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ORIGINAL ARTICLE

Malignant melanoma cells acquire resistance to DNA interstrand cross-linking chemotherapeutics by p53-triggered upregulation of DDB2/XPC-mediated DNA repair

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Malignant melanoma is a cancer characterized by high chemoresistance although p53 is rarely mutated. Here, we show that p53 wild-type melanoma cells acquire resistance to cell death induced by fotemustine (FM), which is a representative of alkylating DNA interstrand cross-linking agents used in melanoma therapy. We show that drug-induced resistance is a result of p53-dependent upregulation of the nucleotide excision repair (NER) genes xeroderma pigmentosum complementation group C (*XPC*) and damaged DNA-binding protein 2 (*DDB2*), which stimulate the repair of DNA interstrand cross-links (ICLs) arising from O^6-chloroethylguanine. Consequently, *TP53* mutated cells are unable to repair ICLs, leading to prolonged ATM, ATR and checkpoint kinase 1 (CHK1) activation, and finally apoptosis. The roles of p53 and NER in ICL-triggered cell death were confirmed by knockdown of p53 and XPC. Upregulation of XPC and DDB2 in p53wt cells following a single drug treatment is a robust and sustained response that lasts for up to 1 week. Pretreatment with an inducing dose followed by a high and toxic dose of FM provoked an adaptive response as the killing outcome of the challenge dose was reduced. Upregulation of XPC and DDB2 was also observed in a melanoma mouse xenograft model following systemic administration of FM. Additionally, XPC and DDB2 induction occurred upon treatment with other cross-linking anticancer drugs, such as cisplatin and mafosfamide, indicating it is a general response of cancer cells to this group of chemotherapeutics. Collectively, the data indicate that p53-dependent upregulation of XPC and DDB2 is a key mechanism upon genotoxic stress, whereby melanoma cells acquire resistance towards DNA cross-linking agents. To our knowledge, this is the first demonstration of upregulation of NER following a single dose of a DNA interstrand cross-linker, which is a robust and long-lasting effect that impacts the killing response of cancer cells to subsequent treatments.

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INTRODUCTION

Malignant melanoma is highly refractory to anticancer drugs despite retaining wild-type p53.[1,2] This may in part be a result of silencing of apoptosis pathways[3,4] and defects in p53-dependent G1 checkpoint activation.[5] This, however, does not explain resistance acquired by tumors during therapy. Once metastasized, the prognosis of melanoma is extremely poor. When metastasizing to the central nervous system, therapy includes alkylating agents such as fotemustine (FM),[6] which acts similar to the nitrosoureas nimustine, carmustine, lomustine and semustine.[7] FM is a chloroethylnitrosourea derivative that undergoes spontaneous hydrolysis at physiological pH to form the reactive intermediate 2-chloroethyldiazohydroxide.[8] 2-chloroethyldiazohydroxide reacts with DNA forming the adduct O^6-chloroethylguanine, among others. The O^6-chloroethyl adduct is removed from the DNA by the repair protein O^6-methylguanine-DNA methyltransferase (MGMT).[9] The level of MGMT in tumors is highly variable. Relative to other tumors, low amounts are expressed in malignant melanomas,[10] which is most likely due to promoter methylation of MGMT.[11] Despite its low expression in melanomas, MGMT is an important resistance marker for melanoma cells exposed to FM,[12,13] and melanoma cells can acquire resistance to FM upon chronic treatment by upregulation of MGMT.[14]

If not repaired, O^6-chloroethylguanine produces the intermediate N1-O^6-ethanoguanine that then reacts with the adjacent cytosine to form a GC DNA interstrand cross-link (ICL).[15] As ICLs can inhibit essential metabolic processes such as RNA transcription and DNA replication, they are thought to be the ultimate killing lesions.[16] ICLs can be repaired in G1 of the cell cycle and employs mechanisms of nucleotide excision repair (NER)[17] and translesion synthesis.[18] During S-phase of the cell cycle, ICLs are repaired by the concerted action of several DNA repair mechanisms, including homologous recombination,[19] NER[20] and translesion synthesis.[21] Consequently, cells defective in homologous recombination[22] and translesion synthesis[23] are hypersensitive to FM compared with wild-type controls, whereas cells defective in NER show increased sensitivity to ICLs.[24,25]

Previously, we showed that FM induces apoptosis in melanoma cells and that p53 wild-type (p53wt) cells are more resistant to FM-induced apoptosis than p53 mutant (p53mt) cells.[13] As most melanomas are p53wt,[1,2] but exhibit defects in the p53-dependent death receptor[3] and mitochondria apoptosis pathways,[4] we endeavored to determine whether p53-dependent

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stimulation of DNA repair contributes to the molecular mechanism whereby p53wt melanomas attain their resistance to DNA interstrand cross-linkers like FM. Here, we show that FM induces robust accumulation of p53 that triggers upregulation of damaged DNA-binding protein 2 (DDB2) and xeroderma pigmentosum complementation group C (XPC) on RNA and protein level, and increases ICL repair, which causes drug resistance. In p53mt, p53 knockdown and XPC knockdown cells, lack of ICL repair resulted in a strong apoptotic response, which was executed by the mitochondrial pathway. The data provide an explanation of why melanoma cells are resistant to this group of alkylating drugs, and highlight the importance of searching for NER inhibitors in order to overcome acquired cancer cell resistance to DNA interstrand cross-linkers.

RESULTS

Melanoma cells containing wild-type p53 are resistant to FM-induced apoptosis

Apoptosis induced by FM was analyzed in a panel of melanoma cells. Seven melanoma cell lines differing in their p53 status were treated with 32 μM FM, which is a dose comparable to that achieved during therapy.[26] In order to facilitate comparison, MGMT was depleted with the MGMT inhibitor O^6-benzyl-guanine,[27] thereby excluding any influence that variable MGMT expression between cell lines might have on cell survival. The apoptotic response differed greatly between the melanoma lines, with p53wt lines clearly more resistant than p53mt lines (Figure 1a, solid symbols p53wt, open symbols p53mt). For further studies, we chose D05 (p53wt) and D14 (p53mt), whose p53 status was confirmed by determining p53 and p21 protein levels. D05 clearly showed p53 stabilization and nuclear localization following FM treatment, whereas D14 cells showed high basal p53 protein expression, as expected (Figure 1b). The transactivation activity of p53 was verified by p21; it was strongly upregulated in D05 cells and undetectable in D14 cells (Figure 1b). Apoptosis determined by annexinV/propidium iodide double-staining showed that D14 has a stronger apoptotic and necrotic (late apoptotic) response than D05 cells (Figure 1c and Supplementary Figure S1A), and that cell death is caspase-dependent, as inhibition of caspases decreased the amount of apoptosis induced by FM (Figure 1d and Supplementary Figure S1B). Additionally, the data showed that apoptosis is executed via the mitochondrial pathway, as FM caused the release of cytochrome c from the mitochondria into the cytoplasm (Figure 1e) and was able to activate caspase-9 (Figure 1f). FM was unable to activate the death receptor apoptosis pathway (Supplementary Figures S2A and B). The caspase dependence of apoptosis in melanoma cells was confirmed by immunoblots of activated caspases-3 and -7 (Supplementary Figure S2C). FM also increased the activity of caspase-3 (Supplementary Figure S2D). Overall, the data show that p53wt melanoma cells are more resistant to FM-induced apoptosis than p53mt cells.

DNA repair of FM-induced lesions is stimulated by p53

To determine the importance of p53 in the resistance of melanoma cells to FM, p53 was stably knocked down in the D05 and A375 (p53wt) cell lines. In Figure 2a, p53 knockdown is shown on protein level in the D05 cells; for the p53 knockdown in A375 cells see Figure 2c, insert. p53 induction by FM was detectable in D05 and D05 empty, whereas D05 p53shRNA showed no induction and a lower p53 basal level. Also, the basal p21 level and FM-triggered p21 induction were lower in D05 p53shRNA cells than in the controls (D05 and D05 empty cells transfected with the plasmid only). The apoptotic response of D05 and A375 cells following p53 knockdown and FM treatment was significantly higher than the corresponding controls (Figures 2b and c). These experiments support the notion that p53 protects against FM-induced apoptosis.

As one of p53's supposed functions is promotion of DNA repair, we determined whether p53 stimulates the repair of FM-induced DNA lesions. FM induces ICLs; therefore the ICL repair capacity of D05 (p53wt) and D14 (p53mt) melanoma cell lines was assayed using a modification of the comet assay that was previously applied to determine cisplatin-induced ICLs.[28] Although 24 h after FM treatment the amount of ICLs (low tail moment due to DNA cross-linking) was comparable in both cell lines, 48 h after the treatment ICLs were removed in D05 cells, but persisted in D14 cells (Figure 2d). It is conceivable that the lack of ICL repair in D14 cells will lead to a block in S-phase progression as DNA polymerases cannot bypass these lesions. To determine whether this is the case, D05 and D14 cells were subjected to FM, and their cell cycle progression was assayed by flow cytometry and ModFit analysis. D14 cells showed a clear S-phase delay 24 and 48 h following FM treatment compared to D05 cells (Figure 2e). The data support the notion that p53 stimulates ICL repair that prevents from S-phase blockage and, thereby, confers resistance of melanoma cells to the DNA alkylator FM.

Lack of ICL repair in p53mt cells corresponds to a strong and prolonged activation of the DNA damage response

Although FM causes similar levels of ICLs in D05 and D14 cells (Figure 2d), the lack of their repair in p53mt D14 cells may lead to a difference in the DNA damage response. In D14 cells, lack of repair of FM-induced DNA lesions caused a strong and sustained activation of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-Rad3 (ATR) protein (Figure 3a) as well as checkpoint kinase 1 (CHK1) (Figure 3b) compared with D05 cells. Enhanced ATM/ATR and checkpoint kinase 1 activity should lead to p53 phosphorylation at serine 15, 20 and 37, which was indeed the case (Figure 3c). There was no increase in phosphorylation at p53 ser6 and 9. Interestingly, serine 15, 20 and 37 phosphorylation coincided with p53 stabilization in both lines, but p21 was only induced in the p53wt cells (Figure 3c), indicating that the mutated protein can be phosphorylated without gaining transactivating activity. The higher amount of unrepaired DNA damage in D14 cells was substantiated by determining the level of phosphorylated H2AX (γH2AX) foci, which is a marker for DNA double-strand breaks, and a target of ATM and ATR. There was a strong and sustained γH2AX formation in p53mt melanoma cells, which was observed up to >96 h following FM treatment (Figures 3d and e). Knockdown of p53 in D05 cells showed similar results: γH2AX formed at higher level in the knockdown cells compared with empty vector control (Figure 3e). These data support the finding that impaired ICL repair caused by the loss of p53 has a strong impact on apoptosis induced by FM.

ICLs trigger p53-dependent upregulation of XPC and DDB2

FM induces ICLs whose repair relies on components of NER. Therefore, the expression of NER genes was determined in melanoma cells. Upon FM treatment, D05 (p53wt) cells showed a robust induction of *DDB2* and *XPC* in a time (Figure 4a) and dose (Figure 4b) dependent manner, while this induction was not observed in p53mt D14 cells. Other repair proteins were not found to be significantly upregulated (Figure 4 and data not shown). D05, but not D14, cells also showed an increase in DDB2 and XPC protein level following FM treatment (Figure 4c). To determine whether this induction is p53-dependent, we examined the response in p53 knockdown D05 cells. While D05 and D05 empty vector cells showed a clear FM-induced upregulation of XPC and DDB2 protein, this upregulation could not be observed in D05 p53shRNA cells (Figure 4d). In order to confirm that DDB2/XPC upregulation is a general response of melanoma cells to FM,

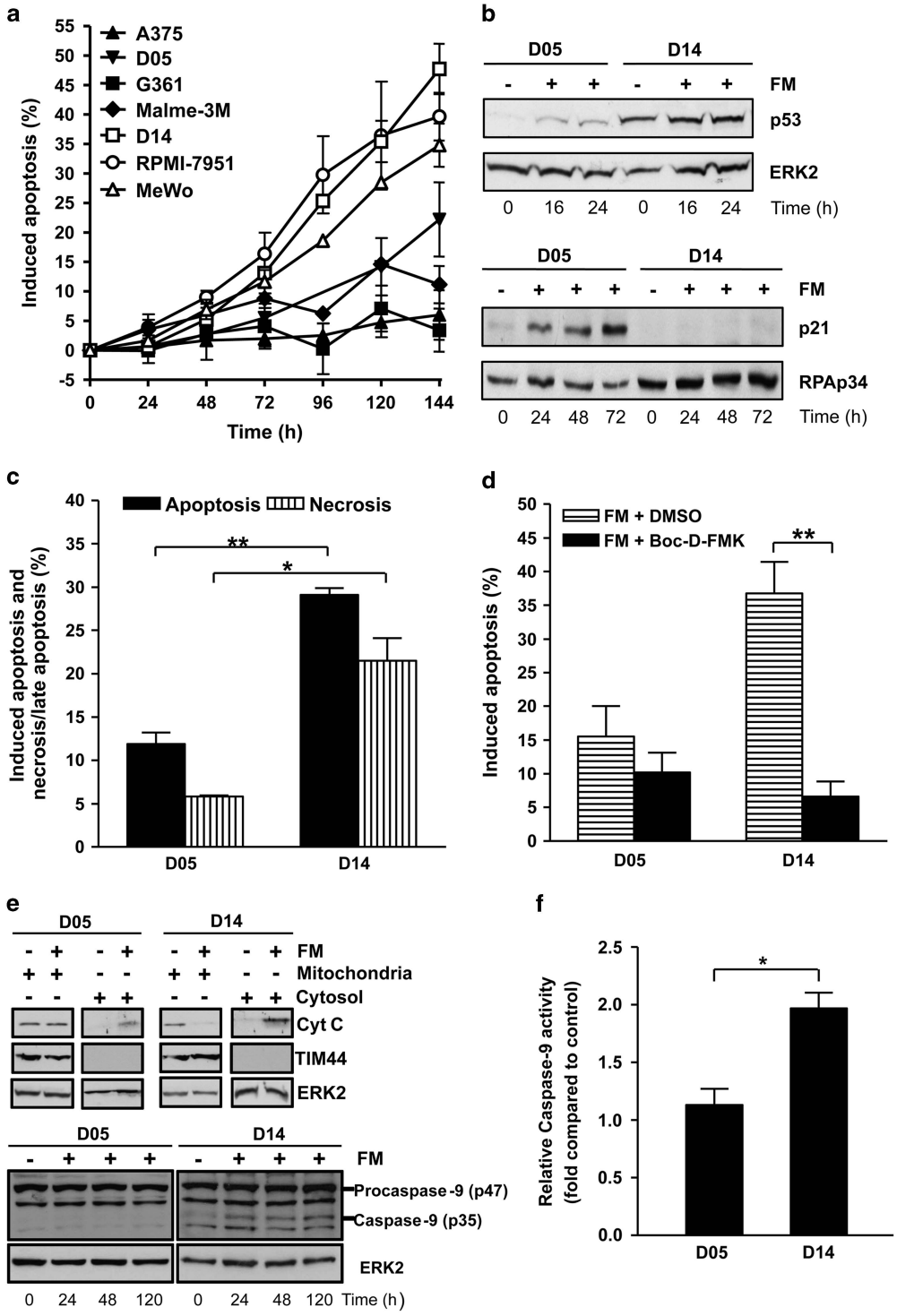

Figure 1. FM triggers apoptosis in melanoma cell lines and causes stabilization and transcriptional activation of p53. (**a**) Kinetics of apoptosis induction in seven different melanoma cell lines 24–144 h after FM addition. Samples were subjected to flow cytometric quantification of the SubG1 fraction. Data represent the mean of three independent experiments. (**b**) Immunoblot of p53 and p21 protein levels in nuclear extracts of D05 and D14 cells at the indicated time points after FM (32 μM) addition. ERK2 was used as loading control for p53 and RPA was used as loading control for p21. (**c**) Quantification of the apoptotic and necrotic/late apoptotic fraction of D05 and D14 cells 144 h after FM (32 μM) treatment by annexinV/propidium iodide (PI) double-staining. Data are the mean of three independent experiments. $*P<0.05$ and $**P<0.01$. (**d**) Quantification of the SubG1 fraction of D05 and D14 cells 96 h after FM (32 μM) treatment. Twenty-four hours after FM addition, the irreversible pan-caspase inhibitor Boc-D-FMK (50 μM) was added. $**P<0.01$. (**e**) Top: Immunoblot analysis of cytochrome c levels in mitochondrial and cytoplasmic fractions 72 h after FM (32 μM) treatment. TIM44 is a mitochondrial marker that served as an indicator for the purity of the cytoplasmic fraction. ERK2 was used as loading control. Bottom: caspase-9 activation determined by immunoblot analysis showing the p35 cleavage fragment (active fragment). Samples were harvested at the indicated time points after FM (32 μM) treatment. (**f**) Relative capase-9 activity 72 h after FM (32 μM) treatment. Data are expressed as relative activation of caspase-9 compared with untreated cells and are the mean of three independent experiments. $*P<0.01$. Where appropriate in aforementioned experiments, MGMT was inhibited by addition of 10 μM O^6-benzylguanine (O^6BG) 1 h before alkylating agent addition.

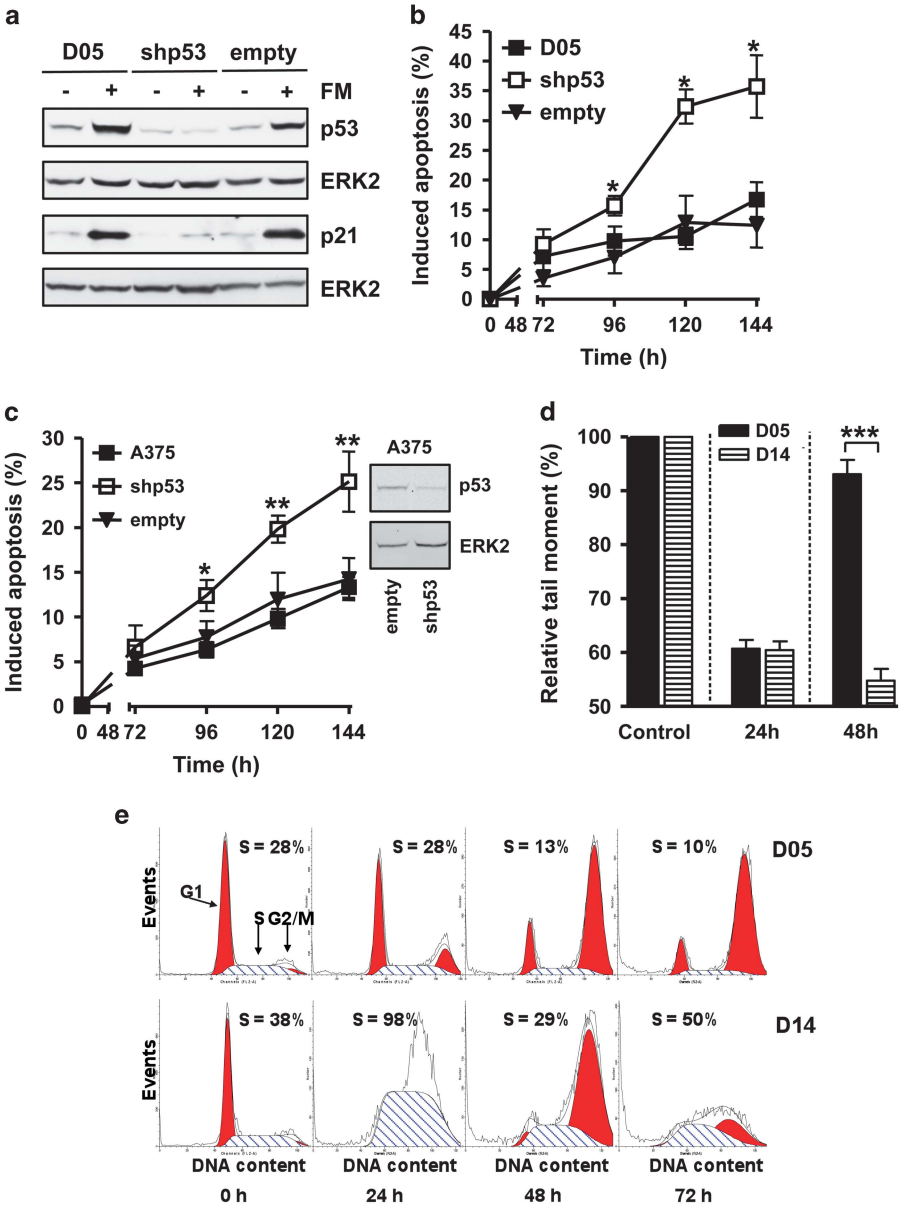

Figure 2. p53 affects cell sensitivity, ICL repair and cell cycle progression of melanoma cell lines in response to FM. (**a**) Verification of p53 status by immunoblot in nuclear extracts of D05, D05 p53shRNA and D05 empty 48 h after FM treatment (64 μM) by analyzing p53 and p21 protein levels**.** (**b**) Induced apoptosis in D05, D05 p53shRNA and D05 empty 72–144 h after FM treatment (32 μM). Samples were subjected to flow cytometric quantification of the SubG1 fraction. Data represent the mean of three independent experiments. *$P<0.05$ (**c**) Induced apoptosis in A375 (p53wt), A375 p53shRNA and A375 empty 72–144 h after FM treatment (32 μM). Samples were subjected to flow cytometric quantification of the SubG1 fraction. Data represent the mean of three independent experiments. * $P<0.05$ and **$P<0.01$. Insert: p53 protein levels in A375 empty and A375 shp53 clones. ERK2 served as loading control. (**d**) ICL repair of D05 and D14 cells at the indicated time points after FM (32 μM) treatment as determined by the single cell gel electrophoresis assay. At least 40 cells were scored for each time point and the experiment was repeated three times. ***$P<0.001$. (**e**) Representative ModFit analysis of flow cytometry histograms analyzed 0, 24, 48 and 72 h after FM (32 μM) addition in D05 and D14 cells. Where appropriate in aforementioned experiments, MGMT was inhibited by the addition of 10 μM O^6BG 1 h before alkylating agent addition.

another melanoma cell pair, namely A375 (p53wt) and RPMI-7951 (p53mt), was tested for FM-triggered increase of p53, p21, DDB2 and XPC protein levels. The data shown in Figure 4e are in line with the results obtained for D05 and D14 cells: A375 responded with an increase in p53 and its transcriptional target p21, as well as with an induction of XPC and DDB2, while RPMI-7951, whose p53 gene carries a nonsense mutation[29] does not show an increase in any of these proteins. Overall, the data revealed that FM treatment results in a p53-dependent upregulation of the NER genes *XPC* and *DDB2* in melanoma cells.

NER protects against FM-induced apoptosis

Next we addressed the biological consequence of NER gene induction. As XPC has been shown to be involved in ICL repair, we created A375 XPCshRNA cells carrying a stable XPC knockdown. XPC protein level of shXPC cells was reduced to 0.3-fold compared with the empty control (Figure 5a). Additionally, mRNA levels in untreated and FM-treated cells were investigated. Results confirmed the successful knockdown (Figure 5b), showing a significant XPC mRNA downregulation in shXPC cells. Even after FM treatment, the XPC mRNA levels of shXPC cells barely reached

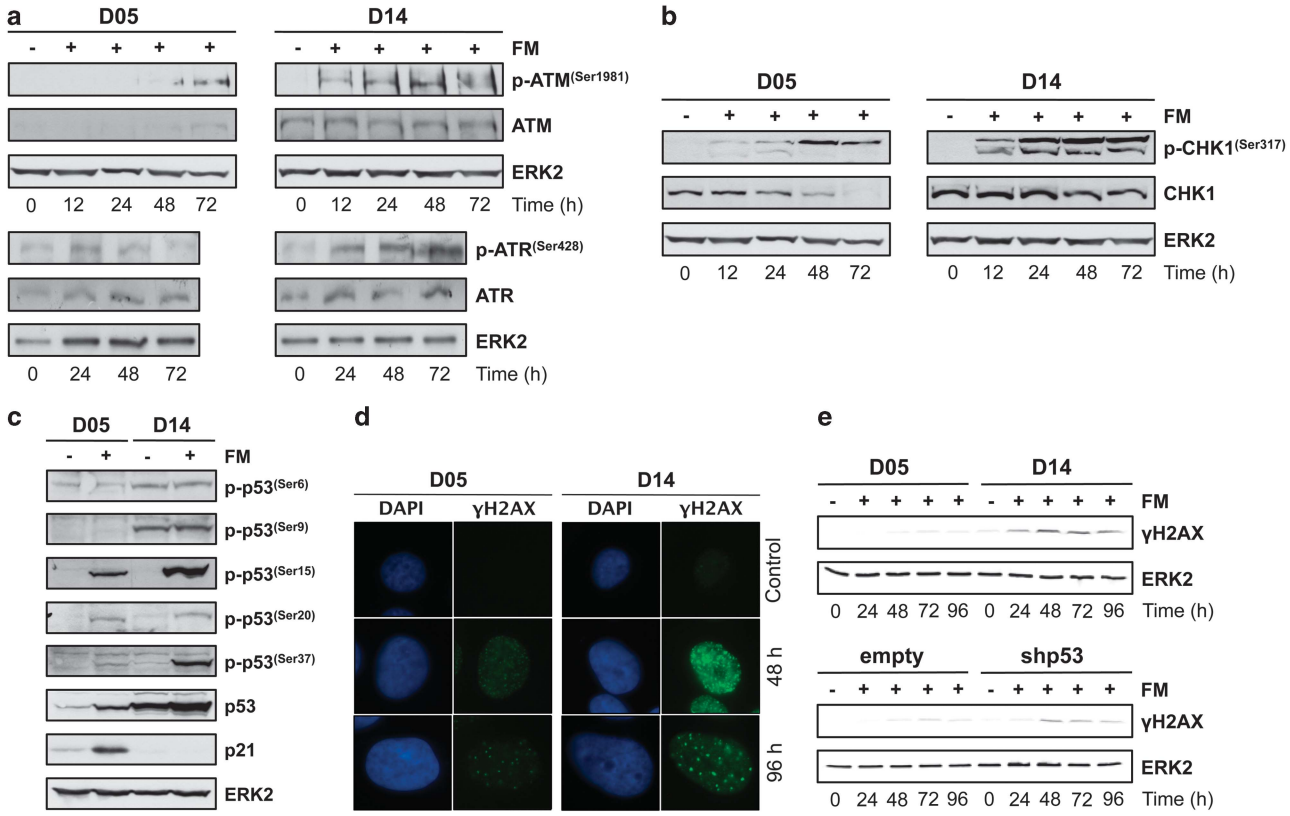

Figure 3. D14 (p53mt) melanoma cells show a stronger FM-induced DNA damage response than D05 (p53wt) cells. (**a**) Total ATM and phosphorylated ATM (serine 1981) and total ATR and phosphorylated ATR (serine 428) shown on immunoblots, (**b**) total checkpoint kinase 1 (CHK1) and CHK1 phosphorylation (serine 317) levels determined in extracts at indicated time points after FM (32 μM) treatment by immunoblotting. (**c**) p53 phosphorylation status at indicated Ser sites 48 h after FM (32 μM) treatment. Representative immunoblots are shown. (**d**) Immunofluorescence analysis of γH2AX in D05 and D14 cells at indicated time points after FM (32 μM) treatment. Nuclear staining was accomplished using 4′,6-diamidino-2-phenylindole (DAPI). Cells were visualized by confocal microscopy. (**e**) Immunoblot analysis of γH2AX in D05, D14, D05 empty and D05 shp53 cells at indicated time points after the addition of FM (32 μM) to the medium. ERK2 served as loading control. Where appropriate in aforementioned experiments, MGMT was inhibited by the addition of 10 μM O^6BG 1 h before alkylating agent addition.

the XPC mRNA levels observed in untreated empty control (Figure 5b). As XPC is indispensable for the efficient removal of ultraviolet (UV) light-induced DNA lesions,[30] XPC knockdown was also confirmed on a functional level by irradiating empty and shXPC cells with UV light. As expected, shXPC cells were more susceptible to UV light-induced apoptosis than empty cells (Supplementary Figure S3). Furthermore, we sought to exclude that sensitization of the XPC knockdown clones is due to loss of p53. To this end, the two clones (empty and XPC knockdown) were subjected to real-time PCR analysis of mRNA levels of two p53-responsive genes, *MDM2* and *CDKN1A* (p21), after FM treatment. Both clones responded with transactivation of the p53 target genes *MDM2* and *CDKN1A* (Figure 5c), but not *TP53* itself, as p53 does not stimulate its own transcription.

Bearing in mind that XPC binds ICLs induced by DNA damaging agents like psoralen,[31] accomplishing the DNA lesion recognition step in ICL repair, we determined whether XPC knockdown has any effect on XPC DNA binding following FM treatment. To this end, the A375 empty and shXPC cell lines were treated with FM and subjected to the trapped in agarose DNA immunostaining assay. The A375 XPCshRNA cell line clearly showed lower levels of XPC protein bound to DNA with or without FM treatment, compared with the empty A375 cell line (Figure 5d). This lends support to the idea that XPC binds to FM-induced ICLs and that this may contribute to their repair. To clarify this, the XPC knockdown and empty vector control cells were subjected to the ICL repair assay. Knockdown of XPC had a significant inhibitory effect on the repair of ICLs 24 and 48 h after FM addition (Figure 5e), confirming the role of XPC in FM-induced ICL repair. This leads to the question: does knockdown of XPC along with reduced ICL repair have an effect on the sensitivity of FM-treated melanoma cells? Quantification of apoptosis induced by FM revealed that shXPC cells are clearly more sensitive to FM than empty cells (Figure 5f), supporting the view that p53-mediated XPC upregulation results in cellular resistance.

Upregulation of XPC and DDB2 confers FM resistance and is a long-lasting effect

Next we addressed the question of the clinical relevance of our findings. First, we sought to elucidate whether a single low-dose treatment with FM would cause the classical adaptive response, thereby conferring resistance to a challenge dose. For this, D05 cells were treated twice with FM: at first with a low (weak toxic) dose, followed by the addition of the threefold higher dose. Data presented in Figure 6a show the induced cell death level after normalizing to the appropriate controls (results obtained with 95 μM were normalized to untreated controls, while samples subjected to the adaptation low dose were normalized to controls treated with the same low dose). This allows for determination of the effectiveness of the challenge dose with or without an adaptive dose. A single pretreatment dose reduced the effect of the challenge dose to zero level (Figure 6a), demonstrating that even a single treatment is sufficient to provoke FM resistance.

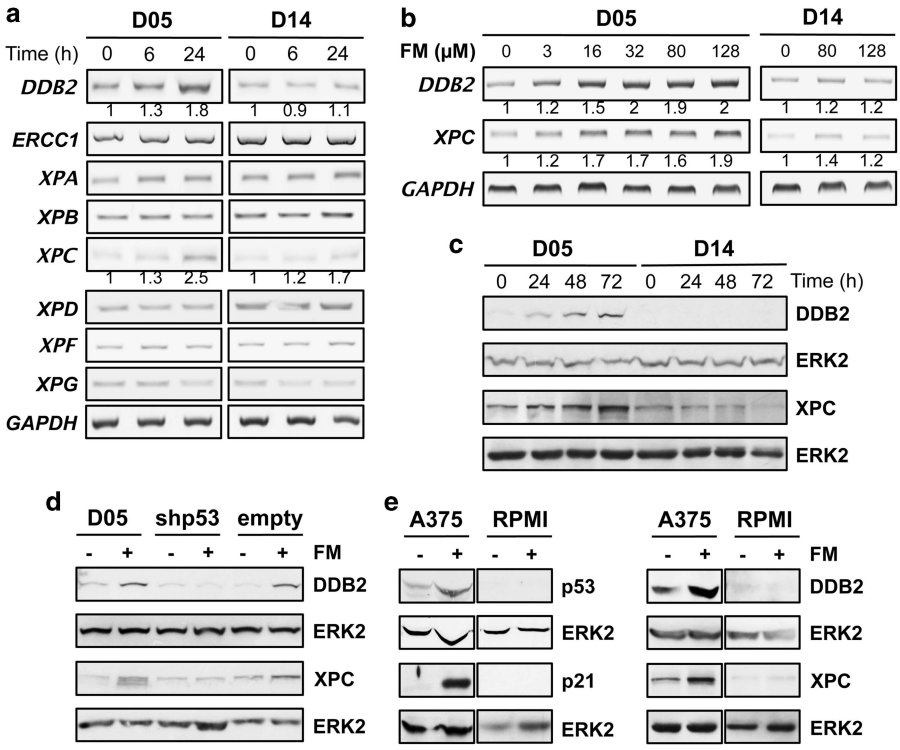

Figure 4. FM treatment induces NER genes in p53wt cells. (**a**) Analysis of NER gene (*DDB2*, *ERCC1*, *XPA*, *XPB*, *XPC*, *XPD*, *XPF* and *XPG*) expression by reverse transcription–PCR (RT–PCR) in D05 and D14 cells 6 and 24 h after FM (32 μM) treatment. (**b**) Analysis of *DDB2* and *XPC* expression by RT–PCR in D05 and D14 cells at 24 h after FM treatment with indicated concentrations. (**c**) Kinetics of DDB2 and XPC protein induction in D05 and D14 cells at indicated time points after FM (32 μM) treatment was analyzed by immunoblotting. (**d**) Immunoblot analysis of DDB2 and XPC protein levels in D05, D05 p53shRNA and p53 empty cells 48 h after FM (64 μM) treatment. (**e**) Immunoblot analysis of DDB2 and XPC protein levels in A375 (p53wt) and RPMI-7951 (p53mt) cell lines 24 h after FM (32 μM) treatment. For PCR, *GAPDH* was used as internal control, while for immunoblot ERK2 was used as loading control. Where appropriate in aforementioned experiments, MGMT was inhibited by the addition of 10 μM O^6BG 1 h before alkylating agent addition.

Treatment schedules with chloroethylating drugs such as FM and lomustine require the administration of the drug once a week for three consecutive weeks.[32] As the adaptive response (demonstrated in Figure 6a) caused resistance of the tumor cells to a subsequent treatment in the following week, we wondered whether the players involved in repair are upregulated in a sustained manner. Therefore, we investigated p53, XPC, DDB2 and p21 protein levels of D05 and A375 melanoma cells up to 144 h after the onset of FM treatment. Interestingly, in D05 cells the levels of all four proteins were elevated for up to 144 h (Figure 6b). In A375 cells, p53 stabilization was observed 96 h after FM treatment; elevation of DDB2 and XPC levels lasted up to 96 h and p21 induction up to 144 h after FM treatment (Supplementary Figure S4). The results indicate that upregulation of DNA repair following a single dose of a DNA interstrand cross-linker is a robust and long-lasting effect that impacts the response of cells to subsequent treatments.

FM-induced upregulation of DDB2 occurs in melanoma xenografts

To address the question of whether FM also stabilizes p53 and induces target DNA repair genes *in vivo*, an A375 (p53wt) xenograft model was established (Figure 6c). After depletion of MGMT with O^6-benzylguanine, mice were injected with FM intraperitoneally. Forty hours later, tumor samples for real-time PCR and immunoblotting were collected. Among the analyzed NER genes (*DDB2, ERCC1, XPA, XPC, XPF and XPG*) FM treatment induced mRNA expression of *DDB2* (induction factor (IF) 5.8) and *XPC* (IF 2.7) as analyzed by real-time PCR (Figure 6d). The positive control gene *MDM2*, a typical p53 target gene, was also induced (IF 7.2), while the p53-coding gene *TP53*, which serves as negative control, remained unaffected (Figure 6d). We should point out that the slight increases of other repair genes *XPG*, *ERCC1*, *XPA* and *XPF* were not significant. The increase of p53 and DDB2 in the mouse xenograft model could also be confirmed on protein level (Figure 6e). An increase in XPC protein could not be detected, although the mRNA level was enhanced. We should note that A375 grown as xenograft exhibited a high basal XPC protein level. Collectively, the upregulation of *DDB2* and *XPC* on RNA level and, for DDB2 on protein level, showed that a single non-toxic dose of FM is able to upregulate DNA repair in the tumor *in situ* following systemic treatment.

Induction of XPC and DDB2 in melanoma cells is also caused by other DNA interstrand cross-linkers

In order to further assess the relevance and validity of this study, we expanded our investigation to other DNA damaging anticancer drugs that induce ICLs. We chose mafosfamide, an activated cyclophosphamide derivative, and cisplatin to analyze *XPC* and *DDB2* expression after the treatment with these drugs. Figure 6f shows induction of *DDB2* and *XPC* in A375 cells by both mafosfamide and cisplatin, which was comparable to the induction caused by FM. As positive control for p53-triggered gene induction *CDKN1A* (p21) and, as negative control, *TP53* were used, which showed the expected response (Supplementary Figure S5). The finding that mafosfamide and cisplatin show similar effectiveness as FM in inducing *XPC* and *DDB2* indicates that the mechanism of acquired resistance to DNA interstrand cross-linkers is not restricted to FM, but has broader implications.

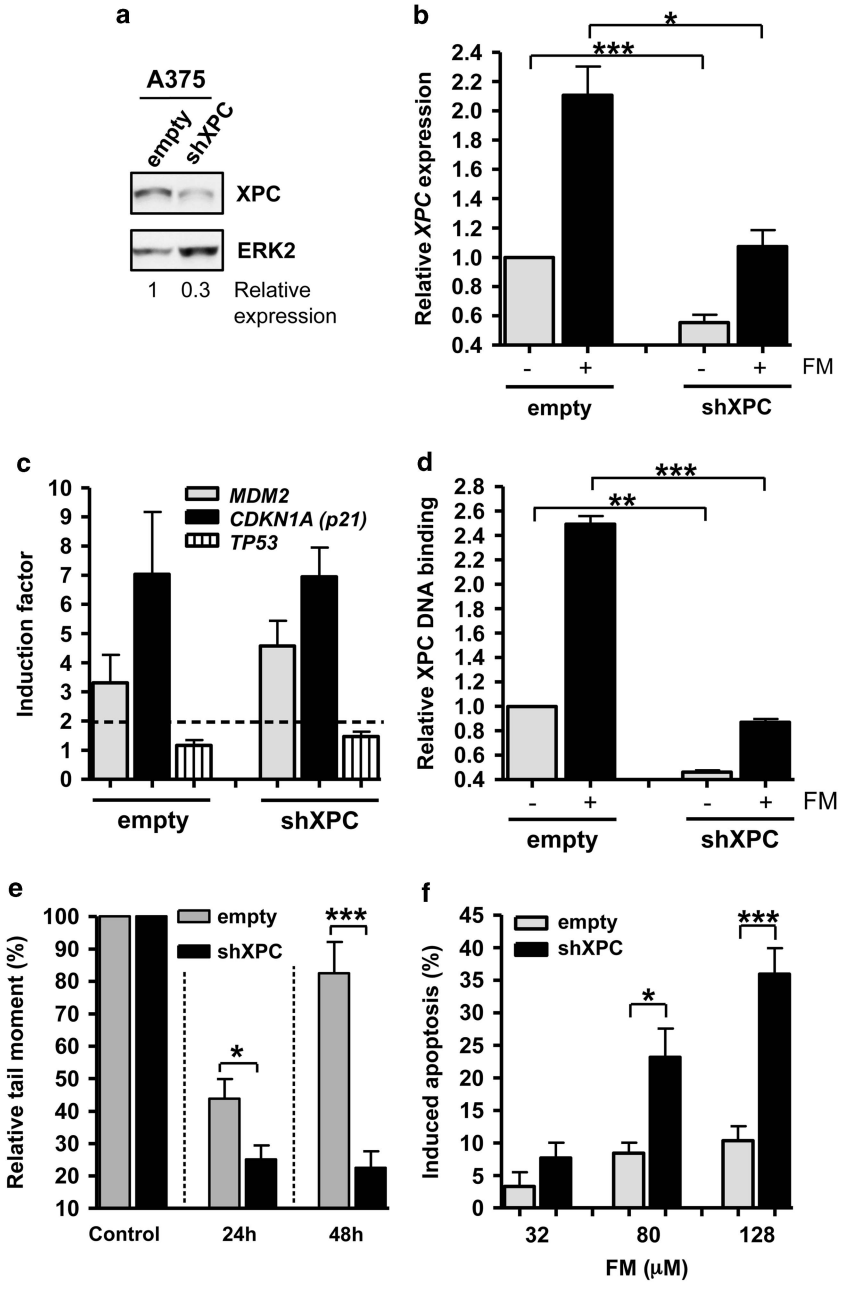

Figure 5. XPC knockdown sensitizes A375 (p53wt) melanoma cells to FM. (**a**) Verification of XPC knockdown in A375 empty and A375 XPCshRNA. Basal XPC protein levels were analyzed by immunoblot. ERK2 was used as loading control. (**b**) XPC mRNA levels in untreated and FM (32 μM, 24 h) treated cells were analyzed by real-time PCR as described in Materials and methods and normalized to the control sample of empty clone. Data represent the mean of three independent experiments. (**c**) Real-time PCR analysis of mRNA levels of the p53 target genes *MDM2* and *CDKN1A* (p21) 24 h after FM (32 μM) treatment in A375 empty and A375 XPCshRNA. *TP53* served as negative control. Data are the mean of three independent experiments. $^{*}P<0.05$, $^{***}P<0.001$. (**d**) DNA binding of XPC to DNA of FM-treated A375 empty and A375 XPCshRNA cells. Cells were treated with 32 μM FM and 24 h later the trapped in agarose DNA immunostaining assay was performed. Data are the mean of three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$. (**e**) ICL repair of A375 empty and A375 shXPC cells at the indicated time points after FM (32 μM) treatment as determined by the single cell gel electrophoresis assay. Fifty cells were scored for each time point and the experiment was repeated three times. $^{*}P<0.05$, $^{***}P<0.001$. (**f**) Induced apoptosis as analyzed by flow cytometric quantification of the SubG1 fraction in A375 empty and XPCshRNA 96 h after FM treatment with indicated doses. Data are the mean of three independent experiments. Where appropriate in aforementioned experiments, MGMT was inhibited by the addition of 10 μM O^6BG 1 h before alkylating agent addition.

DISCUSSION

Metastatic melanoma has a low curability due to their non-responsiveness to radiation and chemotherapy. One of the drugs used in the therapy of this malignancy is the nitrosourea derivative FM. FM induces O^6-chloroethylguanine DNA lesions that are repaired by MGMT; this adduct is the precursor of the main killing lesion of FM and other chloroethylating anticancer drugs, such as carmustine (BCNU), nimustine (ACNU) and lomustine (CCNU).[7] O^6-chloroethylguanine adducts are unstable, forming ICLs several hours after their induction.[33] Previously, we have shown that p53wt melanoma cells are more resistant to FM than p53mt melanoma cells.[13] We demonstrated that although

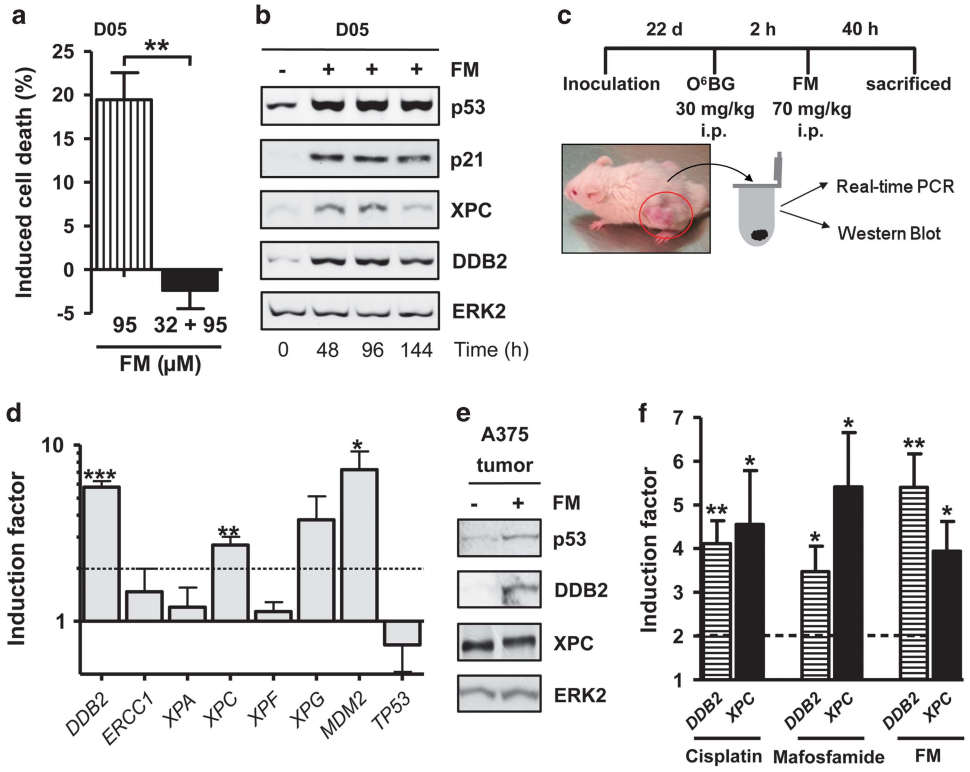

Figure 6. Anticancer drug-induced NER gene expression confers resistance, is long-lasting and occurs in melanoma xenografts *in situ*. (**a**) Analysis of cell death by using annexinV/PI staining. Forty hours after the first FM treatment (32 μM) cells were reseeded. Six hours later a second FM treatment (96 μM) was added. One hundred and twenty hours later cells were analyzed by flow cytometry. (**b**) Monitoring of the duration of FM-induced elevation of p53, XPC, DDB2 and p21 protein levels by immunoblotting in D05 cells. Immunoblot analysis was performed at the indicated time points after FM treatment (64 μM). (**c**) Schematic overview of the experiment. Mice were inoculated with A375 (p53wt) melanoma cells. When tumors reached a suitable size, mice were treated intraperitoneally with O^6BG and 2 h later with FM. Samples for RNA and protein extracts were collected 40 h after FM treatment. (**d**) Analysis of NER gene expression (*DDB2*, *ERCC1*, *XPA*, *XPC*, *XPF* and *XPG*) by real-time PCR in A375 xenograft tumor samples collected 40 h after FM treatment as described in materials and methods. *MDM2* served as positive control, *TP53* as negative control. Data represent the mean of three experiments with randomly cross-paired control and FM samples. $*P<0.05$, $**P<0.01$, $***P<0.001$. (**e**) Immunoblot analysis of p53, DDB2 and XPC protein levels in A375 xenograft tumor samples collected 40 h after FM treatment. ERK2 was used as loading control. Data represent three experiments with randomly cross-paired control and FM samples. (**f**) Expression levels of *DDB2*, *XPC*, *CDKN1A* (p21) and *TP53* after mafosfamide or cisplatin treatment (48 h, 15 μg/ml or 20 μg/ml, respectively) as analyzed by real-time PCR. Where appropriate in aforementioned experiments, MGMT was inhibited by the addition of 10 μM O^6BG 1 h before alkylating agent addition.

p53wt melanoma cells have the ability to upregulate the death receptor FAS/CD95/Apo-1, which is transcriptionally activated by p53,[34] the lack of caspase-8 frequently observed in melanomas partially prevented the activation of apoptosis.[3] This would explain at least in part why melanomas, which exhibit mostly the p53wt status,[1,2] are extremely drug resistant.

Lack of apoptosis activation is, however, only partly responsible for the resistance of melanoma cells to ICLs. Here, we report that an additional factor comes into play, which rests on p53-triggered upregulation of components of NER. Initially, we ascertained the expression of DNA repair genes following FM treatment and observed upregulation of two DNA repair genes, *DDB2* and *XPC*, both on RNA and protein level in p53wt, but not p53mt cells. These genes have previously been shown to be inducible in human fibroblasts and glioma cells by ultraviolet light, carmustine and ninmustine in a p53-dependent manner.[35–38] For malignant melanoma cells this is the first report demonstrating alkylating drug-induced stimulation of NER by upregulation of *DDB2* and *XPC*.

The finding that XPC has a role in the recognition step of ICL repair[17,31,39] prompted us to assay for differences in the repair of ICLs induced by FM in p53wt versus p53mt melanoma cells. ICLs were removed in p53wt but not in p53mt cells, indicating that p53wt melanoma cells have the capacity for repairing ICLs, which are most critical cytotoxic DNA lesions. Consequently, p53wt melanoma cells responded with lower ATM and ATR activation and subsequently with less H2AX phosphorylation compared with p53mt cells. This response occurred concomitant with p53 stabilization, and elevated p53 levels after FM treatment correlated with enhanced phosphorylation of p53 at serine 15, 20 and 37, which are known to decrease p53 interaction with MDM2, and increase its transactivation potency and affinity for DNA.[40,41] This would account for the efficient induction of XPC and DDB2 in p53wt melanoma cells and an increased repair capacity for ICLs.

Cell cycle analysis revealed that p53mt melanoma cells that are unable to repair ICLs are inhibited in S-phase progression and accumulate in G2/M from where they undergo apoptosis. It is important to note that p53 activation and DDB2/XPC upregulation following a single dose of FM is a sustained effect, lasting for up to 144 h, which is clearly different from the well-described immediate-early responses following ultraviolet light resulting in transient gene activation for only several hours.[42] We should also stress that DDB2/XPC upregulation had a dramatic impact on a second dose of the alkylating drug, reducing the killing response significantly. Although it is difficult to assess the expression of repair genes in

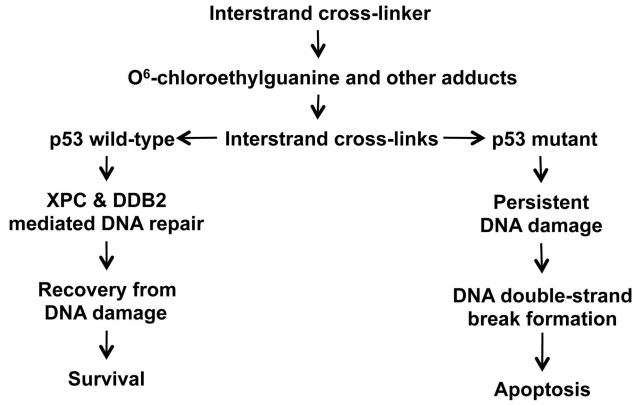

Figure 7. Model for acquired resistance of p53wt cancer cells exposed to interstrand cross-linking chemotherapeutics. Chloroethylnitrosoureas cause the formation of O^6-guanine DNA monoadducts, which spontaneously convert to G-C ICLs. In p53wt cancer cells, this leads to p53 stabilization and transactivation of the genes encoding the DNA repair proteins XPC and DDB2 that facilitate the removal of DNA ICLs. This reduces the level of DNA double-strand breaks (DSBs) formed, attenuates DNA damage signaling upstream of the apoptotic machinery, and thus provokes a resistant phenotype. In p53mt cancer cells, ICLs are not repaired leading to collapse of replication forks, DNA DSB formation and apoptotic cell death executed by the mitochondrial caspase-dependent pathway. The cells, therefore, exhibit a sensitive phenotype.

the tumor in patients, it is reasonable to posit that p53wt melanomas quickly acquire drug resistance following genotoxic therapy because of sustained upregulation of DDB2 and XPC during interstrand cross-linker-based therapy.

It is important to note that the induction of NER and drug resistance was observed with a low dose of FM, which is therapeutically relevant (32 μM that is comparable to plasma levels achieved during intravenous FM therapy, that is, between 5 and 25 μM). Additionally, we demonstrated that the induction of NER by upregulation of DDB2 and XPC in melanoma cells also pertains to other interstrand cross-linkers that are often used in cancer therapy, namely cyclophosphamide (we used the activated form mafosfamide) and cisplatin. In a translation approach, we showed that DDB2 together with p53 is also upregulated in melanoma cells grown as xenograft following a single systemic treatment of mice with a non-toxic, therapeutic dose of FM. It is thus reasonable to conclude that upregulation of NER occurs during tumor therapy, making malignant melanoma cells refractory to subsequently applied doses of these types of drugs. To our knowledge, this is the first demonstration of acquired anticancer drug resistance that is dependent on upregulation of NER/ICL repair.

Collectively, the data revealed that an important mechanism of drug resistance of melanoma cells is p53-driven induction of *DDB2* and *XPC*. This ameliorates processes in which XPC and DDB2 are involved, namely ICL repair and NER. For DNA interstrand cross-linking anticancer drugs, this results in a more efficient removal of ICLs and better survival (see Figure 7). The resistance of p53wt melanoma cells to FM due to ICL repair may further be increased by the possible upregulation of MGMT, as upregulation of MGMT is also controlled by p53.[43,44] We should stress, however, that upregulation of MGMT has not yet been shown to occur with a single dose of FM and other anticancer drugs in melanoma cells *in vitro* and, because of unavailability of tumor biopsies, in a clinical setting. We should also note that upregulation of DDB2 and XPC does not impact on the response of cells to methylating anticancer drugs such as dacarbazine (DTIC) and temozolomide, which are also applied in melanoma therapy.[45] In conclusion, the stimulation of ICL repair reported in this manuscript along with silencing of apoptosis pathways[3,4] provides a mechanistic explanation of why melanoma cells are highly refractory to genotoxic (ICL-based) chemotherapy. As the majority of malignant melanoma are wild-type for p53[1,2], the data bear clinical relevance pointing to the need for strategies of silencing XPC/DDB2 mediated DNA repair.

MATERIALS AND METHODS

Cell culture and drug treatment

All cell lines were grown at 37 °C under a humidified atmosphere with 5% CO_2. The melanoma cell lines D05-Mel, D14-Mel,[13,46,47] A375 and RPMI-7951[29] were described previously. A375 was from American Type Culture Collection, RPMI7951 from The German Cell Culture Depository (DSMZ), and D05-Mel and D14-Mel were from CW Schmidt (Queensland Institute of Medical Research, Queensland, Australia) and checked in the laboratory of Dr Wölfel (Mainz, Germany). D05-Mel and D14-Mel were maintained in RPMI 1640 medium, A375 and RPMI-7951 in Dulbecco's modified Eagle medium. Media contained 10% fetal bovine serum, 10 U/ml penicillin and 10 mg/ml streptomycin. Fotemustine (FM, diethyl1-[3-(2–chloroethyl)-3-nitrosoureido]ethylphosphate; Muphoran, Servier Research International, Neuilly-sur-Seine, France) was freshly prepared for each treatment in 100% ethanol and added to the cell's medium at the indicated concentration. Mafosfamide was provided by ASTA Medica (Frankfurt, Germany) dissolved in water, stored at − 20 °C and added to the cell's medium at the indicated concentration. For cisplatin (CDDP, Platinex, Bristol-Myers Squibb, Munich, Germany) treatment, cells were incubated with the drug for 1 h, followed by a medium change to remove the drug. The MGMT inhibitor O^6-benzylguanine (O^6BG, Sigma-Aldrich, Munich, Germany) was added 1 h before FM and mafosfamide treatment to a final concentration of 10 μM.

Plasmids and stable transfections

A pSuper (OligoEngine, Seattle, WA, USA) construct was generated to express short hairpin RNA targeting XPC mRNA using the previously described sequence (5′-GCTCGAGATGATGAGGAATTG-3′).[48] The pSupershp53 vector was purchased from OligoEngine. Plasmid DNA were transfected using Effectene (Qiagen, Darmstadt, Germany). Transfected cells were selected with 2.2 mg/ml and 8.8 mg/ml puromycin (Invitrogen), for D05 and A375, respectively, until clones formed.

Measurement of apoptosis by flow cytometry

Determination of apoptosis by SubG1 measurement and annexinV/propidium iodide double-staining was performed as described.[3]

Statistics

Statistical analysis was performed using the computer-based program GraphPad Prism. For comparing differences between two populations the unpaired *t*-test was performed.

Preparation of protein extracts

Phosphoprotein extracts. Protein extracts that retained their phosphorylated states were prepared as described.[49]

Whole-cell extracts. Whole-cell protein extracts were prepared as described.[50]

Sub-cellular fractionation. Sub-cellular fractionation of protein extracts was prepared as described.[51]

Cytochrome c extracts. Protein extracts from mitochondria were prepared as described.[52] Protein concentration was determined by Bradford's method.[53] Extracts were stored at − 80 °C.

Immunoblotting

Immunoblotting (western blots) was performed as described.[37] The following antibodies were used: anti-ATM, anti-phospho-Chk1 Ser317 (Bethyl Laboratories, Montgomery, TX, USA); anti-phospho-ATM Ser1981; anti-phospho-ATR Ser428; anti-Caspase-3; anti-Caspase-7, anti-Caspase-8, anti-Caspase-9, anti-Chk1, anti-phospho p53 Ser6/9/15/20/37 (Cell

Signaling Technology, Dallas, TX, USA); anti-ATR, anti-cytochrome c, anti-ERK2, anti-p21, anti-FAS (Santa Cruz Biotechnology); anti-p53 (Dianova, Hamburg, Germany); anti-RPA p34 (Neomarkers, Fremont, CA, USA); anti-Tim44 (BD Pharmingen, Heidelberg, Germany); anti-γH2AX (Upstate, Billerica, MA, USA); anti-XPC (Abcam, Cambridge, UK); anti-mouse and anti-rabbit, horseradish peroxidase-conjugated (Amersham Biosciences, Uppsala, Sweden); anti-goat horseradish peroxidase-conjugated antibody (Santa Cruz). DDB2 antibody was a gift from Vesna Rapic-Otrin, University of Pittsburgh, Cancer Institute.

RNA isolation, reverse transcription polymerase chain reaction and gene expression analysis

RNA from cultured cells was isolated using the Nucleospin RNA II–Kit (Macherey-Nagel, Düren, Germany). One microgram RNA was transcribed into complementary DNA using the Verso cDNA Kit (Thermo scientific, St Leon-Rot, Germany). For complementary DNA amplification by PCR, specific primers targeting the mRNA of interest were used by employing Ampliqon PCR Mastermix (Biomol, Lörrach, Germany). *DDB2*-up:-5′-GGGGCTCCAGCAGTCCTTTTT-3′, *DDB2*-low:-5′-GGGCCACATGCGTCACTTTCTTTT-3′, *ERCC1*-up:-5′-AGGGCCGCCAGCAAGGAAGAAAT-3′, *ERCC1*-low:-5′-TGCAGTCGGCCAGGATACACAT-3′, *GAPDH*-up:-5′-CCCCTCTGGAAAGCTGTGGCGTGAT-3′, *GAPDH*-low:-5′-GGTGGAAGAGTCGGAGTTGCTGTTGA-3′, *XPA*-up:-5′-ATGCGAAGAATGTGGCAAAG-3′, *XPA*-low:-5′-TCACCGCATTGTGAATGATG-3′, *XPB*-up:-5′-ACCGGGAATATGTGGCAATCAAAA-3′, *XPB*-low:-5′-AAACGCCAAGTCTTCCTCCTCCAT-3′, *XPC*-up:-5′-CTTTGATTTCCATGGCGGCTACTC-3′, *XPC*-low:-5′-GCTGCTGCTTTCTTTTCCCTTTTG-3′, *XPD*-up:-5′-GACCCGGGAGGATATTGCTGTGAT-3′, *XPD*-low:-5′-ACCATGAGGCCGTAGTCCGTCTTG-3′, *XPF*-up:-5′-TCCCGTGCTTCTGATTGAGTTTGA-3′, *XPF*-low:-5′-AGGGCTGCTAATTCTGCGATGTTC-3′, *XPG*-up:-5′-AAGAAGCAGCAGCCAGCGAAATAG-3′ and *XPG*-low:-5′-AAACACAGATCTGGCGGTCACGAG-3′.

Caspase activity assays

The activity of caspase-3, -8 and -9 was determined with a colorimetric caspase activity assay from R&D Systems and used according to the manufacturer's instructions.

Immunofluorescence staining of γH2AX

Immunofluorescence was performed as described.[54] γH2AX antibody (Upstate) primary and Alexa488-conjugated secondary antibodies (Invitrogen) were used.

Cross-link repair measured by the single cell electrophoresis assay

The assay was performed as described.[28] The results are expressed as relative tail moment (%) = fluorescence intensity tail (%) x distance head center to tail end comparing ionizing radiation irradiated cells with FM and ionizing radiation treated cells. Fragmentation of genomic DNA by ionizing radiation leads to comet formation, while DNA ICLs reduce the mobility of DNA.

Trapped in agarose DNA immunostaining assay

The assay is based on the fact that genomic DNA imbedded in agarose does not diffuse, while proteins do. Therefore, any protein bound to DNA will also be trapped in the agarose. FM treated and untreated cells were exposed to 1% formaldehyde in phosphate-buffered saline for 10 min at room temperature. Following cross-linking of XPC to the DNA, the assay was performed as described.[55] XPC fluorescence intensity was determined by laser scanning microscopy (LSM710, Carl Zeiss MicroImaging, Jena, Germany). At least 50 nuclei per treatment were analyzed and the experiment was repeated twice.

In vivo experiments and real-time PCR

Immunodeficient mice (NOD.CB17-Prkdcscid/J) were housed in a sterile environment and allowed free access to food and water. A375 cells (8×10^6) were injected in the left and the right flank. Control and FM group comprised two animals each. When tumors reached a suitable size, all animals were injected with O^6-benzylguanine (30 mg/kg i.p., dissolved in 1/3 PEG 400 and 2/3 phosphate-buffered saline). Two hours later vehicle or FM (70 mg/kg i.p., dissolved in $^1/_4$ Ethanol and $^3/_4$ phosphate-buffered saline) were administered. Forty hours later the mice were killed, tumors were isolated, immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}C$. For gene expression analysis by real-time PCR, 20–30 μg of tumor tissue was disintegrated by using a tissue lyser, applying 25 Hz for ~3 min, before purifying the RNA with the Nucleospin RNA II–Kit (Macherey-Nagel). For complementary DNA synthesis with 2 μg of RNA, the Verso cDNA Kit (Thermo scientific) was used. Three control and treated samples of the four available tumors were randomly cross-paired and subjected to real-time PCR analysis, which was carried out in triplicates. The PCR was conducted with the SYBRGreenER *GAPDH*-low qPCR SuperMix and a MyIQ Thermal Cycler (Bio-Rad, Munich, Germany). *GAPDH*-up:-5′-CATGAGAAGTATGACAACAG-3′, *GAPDH*-low:-5′-ATGAGTCCTTCCACGATA-3′, *ACTB*-up:-5′-TGGCATCCACGAAACTACC-3′, *ACTB*-low:-5′-GTGTTGGCGTACAGGTCTT-3′, *DDB2*-up:-5′-TTTAACCCTCTCAATACCA-3′, *DDB2*-low:-5′-CTACTAGCAGACACATCC-3′, *ERCC1*-up:-5′-AGGAAGAAATTTGTGATAC-3′, *ERCC1*-low:-5′-TGTGTAGATCGGAATAAG-3′, *MDM2*-up:-5′-ATCTTGATGCTGGTGTAA-3′, *MDM2*-low:-5′-AGGCTATAATCTTCTGAGTC-3′, *XPA*-up:-5′-AAGGAAGTCCGACAGGAA-3′, *XPA*-low:-5′-ACACGCTGCTTCTTACTG-3′, *XPC*-up:-5′-ACACCTACTACCTCTCAA-3′, *XPC*-low:-5′-TAAATAGCAAATCTCCTTTCC-3′, *XPF*-up:-5′-GGTTGACTTCTTGACTGATA-3′, *XPF*-low:-5′-AGATGAATGCTTCTTGACA-3′, *XPG*-up:-5′-CTGGCTGATGAGTGTGTA-3′ and *XPG*-low:-5′-CAAGTGCTGTCTGATTCC-3′. mRNA levels of GAPDH and ACTB were used for normalization. After the PCR, melting curves of the PCR products were surveyed for amplification of the specific product. For protein analysis by immunoblot, 10–13 mg of the tumor tissue were lysed in ~60 μl per mg tissue of sample buffer (Roti-Load, Roth, Karlsruhe, Germany) by using a tissue lyser (Retsch, Haan, Germany), applying 25 Hz for ~3 min. Samples were centrifuged at room temperature for 10 min at maximum speed. The supernatant was transferred to another tube and boiled for 5 min at 95 °C. The immunoblot was performed as described above.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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