). U87MG cells stably transduced with control vector were preincubated or not with 4 mM 3-AB for 40 min. followed by 5 min. treatment with 250 μM H_2O_2. Scale bar: 10 μM.

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