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The chloroethylating anticancer drug ACNU induces FRA1 that is involved in drug resistance of glioma cells

Ruth Meise, Maja T. Tomicic, Bernd Kaina, Markus Christmann *

Department of Toxicology, University Medical Center Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

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ABSTRACT

FRA1 belongs, together with c-Fos and FosB, to the family of Fos proteins that form with members of the ATF and Jun family the transcription factor AP-1 (activator protein 1). Previously we showed that c-Fos protects mouse embryonic fibroblasts against the cytotoxic effects of ultraviolet (UV) light by induction of the endonuclease XPF, leading to enhanced nucleotide excision repair (NER) activity. Here, we analyzed the regulation of FRA1 in glioma cells treated with the anticancer drug nimustine (ACNU) and its role in ACNU-induced toxicity. We show that FRA1 is upregulated in glioblastoma cells following ACNU on mRNA and protein levels. Knockdown of FRA1 by either siRNA or shRNA clearly sensitized glioma cells towards ACNU-induced cell death. Despite decreased AP-1 binding activity upon FRA1 knockdown, this effect is independent on regulation of the AP-1 target genes *fasL*, *ercc1* and *xpf*. In addition, FRA1 knockdown does not affect DNA repair capacity. However, lack of FRA1 attenuated the ACNU-induced phosphorylation of CHK1 and led to a reduced arrest of cells in G2/M and, thereby, presumably leads to enhanced cell death in the subsequent cell cycle.

1. Introduction

The cellular genome is permanently attacked by endogenous and exogenous DNA damaging insults. To maintain genomic integrity, DNA repair mechanisms have evolved [1]. DNA repair is however not only beneficial for the cell; it can also endanger the integrity of the genome itself. Thus, DNA repair nucleases may lead, via incision of the DNA, to DNA single-strand breaks (SSBs). If not processed properly, these breaks can be converted into lethal DNA double-strand breaks (DSBs) during replication. Therefore, these enzymes have to be tightly regulated.

An important factor in transcriptional regulation of DNA repair genes is the dimeric transcription factor AP-1 (activator protein 1), which consists of different proteins belonging to the Fos, Jun or ATF family [2,3]. AP-1 has been shown to stimulate a broad spectrum of genes harbouring AP-1 sites in the promoter and thereby regulates different cellular mechanisms like apoptosis and proliferation [4]. In addition, AP-1 complexes consisting of c-Fos are involved in the regulation of several DNA repair genes like *trex1*, *xpf* and *xpg* and thereby stimulate DNA repair activity and protect from genotoxin-induced cell death [5-7]. Given a key role for c-Fos in genotoxic defense, the question arises whether other members of the c-Fos family also play a role in protection against genotoxic stress. Besides c-Fos, the Fos-family comprises FosB, FRA1 and FRA2. In contrast to c-Fos and FosB, FRA1 has no transactivation domain and thereby its transcriptional regulatory activity is entirely dependent on its binding partner.

FRA1 is overexpressed in several types of tumors like breast cancer [8,9] and regulates proliferation and invasiveness [10]. In addition, FRA1 has been shown to play a role in the maintenance/progression of malignant gliomas [11]. In a study performed with glioblastoma cells, it was shown that FRA1 rather mediates sensitivity than protection against the anticancer drug cisplatin [12]. Thus, knockdown of FRA1 resulted in decreased cisplatin-induced apoptosis, which was explained by a reduced S-phase arrest through modulation of the CHK1–Cdk2 pathway. However cisplatin is not used routinely in the treatment of gliomas.

Another anticancer drug leading, similar to cisplatin, to the formation of DNA crosslinks is the chloroethylating agent nimustine (1-[4-amino-2-methyl-5-pyrimidinyl] methyl-3-[2-chloroethyl]-3-nitrosourea hydrochloride, ACNU). ACNU belongs to the nitrosureas (other clinical used drugs of this group are BCNU, CCNU and fotesmustine) and is used as 2nd line anticancer agent in the treatment of glioblastoma. Here we asked the question of whether FRA1 modulates the sensitivity of glioblastoma cells to ACNU. We observed a protective effect of FRA1 on ACNU-induced cell death. This effect is most likely independent of regulation of DNA repair genes and genes involved in apoptosis; it likely rests on the effect of FRA1 on cell cycle progression. The data suggest FRA1 as a factor of drug resistance in glioma and possible also other tumor groups.

Abbreviations: AP-1, activator protein 1; FRA1, Fos-related antigen 1; DSB, DNA double-strand breaks; SSB, DNA single-strand breaks; ACNU, nimustine, (1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride

* Corresponding author. Tel.: +49 6131 17 9066; fax: +49 6131 393 3421.
E-mail address: mchristm@uni-mainz.de (M. Christmann).

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2. Material and methods

2.1. Cell lines and anticancer drug treatment

The glioma cell lines LN229, U87, and U138 were described previously [13]. The cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), in 7% CO_2 at 37 °C.

2.2. Preparation of cell extracts and Western blot analysis

Whole cell extracts and nuclear cell extracts were prepared as described previously [14]. Samples of 25 μg protein extract were separated by 10% SDS-PAGE and electro-blotted onto nitrocellulose membranes, which were then incubated with specific antibodies. Polyclonal antibodies raised against FRA1 (N17 and sc-605, Santa Cruz Biotechnology), ERCC1 (rwo 18 [15]), p-cJun (3270, Cell Signaling), cJun (9165, Cell Signaling), p-CHK1 (2341S, Cell Signaling), CHK1 (2345, Cell Signaling) and monoclonal antibodies raised against $\gamma H2AX_{Ser139}$ (JBW301, Millipore), ß-Actin (C4, Santa Cruz Biotechnology), FasL (G247-4, BD Pharmingen), XPF (ab-5 clone 51, NeoMarkers/ThermoScientific), and XPG (8H7, Santa Cruz Biotechnology) were diluted 1:500–1:2000 in 5% non-fat dry milk and 0.2% Tween/PBS and incubated overnight at 4 °C. The protein–antibody complexes were visualized by ECL reagent (GE Health care). Quantification of signal intensity was performed by use of the Multianalyst Software and the Gel Doc 1000 system from Biorad. Signal intensity of ß-Actin, which is not regulated served as loading control. For quantification, the expression of the analyzed proteins was normalized with ß-Actin and the untreated control was set to one.

2.3. Preparation of RNA, RT-PCR and real-time RT-PCR

Total RNA was isolated using the RNA II Isolation Kit from Machery and Nagel. One μg RNA was transcribed into cDNA by Superscript II (Invitrogen) in a volume of 50 μl and 3 μl was subjected to RT-PCR. RT-PCR was performed by the use of specific primers (MWG Biotechnology) and Red-Taq Ready Mix (Sigma-Aldrich). The PCR program used was: 1.5 min 94 °C, [(denaturation: 45 s, 94 °C; annealing: 1 min 56–62 °C; elongation: 1 min, 72 °C) 25–30 cycles], 10 min 72 °C. Real-time RT-PCR was performed using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) and the light cycler of Roche Diagnostics.

2.4. Cloning and transfection

To generate a FRA1-shRNA vector, a 60mer hairpin-nucleotide containing the FRA1 siRNA sequence CACCATGAGTGGCAGTCAG and the corresponding antisense sequence was cloned into the Bgl II/Hind III restriction sites of the pSUPER.basic vector (Oligo Engine). For stable transfection, 3×10^5 LN229 cells were seeded per 6-cm dish. One day later, cells were transfected with 1 μg of *pSuper-FRA1-shRNA* vector or the empty *pSuper* vector together with 0.1 μg pSV2neo plasmid for selection, by use of Effectene reagent as described by the manufacturer (Qiagen). For transient down-regulation of FRA1, 20 nM siRNA specific for FRA1 (5′-CACCATGAGTGGCAGTCAG-3′) or 20 nM non-silencing siRNA (AllStar neg. control siRNA, Qiagen) was transfected in LN229 cells by using Lipofectamin RNAi MAX reagent as described by the manufacturer (Invitrogen). Experiments were performed 24 h after transfection.

2.5. Single cell gel electrophoresis (SCGE, comet assay)

For the alkaline comet assay, exponentially growing cells were pulse-treated for 1 h with 50 μM ACNU and, after the indicated time periods, trypsinized and washed with ice-cold PBS. Alkaline cell lyses and electrophoresis were essentially performed as described previously [16]. For the modified comet assay cells were initially pulse-treated for 1 h with 25 μM ACNU, trypsinized after indicated time points and washed with ice-cold PBS. After adjustment to a cell concentration of 10^6 cells/ml, cells were irradiated by 8 Gy IR. Alkaline cell lyses and electrophoresis was essentially performed as described previously [16].

2.6. Determination of apoptosis and cell cycle distribution

For monitoring drug-induced apoptosis and cell cycle distribution, ethanol-fixed cells were treated with DNAse free RNAse and stained with propidium iodide (PI). The Sub-G1, G1, S and G2 fraction was determined by flow cytometry. The protocol was described previously [17,18]. Experiments were repeated at least three times, mean values ± SD are shown and data were statistically analyzed using Student's *t* test.

2.7. Preparation of nuclear extracts and EMSA

Nuclear cell extracts were prepared and subjected to electromobility shift assay (EMSA) or Western blot analysis as described previously [14]. The sequence of the oligonucleotides specific for the AP-1 binding site of the *mmp1* promoter was 5′-AGTGGTGACTCATCACT-3′. For supershift analysis, 2 μg of the specific antibody was added and the mixture was additionally incubated for 1 h at 4 °C.

3. Results

3.1. Expression of FRA1 is induced following ACNU treatment

The expression of FRA1 was analyzed upon exposure of LN229 glioblastoma cells to the chloroethylating anticancer drug ACNU. A significant induction of *fra1* mRNA was observed by semi-quantitative real-time RT-PCR using primers located in the coding region of the gene. An increased expression of *fra1 mRNA* was observed 8–24 h after exposure of LN229 cells to 25 μM ACNU (Fig. 1A). To identify the mechanism responsible for the increase in *fra1* mRNA level upon ACNU treatment, mRNA *de novo* synthesis was determined. To this end, LN229 cells were exposed to ACNU in the presence or absence of the transcription-blocking agent actinomycin D (Fig. 1B). Treatment of LN229 cells with the inhibitor for 7 h reduced the basal expression of *fra1* mRNA, indicating its high instability. In addition, treatment with actinomycin D also abrogated the ACNU-triggered induction of *fra1* mRNA, which suggests that the observed accumulation of *fra* mRNA is mainly dependent on RNA *de novo* synthesis. To analyze whether the increased expression of *FRA1* mRNA leads to an increased protein level, the amount of FRA1 protein was studied by Western blot analysis. As shown in Fig. 1C, a strong time-dependent accumulation of FRA1 protein was determined in whole cell extracts. Increase in FRA1 protein followed the mRNA upregulation, starting 16 h after ACNU exposure. Besides transcriptional regulation, also post-translational modifications by members of the mitogen-activated protein kinase (MAPK)-cascade are important for expression of FRA1. Since only nuclear FRA1 bound to its binding partner c-Jun represents the active AP-1 complex, we additionally analyzed the expression of nuclear FRA1 and c-Jun protein (Fig. 1D). Whereas the overall amount of c-Jun was changed only slightly, a strong nuclear accumulation of FRA1 was observed, starting 8 to 16 h after ACNU exposure. This accumulation paralleled the activation of c-Jun by phosphorylation, indicating a possible interaction between both proteins, forming an active AP-1 complex.

To analyze the impact of the different MAPK on ACNU-induced FRA1 expression, we utilized different kinase-specific inhibitors, which were added to the medium 1 h prior to ACNU exposure. As shown in Fig. 1E, inhibition of p38K by SB203580 and inhibition of ERK1/2 activity using U0126 (an inhibitor of the upstream MEK1/2; both panels) resulted in significant reduction of ACNU-induced

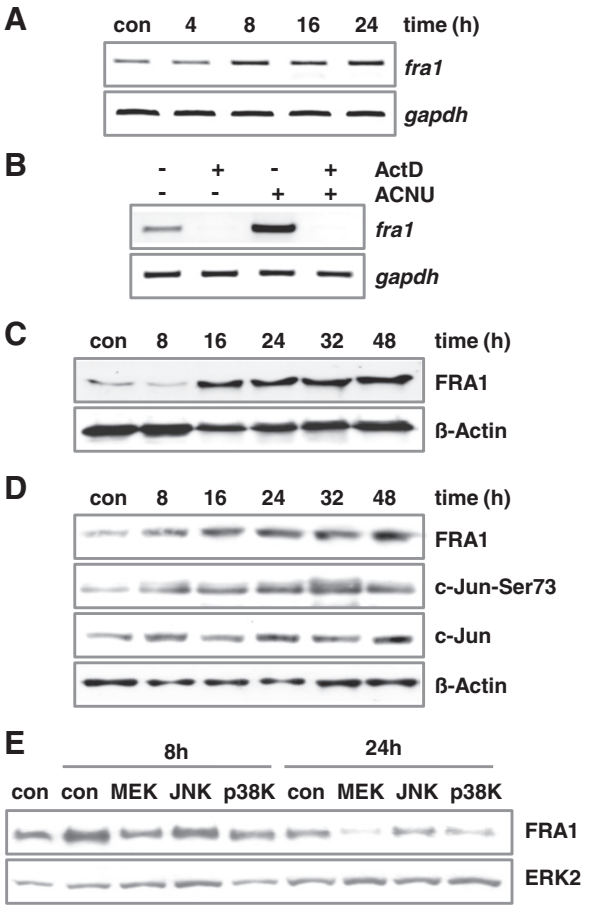

Fig. 1. ACNU-mediated induction of FRA1. (A) LN229 glioblastoma cells were exposed to 25 μM ACNU. At different time points after exposure, total RNA was isolated and semi-quantitative RT-PCR was performed using *fra1* or, as loading control, *gapdh* specific primers. (B) LN229 glioblastoma cells were either not incubated or pre-incubated for 1 h with the transcription blocking agent actinomycin D (ActD). Thereafter cells were exposed to 100 μM ACNU or kept unexposed. 6 h later total RNA was isolated and semi-quantitative RT-PCR was performed using *fra1* or, as loading control, *gapdh* specific primers. (C) LN229 cells were exposed to 50 μM ACNU for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis. The membrane was incubated with FRA1 specific antibody or, as loading control, with β-Actin specific antibody. (D) LN229 cells were exposed to 50 μM ACNU for the indicated times. Nuclear extracts were prepared and subjected to Western blot analysis. The membrane was incubated with antibodies against c-Jun, phospho-c-Jun (c-Jun-Ser73), FRA1 or, as loading control, with β-Actin specific antibody. (E) LN229 cells were pre-incubated for 1 h with a specific inhibitor for JNK1/2/3 (SP600125), p38K (SB103580) and MEK1/2 (U0126) and 8 h or 24 h after treatment with 50 μM ACNU, whole cell extracts were prepared and subjected to Western blot analysis. The membrane was incubated with FRA1 and, as loading control, with ERK2 specific antibodies.

FRA1 expression 8 h after ACNU exposure. Inhibition of these kinases nearly completely abrogated ACNU-induced FRA1 expression after 24 h. In contrast, inhibition of the JNK activity by the JNK1/2/3 inhibitor SP600125 had no impact on FRA1 expression. The data suggest that p38K and ERK1/2 are involved in FRA1 upregulation following ACNU treatment.

3.2. Silencing of FRA1 confers sensitivity to ACNU

To analyze whether induction of FRA1 impacts the sensitivity of glioblastoma cells to ACNU, FRA1 was transiently or stably down-regulated via siRNA and shRNA, respectively. Transient downregulation of FRA1 using siRNA was verified on protein level. Thus a strongly reduced expression of FRA1 was observed in ACNU-exposed LN229 cells 48 and 72 h after siRNA transfection (Fig. 2A). To compare parental LN229 and FRA1-depleted LN229 cells as to their sensitivity to ACNU, cell death was measured by flow cytometry as Sub-G1 fraction (Fig. 2B). Cell death occurred at a significant higher frequency in LN229 cells transfected with FRA1-siRNA than in those transfected with a nonsense siRNA (Fig. 2B). To substantiate the data, we established stable FRA1 knockdown cells. The most efficient down-regulation was observed in the clone sh-10 (Fig. 2C). This clone was designated LN229-FRA1-shRNA and used for further experiments. Besides basal expression, also ACNU-induced FRA1 induction was clearly reduced in the knockdown cells in comparison to the vector-only control (LN229-vec) (Fig. 2D). Using these cell lines we again measured cell death (Sub-G1 fraction) as a function of dose (5–100 μM ACNU) 96 h after ACNU treatment (Fig. 2E). Also in these experiments, cell death occurred at a higher frequency in FRA1-depleted LN229-FRA1-shRNA cells. However, the increased sensitivity of LN229-FRA1-shRNA cells was only observed at dose levels above 50 μM ACNU (Fig. 2E), indicating a threshold for the protective effect of FRA1. Overall, the data suggest a functional role of FRA1 induction in the protection against the anticancer drug ACNU. To substantiate the findings obtained with LN229 cells, additional experiments were performed with the glioma lines U87 and U138, which were transfected with FRA1 siRNA (Fig. 2F). In these cells, cell death (Sub-G1 fraction) was measured 96 h upon exposure to 50 μM ACNU (Fig. 2G). Also in U87 and U138 cells, death occurred at a higher frequency upon FRA1 knockdown, suggesting that the effect of FRA1 on ACNU-mediated cell death in glioma cells is not limited to a specific cell line, but represents a more general effect.

3.3. FRA1-mediated resistance to ACNU is independent of FasL regulation

AP-1 dependent gene regulation has been shown to be important for both apoptosis and DNA repair. AP-1 regulates *fasL*, an important factor of the extrinsic apoptotic pathway [19], as well as the DNA repair genes *ercc1*, *xpf* and *xpg* [6,7,20]. To analyze the effect of FRA1 downregulation on ACNU-induced activation of AP-1, electromobility shift assays (EMSA) were performed using radioactively labeled oligonucleotides harboring the AP-1 binding site of the collagenase 1 (matrix metalloproteinase-1, *mmp1*) promoter. The oligonucleotides were incubated with nuclear extracts obtained from LN229-vec and LN229-FRA1-sh cells, either untreated or treated with 50 μM ACNU for different times. As shown in Fig. 3A, a time-dependent induction of AP-1 binding activity was observed using the *mmp1* AP-1 binding site. The AP-1 binding activity was at maximum 16–24 h after ACNU exposure. In LN229-FRA1-sh cells a strong reduction of the AP-1 binding activity was observed, indicating that reduction of FRA1 protein expression directly translates into reduced AP-1 binding activity. To verify that FRA1 is a component of the AP-1 complex upon ACNU exposure, supershift experiments were performed. As shown in Fig. 3B, FRA1 and its binding partner c-Jun are present in the AP-1 complex in LN229-vec cells, 24 h after ACNU exposure, however in LN229-FRA1-shRNA cells only c-Jun can be detected.

One of the most important AP-1 target genes encodes the Fas ligand (*fasL*). To analyze whether reduced AP-1 activity leads to a differential regulation of *fasL* mRNA upon ACNU treatment, semi-quantitative RT-PCR and real-time RT-PCR were performed. A slight induction of *fasL* was observed 0–24 h after treatment in LN229-vec, but not in LN229-FRA1-shRNA cells, as determined by semi-quantitative RT-PCR (Fig. 3C). Real-time RT-PCR showed a two times higher basal expression in LN229-vec cells and a comparable induction of *fasL* mRNA upon ACNU exposure in both LN229-vec and LN229-FRA1-shRNA cells (Fig. 3D). In addition, a slightly higher expression of the membrane-bound and the soluble FasL protein was observed in LN229-vec compared to LN229-FRA1-shRNA cells (Fig. 3E), indicating that FRA1 down-regulation only marginally impacts the regulation of FasL. In contrast to *fasL* mRNA, the mRNA coding for the Fas receptor (*fasR*) was increased in both cell lines following ACNU treatment (Fig. 3C).

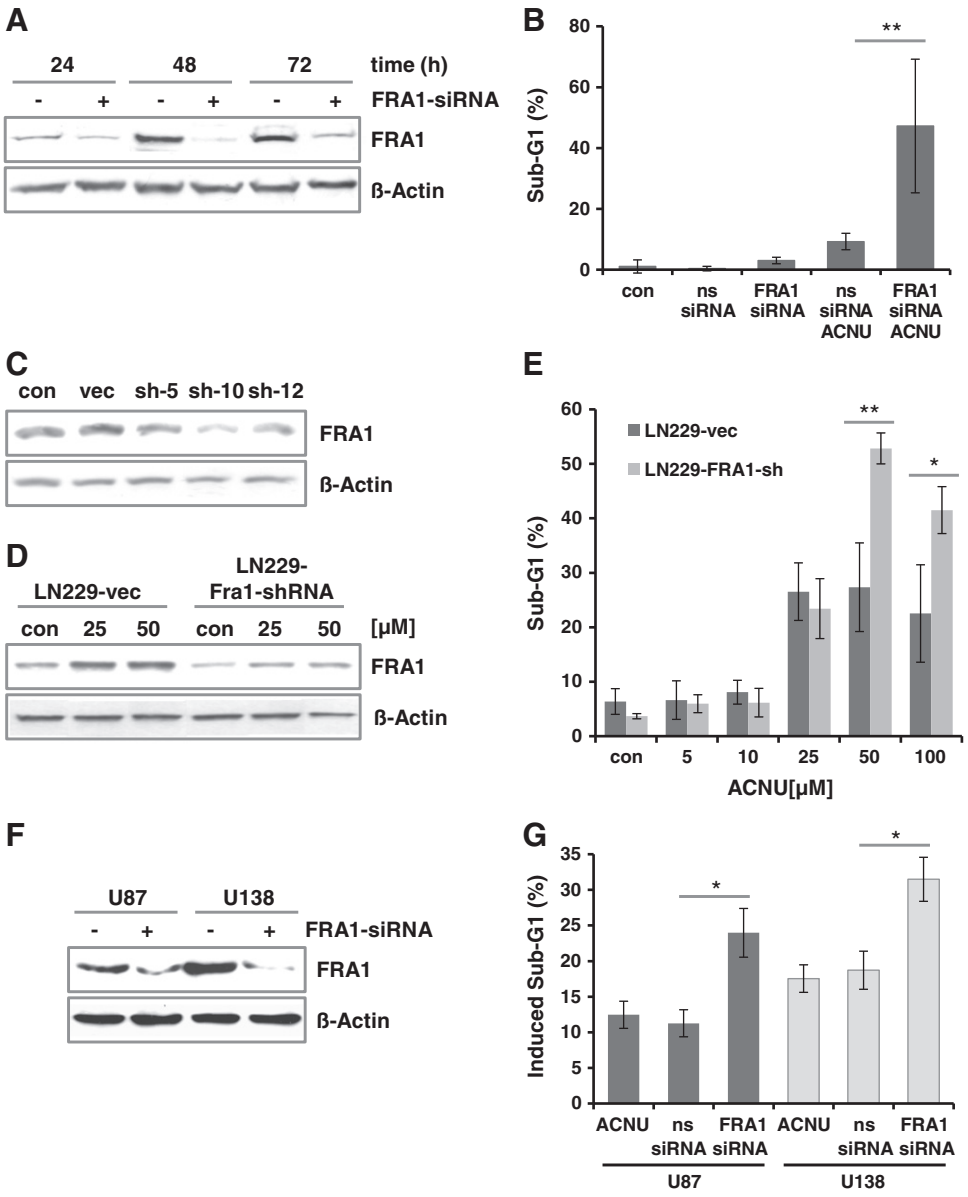

Fig. 2. Effect of FRA1 knockdown on sensitivity to ACNU. (A) FRA1 expression was analyzed by the use of specific antibodies in LN229 cells, 24 to 72 h after transfection with FRA1 siRNA. To analyze the effect of the downmodulation on the ACNU-induced FRA1 level, cells were exposed to 50 µM ACNU 6 h before harvest. (B) To monitor the impact of FRA1 knockdown on sensitivity to ACNU, LN229 cells were transfected with FRA1 specific siRNA (FRA1-si) or nonsense-siRNA (ns-si). 24 h later, cells were exposed to 25 µM ACNU and incubated for 96 h. Cells were stained with PI and the sub-G1 fraction was determined by flow cytometry. (*$p<0.05$). (C) FRA1 expression was analyzed by the use of specific antibodies in different LN229 clones, stably transfected with FRA1-shRNA. (D) FRA1 expression was analyzed by the use of specific antibodies in LN229 cells stably transfected with FRA1 specific shRNA (LN229-FRA1-shRNA = sh10) or a vector only clone (LN229-vec) 24 h after exposure to 25 or 50 µM ACNU. (E) LN229-FRA1-shRNA cells and LN229-vec cells were exposed to different ACNU doses and incubated for 96 h. Cells were stained with PI and the sub-G1 fraction was determined by flow cytometry. (*$p<0.05$, **$p<0.01$). (F) FRA1 expression was analyzed by the use of specific antibodies in U87 und U138 cells, 24 h after transfection with FRA1 siRNA. (G) U87 and U138 cells were transfected with FRA1 specific siRNA (FRA1-si) or nonsense-siRNA (ns-si). 24 h later, cells were exposed to 50 µM ACNU and incubated for 96 h. Cells were stained with PI and the Sub-G1 fraction was determined by flow cytometry. (*$p<0.05$).

3.4. FRA1-mediated resistance to ACNU is independent of AP-1-associated regulation of DNA repair

Several proteins of the NER (nucleotide excision repair) pathway have been described to be transcriptionally regulated, e.g. XPF and XPG, which are induced by AP-1 upon exposure of fibroblasts to UV-light [7]. Little is known, however, about the regulation of the corresponding genes in tumor cells after exposure to anticancer drugs. To analyze the regulation of these genes upon ACNU treatment, semi-quantitative RT-PCR was performed. No alterations in the expression of *xpf* mRNA were observed, neither in LN229-vec nor in LN229-FRA1-shRNA cells (Fig. 4A). For *ercc1* a slight induction was observed 16–48 h upon ACNU treatment in LN229-vec but not in LN229-FRA1-shRNA cells (Fig. 4A). However, no differences in the expression were observed on protein level (Fig. 4B).

To measure the impact of FRA1 downmodulation on DNA repair, DNA damage was determined using the alkaline comet assay, which detects mostly DNA single-strand breaks. Additionally, a modified alkaline comet assay, which detects DNA crosslinks [21], was performed. To this end, LN229 cells were transfected with FRA1-siRNA and 24 h later exposed to 25 or 50 µM ACNU. At different time points, cells were collected and subjected to the comet assay. As shown in

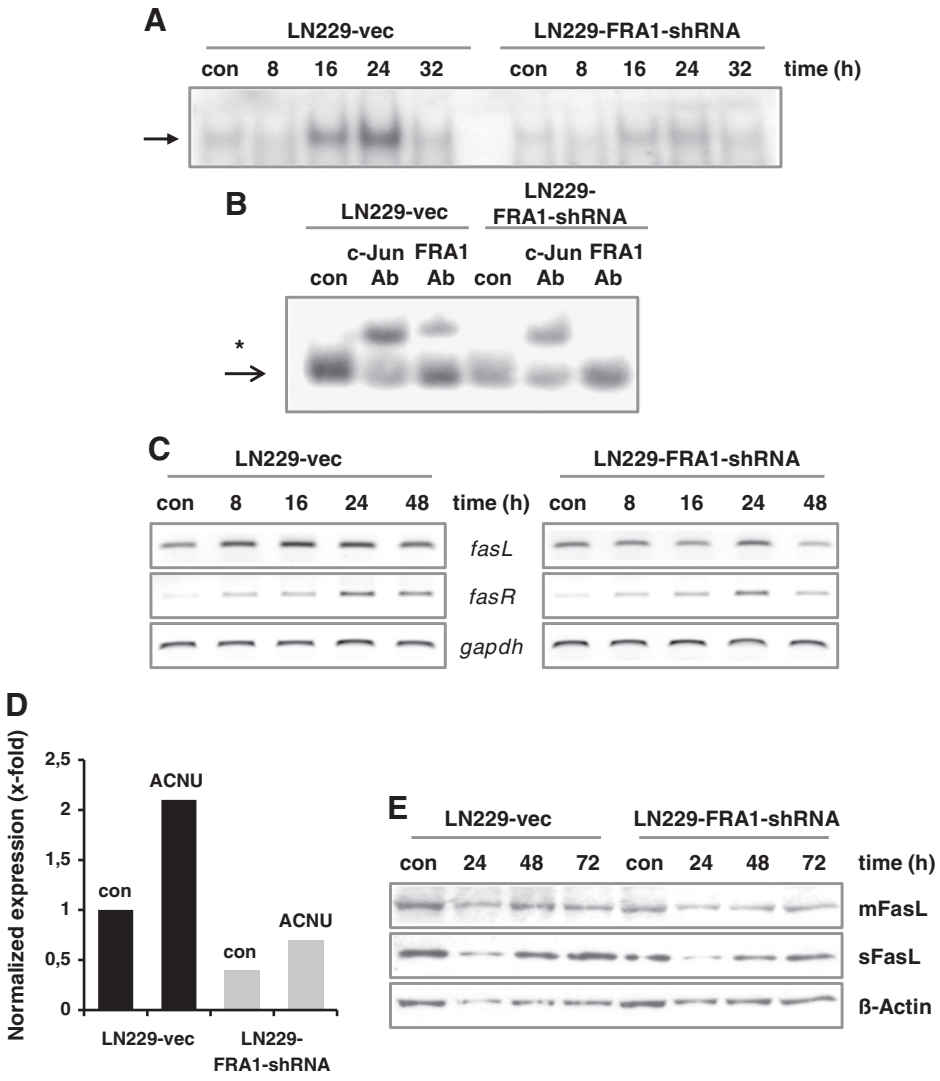

Fig. 3. Impact of FRA1 down-modulation on AP-1 activity and FasL expression. (A) LN229-vec cells and LN229-FRA1-shRNA cells were exposed to 50 μM ACNU and harvested at indicated time points. Nuclear extracts were isolated and incubated with radioactively labeled oligonucleotides containing the AP-1 binding site of the collagenase promoter (*mmp1*). The binding of AP-1 was visualized by EMSA and the specific complex is indicated by an arrow. (B) EMSA supershift assay: The presence of c-Jun and FRA1 in the AP-1 factor bound to oligonucleotides containing the AP-1 binding site of the collagenase promoter (*mmp1*) was analyzed by the addition of specific antibodies to the reaction; the specific complex is indicated by an asterisk. Nuclear extracts were prepared 24 h after exposure to 50 μM ACNU. (C/D) LN229-vec and LN229-FRA1-shRNA cells were exposed to 50 μM ACNU. (C) At different time points after exposure, total RNA was isolated and semi-quantitative RT-PCR was performed using *fasL*, *fasR* or, as loading control, *gapdh* specific primers. (D) 24 h after exposure, total RNA was isolated and real-time RT-PCR was performed using *fasL* specific primers. Expression was normalized on *gapdh* and *β-actin*, and the *fasL* expression of untreated LN229-vec cells was set to one. (E) LN229-vec and LN229-FRA1-shRNA cells were exposed to 50 μM ACNU for the indicated times. Protein extracts were prepared and subjected to Western blot analysis. The membrane was incubated with FasL specific antibody or, as loading control, with β-Actin specific antibody.

Fig. 5, no differences in the formation and repair of DNA single-strand breaks (Fig. 5A) or crosslinks (Fig. 5B) were observed between FRA1 expressing and FRA1 downmodulated cells.

Next we analyzed the generation of DNA double-strand breaks (DSB) by measuring the phosphorylation of H2AX (γH2AX), a sensitive marker for DSB. A strong time-dependent phosphorylation of H2AX occurred following ACNU treatment, which was comparable in FRA1 proficient cells and cells transfected with FRA1-siRNA (Fig. 5C) as well as in LN229-vec and LN229-FRA1-shRNA cells (Fig. 5D). The data indicate that FRA1 neither impacts DNA damage formation nor repair.

3.5. FRA1 mediated resistance to ACNU is associated with delayed cell cycle progression

It was previously shown that FRA1 confers sensitivity to cisplatin by its effect on cell cycle checkpoints. Thus, knockdown of FRA1 resulted in a reduced S-phase arrest, which was signaled through modulation of the CHK1–Cdk2 pathway [12]. To analyze the effect of FRA1 on cell cycle checkpoints upon exposure of glioblastoma cells to ACNU the cell cycle distribution was analyzed via flow cytometry 96 h after exposure (Fig. 6A). Following exposure to ACNU, a strong accumulation of cells in the G2-phase was observed. The amount of LN229-vec cells in the G2-phase increased from approximately 20% in untreated cells to 80% in cells exposed to 50 μM ACNU. In untreated LN229-FRA1-shRNA cells the cell cycle distribution was similar to untreated LN229-vec cells, whereas upon exposure to 50 μM ACNU only 50% of the cells were arrested in G2. The amount of cells either in G1 and S-Phase was increased to 25%. The data indicate that at this dose the FRA1 knockdown leads to a reduced G2-phase arrest or to an accumulation of the cells in the G1/S-phase before entering the G2-phase. Similar results were obtained in U87 and U138 cells (Fig. 6B). In both cell lines, knockdown of FRA1 leads to an increased S-phase population and a decrease of cells in the G2 phase.

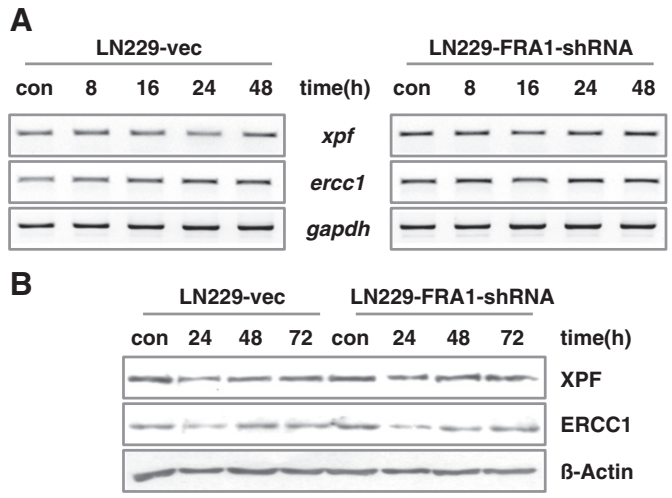

Fig. 4. Impact of FRA1 down-modulation on the expression of NER genes. (A) LN229-vec and LN229-FRA1-shRNA cells were exposed to 50 μM ACNU. At different time points after exposure, total RNA was isolated and semi-quantitative RT-PCR was performed using *xpf*, *ercc1* or, as loading control, *gapdh* specific primers. (B) LN229-vec and LN229-FRA1-shRNA cells were exposed to 50 μM ACNU for the indicated time points. Protein extracts were prepared and subjected to Western blot analysis. The membrane was incubated with XPF or ERCC1 specific antibodies. β-Actin was used as loading control.

Upon exposure to DNA damaging agents, the G2/M cell cycle arrest is mediated via the ATR–CHK1–Cdc25c axis [1]. To investigate the activation of this DNA damage response cascade, the phosphorylation of the serine/threonine-protein kinase CHK1 was analyzed using specific antibodies (Fig. 6C and D). In both LN229-FRA1-shRNA and LN229-vec cells, similar CHK1 phosphorylation was observed 24 h after ACNU exposure (Fig. 6C). However, additional 24 h later, phosphorylated CHK1, was only detectable in LN229-vec cells. In LN229-FRA1-shRNA cells not only the expression of the phosphorylated but also of the non-phosphorylated CHK1 protein was strongly reduced, which could attribute to the observed reduced accumulation of the cells in the G2/M phase. To substantiate our data concerning the reduced CHK1 activation in the absence of FRA1 we further utilized siRNA mediated knockdown of FRA1 in U87 and U138 cells. Similar to the data obtained in LN229 cells, knockdown of FRA1 resulted in a reduced phosphorylation of CHK1 48 h after ACNU exposure in both U87 and U138 cells (Fig. 6D).

4. Discussion

The dimeric transcription factor AP-1 is involved in the regulation of several cellular key mechanisms like angiogenesis, apoptosis, differentiation, hypoxia, invasion, metastasis and proliferation [4]. Furthermore, AP-1 dimers containing c-Fos are involved in the regulation of several DNA repair genes. Thus the UV-induced upregulation of the DNA repair genes *trex1*, *xpf* and *xpg* is mediated by c-Fos, which stimulates DNA repair activity and protects against genotoxin-induced cell death [5-7].

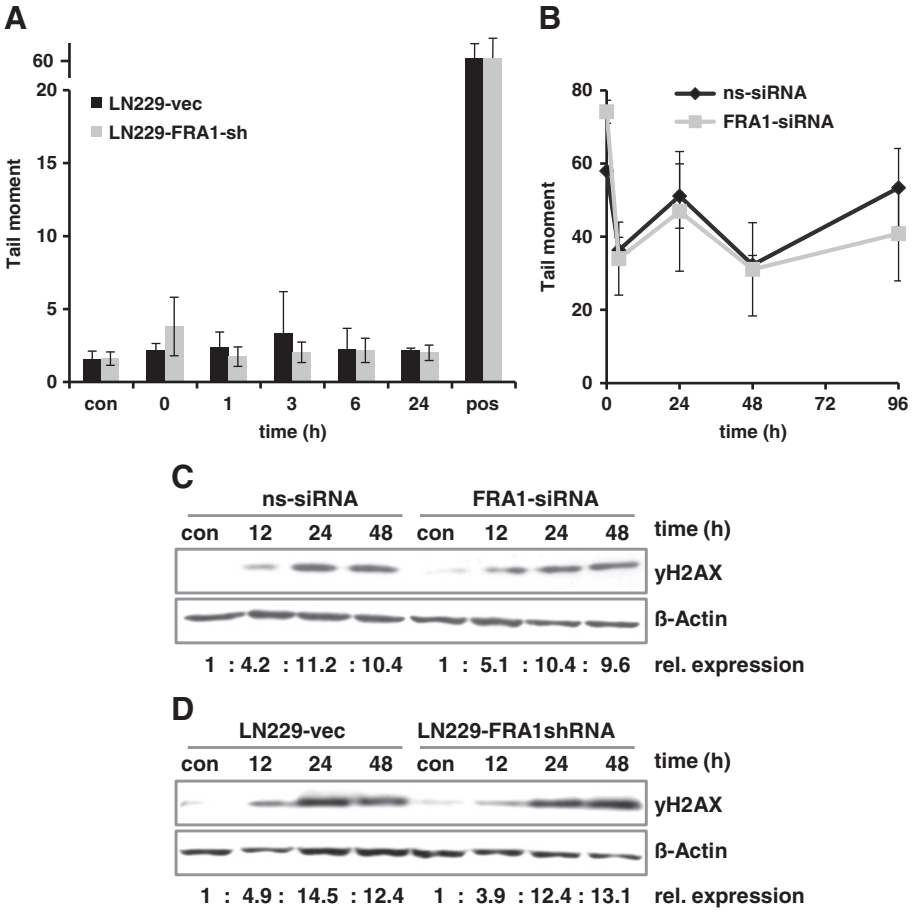

Fig. 5. Effect of FRA1 knockdown on damage formation and DNA repair capacity. To analyze repair capacity upon FRA1 knockdown, LN229 cells were either transfected with FRA1 specific siRNA (si) or nonsense-siRNA (ns-si), or stably transfected cells were used. (A) LN229-vec and LN229-FRA1-shRNA cells were exposed for 1 h to 50 μM ACNU and at different time points following exposure the alkaline comet assay was performed. (B) 24 h after transfection, the cells were exposed for 1 h to 25 μM ACNU, harvested at indicated time points after exposure and subsequently irradiated with 8 Gy ionizing radiation. Thereafter, the alkaline comet assay was performed. (C/D) Expression of phosphorylated H2AX (γH2AX) was analyzed at different time points after exposure to 50 μM ACNU in FRA1 proficient cells and cells transfected with FRA1-siRNA (C) and in LN229-vec and LN229-FRA1-shRNA cells (D). β-Actin was used as loading control.

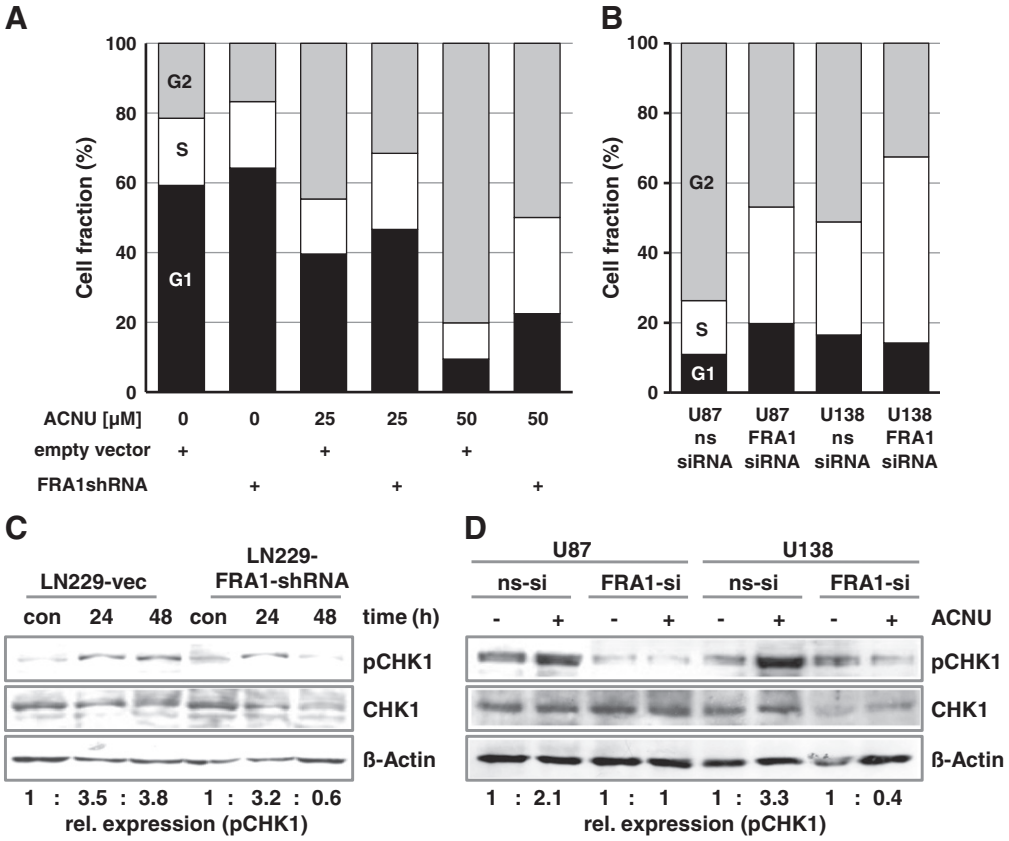

Fig. 6. Effect of FRA1 knockdown on cell cycle checkpoints. (A) LN229-vec cells and LN229-FRA1-shRNA cells were exposed to 25 and 50 µM ACNU and incubated for 96 h. Cells were stained with PI and the G1, S and G2/M fraction was determined by flow cytometry. (B) U87 and U138 cells were transfected with FRA1 specific siRNA (FRA1-si) or nonsense-siRNA (ns-si). 24 h later, cells were exposed to 50 µM ACNU and incubated for 96 h. Cells were stained with PI and the G1, S and G2/M fraction was determined by flow cytometry. (C) LN229-vec and LN229-FRA1-shRNA cells were exposed to 50 µM ACNU for 24 or 48 h. Protein extracts were prepared and subjected to Western blot analysis. The filter was incubated with phosphospecific and non-phosphospecific antibodies against CHK1 and, as loading control β-Actin. (D) U87 and U138 cells were transfected with FRA1 specific siRNA (FRA1-si) or nonsense-siRNA (ns-si). 24 h later, cells were exposed to 50 µM ACNU and incubated for 48 h. Protein extracts were prepared and subjected to Western blot analysis. The membrane was incubated with phosphospecific and non-phosphospecific antibodies against CHK1 and, as loading control, β-Actin.

Other members of the Fos-family are FosB, FosL1 (FRA1) and FosL2 (FRA2). Contrary to c-Fos, FRA1 has no transactivation domain and in glioblastoma cells its transcriptional activity is dependent on its interaction with JunB [22]. In glioblastoma, FRA1 has been suggested to be involved in the maintenance and progression of the disease [11].

Here, we analyzed the role of FRA1 in the sensitivity of the glioblastoma cell line LN229 to the anticancer drug ACNU that acts similar to BCNU (carmustine), CCNU (lomustine), MeCCNU (semustine) and fotemustine [23]. These drugs are used in the therapy of malignant glioma, metastatic melanoma and other tumors. We show that upon ACNU exposure FRA1 is transcriptionally induced within 8–16 h. Transcriptional activation of FRA1 was described for different mitogens, but not yet for genotoxicants, and was shown to be mediated via serum and TPA response elements [24]. Upon ACNU exposure, induction of *FRA1* mRNA is accompanied by a strong increase in the corresponding protein level. FRA1 is additionally post-translationally modified by members of the MAPK cascade since inhibition of p38K and ERK2 led to a strong decline in FRA1 protein expression, which is in line with previously published data on the regulation of FRA1 [25,26].

Using transient (siRNA) and stable (shRNA) knockdown strategies, we showed that FRA1 is involved in the protection against ACNU-induced cytotoxicity. Downregulation of FRA1 resulted in decreased AP-1 binding activity as measured by EMSA using the *mmp1* AP-1-binding site. However, in contrast to data obtained in melanoma cells where FRA1 is important for the basal expression of *mmp1* [27], the downregulation of FRA1 in glioblastoma cells did not change the basal AP-1 binding activity to *mmp1* specific AP-1 binding site, but rather reduced the ACNU-induced AP-1 binding activity.

One of the most prominent targets of AP-1 is the gene encoding the Fas ligand (*fasL*) [19,28]. Fas ligand binds to the Fas receptor and thereby activates the extrinsic apoptotic pathway [29,30], leading to cell death. Induction of the *fasL* gene is a key component of the apoptotic pathway, which is mediated via the SAPK/JNK signaling cascade [19,31] and activation of AP-1. Whereas the participation of c-Jun, ATF2 and FosB in the induction of *fasL* is well established [19,32,33], the role of c-Fos and FRA1 is not clear. Previously we showed that upon UV exposure, c-Fos is not involved in the induction of FasL [34]. Upon ACNU exposure of LN229 cells induction of *fasL* mRNA was observed, which was not the case in cells knockdown for FRA1. Concomitant with *fasL* mRNA a slightly higher expression of membrane-bound and soluble FasL protein was observed in FRA1 expressing cells, indicating that FRA1 is involved in the transcriptional regulation of *fasL*. However, since FRA1 knockdown cells that show no *fasL* induction and lower FasL expression, are more sensitive to ACNU it is pertinent to posit that the protective effect of FRA1 is not related to the regulation of the FasL. It appears that FRA1 regulates in addition to the FasL another function that neutralizes FasL upregulation, thus exerting strong killing protection.

Besides the *fasL*, several DNA repair genes involved in the NER pathway like *ercc1*, *xpf*, and *xpg* are regulated by AP-1 [6,7,20]. NER and especially the proteins ERCC1 and XPF are involved in the repair of mono-adducts and crosslinks induced by chloroethylating anticancer drugs [35]. Therefore, differential regulation of NER genes could attribute to the observed sensitizing effect of FRA1 knockdown. However, opposite to data obtained in fibroblasts following UV exposure [6,7], ACNU did not lead to the induction of *xpf* in glioblastoma cells.

For *ercc1* a slight induction was observed in the FRA1 proficient, but not the FRA1 downmodulated cells. However, similar to *fasL*, the induction of *ercc1* mRNA did not lead to a significant change in the expression level of the corresponding ERCC1 protein. Also in the case of XPF no ACNU-induced upregulation of the protein was observed. To further analyze the effect of FRA1 downmodulation on DNA repair, the formation and repair of SSB, DSB and crosslinks was analyzed, showing in all cases no differences between FRA1 proficient and FRA1 downregulated cells, again indicating that the effect of FRA1 on ACNU-triggered toxicity is not based on modulation of DNA repair.

In contrast to the missing effect of FRA1 on the regulation of DNA repair we observed that FRA1 modulates cell cycle progression upon ACNU exposure. Thus, in FRA1 downregulated cells sensitization to ACNU was accompanied by a reduced G2 arrest. An important factor in the regulation of the G2/M checkpoint is CHK1. This kinase phosphorylates Cdc25c which induces binding of Cdc25c to the 14-3-3 protein [36,37]. Within this complex, Cdc25c is transported out of the nucleus and therefore unable to dephosphorylate/activate Cdk1–CyclinB, which finally leads to G2/M arrest [38]. In our experiments, we observed a comparable initial activation of CHK1 24 h after exposure of cells to ACNU. However 48 h after exposure, phosphorylated CHK1 was observed only in FRA1 proficient but not FRA1 downregulated cells. From these data we conclude that reduced CHK1 phosphorylation observed in FRA1 downregulated cells leads to an abrogation of the G2/M cell cycle check point. Interestingly a decreased phosphorylation of CHK1 was also observed upon cisplatin treatment of glioma cells downregulated for another member of the AP-1 family, namely ATF3 [12]. Also in this case, downregulation of ATF3 conferred sensitivity to anticancer drug treatment. The abrogated G2/M arrest observed in FRA1 downregulated cells may lead to progression of cells through mitosis before the DNA damage is repaired, finally leading to cell death in the subsequent cell cycle.

What are the clinical implications? The treatment of gliomas suffers from poor response of the tumor and, therefore, most patients succumb to the disease. Only a few factors that determine resistance of glioblastoma cells to chloroethylating anticancer drugs have been identified so far. One is the DNA repair protein MGMT, which removes chloroethyl groups from the O^6-position of guanine ([23,39]). In addition, the FA pathway, especially FANCD1/BRCA2 confers glioma resistance to BCNU [40] and ACNU [41]. It has also been shown that p53 mediates resistance to chloroethylating agents, by induction of the nucleotide excision repair proteins DDB2 and XPC [42]. Our data suggests FRA1 to be a novel player in the resistance of glioma cells to chloroethylating anticancer drugs. A concept in tumor chemotherapy rests on the use of inhibitors of DNA damage signaling and repair, either as monotherapy or in combination with anticancer drugs [43]. However, inhibition of transcription factors like FRA1 seems not to be feasible due to their broad involvement in different cellular functions. Our data indicate that the protective effect of FRA1 is associated with CHK1. CHK1 inhibitors like UCN-01 or XL844 are used already in the clinic in phase I and II studies alone or in combination with anticancer drugs like topoisomerase inhibitors [44]. Therefore, inhibition of CHK1 seems to be a reasonable strategy to sensitize glioma cells to ACNU.

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