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## The RNA helicase p68 (DDX5) is selectively required for the induction of p53-dependent p21 expression and cell cycle arrest after DNA damage

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### Abstract

The RNA helicase p68 (DDX5) is an established co-activator of the p53 tumour suppressor that itself plays a pivotal role in orchestrating the cellular response to DNA damage. Although several factors influence the biological outcome of p53 activation, the mechanisms governing the choice between cell cycle arrest and apoptosis remain to be elucidated. In the present study we show that, while p68 is critical for p53-mediated transactivation of the cell cycle arrest gene *p21<sup>WAF1/CIP1</sup>*, it is dispensable for induction of several pro-apoptotic genes in response to DNA damage. Moreover, p68 depletion results in a striking inhibition of recruitment of p53 and RNA Pol II to the *p21* promoter but not to the *Bax* or *PUMA* promoters, providing an explanation for the *selective* effect on *p21* induction. Importantly, these findings are mirrored in a novel inducible p68 knockout mouse model in which p68 depletion results in a selective inhibition of p21 induction in several tissues. Moreover, in bone marrow, p68 depletion results in an increased sensitivity to  $\gamma$ -irradiation, consistent with an increased level of apoptosis. These data highlight a novel function of p68 as a modulator of the decision between p53-mediated growth arrest and apoptosis *in vitro* and *in vivo*.

### Keywords

p68 (DDX5) RNA helicase; p53; DNA damage; cell cycle arrest; apoptosis

### Introduction

The p53 tumour suppressor protein is a tightly regulated transcription factor that plays a pivotal role in orchestrating the cellular response to tumorigenic agents such as activated oncogenes or DNA damage (1). The outcome of inducing and activating p53 varies from the

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instigation of a reversible growth arrest that permits repair and recovery, to the exclusion of the cell from the cycling population by senescence (permanent arrest) or its elimination by apoptosis. Several mechanisms/agents have been identified as mediators of the decision between growth arrest and programmed cell death and two alternative models, involving either selective *binding* of p53 to specific pro-arrest or pro-apoptotic promoters or selective *activation (context)* of bound p53 through modifications or recruitment of co-factors at specific promoters, have been proposed to achieve the flexibility of the p53 response (2). Furthermore, it is well established that there is considerable tissue-specific heterogeneity in the p53 response to stress *in vivo*; the combination of factors present in different tissue/cell environments has been likened to a “barcode” that governs p53 activity (3) and is likely to be critical in the choice between cell cycle arrest and apoptosis. However there is relatively little known about the factors underpinning this selectivity *in vivo*.

p68 (DDX5) is a prototypic member of the DEAD box family of RNA helicases that can unwind RNA in an ATP-dependent manner (4). p68 is a multi-functional protein known to be involved in several cellular processes requiring manipulation of RNA structures, including processing of pre-mRNA, rRNA, and miRNA, and has been shown to be aberrantly expressed/modified in a range of cancers (5). Studies from several groups, including our own, have demonstrated that p68 also plays an important, and apparently RNA helicase-independent, role as a co-activator of several transcription factors that are critical to cancer development, including Estrogen Receptor alpha (6), Androgen Receptor (7), and the tumour suppressor p53 (8). p68 is recruited to the promoters of responsive genes under conditions in which these transcription factors are activated, consistent with a role in transcription initiation (9).

We have previously demonstrated that p68 is important for the p53 response to DNA damage (8). In the present study we show that, while p68 is critical for p53-mediated transactivation of the pro-survival cell cycle arrest gene *CDKN1 (p21<sup>WAF1/CIP1</sup>* - herein referred to as *p21*) and for the G1/S cell cycle checkpoint, it is dispensable for the expression of several pro-apoptotic genes, or the induction of apoptosis, in cell lines in response to DNA damage. We also demonstrate that siRNA depletion of p68 results in a striking inhibition of recruitment of p53 and RNA Pol II to the *p21* promoter but not to the *Bax* or *PUMA* promoters, providing a means by which the *selective* inhibition of *p21* induction could be achieved. Finally, using our novel inducible p68 knockout mouse model, we show that p68 is required for p53-dependent induction of p21 in several, although not all, tissues in response to  $\gamma$ -irradiation and does not appear to be required for induction of pro-apoptotic genes. Taken together, our data highlight an important function of p68 in selectively regulating p53-dependent p21 expression and thus potentially influencing the decision between p53-mediated growth arrest and apoptosis *in vitro* and *in vivo*. Importantly, such a role for p68 *in vivo* may provide a mechanism by which elevated p68 in cancer cells could undermine p53-dependent cell death.

## Results

### **p68 is required for the DNA damage-induced G1/S cell cycle checkpoint but has no effect on the induction of apoptosis**

Our previous work demonstrated that p68 is critical for p53-dependent p21 induction in response to DNA damage (8). We therefore considered whether p68 depletion affects DNA damage-induced, p53-dependent, cell cycle arrest. To this end MCF-7 cells (wt p53) were transfected with either non-specific, p68 or p21 siRNAs, treated with the chemotherapeutic drug etoposide to induce cell cycle arrest and analysed by flow cytometry. As Figure 1A shows, in the absence of etoposide, approximately 50% of the cells are in G1, 40% in S and 10% in G2 and the cell cycle profiles are similar regardless of p68 or p21 depletion. In cells

transfected with a non-specific siRNA, etoposide treatment does not cause a significant change in the G1 population but results in a reduction in the proportion of S-phase cells and an increase in the proportion of G2 cells, consistent with the induction of a G2/M arrest under these conditions of DNA damage. In contrast, in cells transfected with the p68 siRNA, while the etoposide-induced G2/M arrest is maintained, and indeed enhanced, the proportion of G1 cells is significantly reduced suggesting that depletion of p68 *selectively* results in a failure of the G1/S checkpoint, allowing cells to progress through S to G2/M. Strikingly this defect is similar to that observed in cells transfected with a p21 siRNA, consistent with the idea that the G1/S checkpoint failure in cells depleted of p68 is due to their failure to induce p21. Flow cytometry profiles are shown in Supplementary Figure S1A.

Analysis of *p68*, *p53* and *p21* mRNA levels (Figure 1B-D) confirmed the p68/p21 knockdown and showed no effect on *p53* mRNA. Strikingly p68 depletion resulted in a significant reduction of both basal and etoposide-induced *p21* mRNA levels while there was no reduction in the expression of *Bax* mRNA in cells treated with p68 or p21 siRNA (Figure 1E); instead there was a minor but reproducible and statistically significant increase in etoposide-induced *Bax* mRNA levels. Corresponding western blots showing p68, p53, p21 and Bax protein levels are shown in Figure 1F. A small increase in p68 mRNA was observed upon etoposide treatment (Figure 1B-note different scales in graphs); however no corresponding increase was seen in p68 protein. These observations suggest that p68 siRNA knockdown does not affect the expression of all p53 target genes. In order to investigate this further we examined the induction of *GADD45*, which is a mediator of the G2/M checkpoint (10) and of several other pro-apoptotic genes. Interestingly, p68 or p21 knockdown resulted in a significant increase in the induction of *GADD45* (consistent with the observed increase in the population of G2 cells) and the pro-apoptotic genes *PUMA*, *Noxa*, *Fas* and *PIG3* (Supplementary Figure S2). Interestingly, in the case of *GADD45*, *PUMA* and *Noxa*, the baseline (uninduced) mRNA levels are also increased upon p68 or p21 knockdown. These findings suggest that p68 is *selectively* required for p21 expression and that, under certain conditions, may indeed protect against apoptosis.

To test this further we used an established U2OS cell line model of apoptosis: treatment of these cells with 1.5 $\mu$ M doxorubicin for 1 h results in a marked induction of apoptosis (measured 72 h later-Figure 2A). Flow cytometry profiles are shown in Supplementary Figure S3A. Analysis of p68, p53 and p21 mRNA and protein levels (Figure 2B-F) confirmed the p68/p21 knockdown and, as in the MCF-7 cells (above), showed that p68 depletion resulted in a significant reduction of both basal and doxorubicin-induced p21 but no difference in Bax induction. Under these conditions p68 siRNA knockdown did not significantly alter the induction of apoptosis by doxorubicin while p21 knockdown resulted in a marked enhancement of apoptosis induction (Figure 2A), consistent with the idea that p21 is protective against apoptosis. (Note: p21 siRNA results in almost complete abrogation of p21 as opposed to the ~50% reduction observed with the p68 siRNA). We observed a minor but statistically significant increase *GADD45*, *PUMA* and *Noxa* induction but no significant effect on the induction of *Fas* or *PIG3* (Supplementary Figure S4). For both cell cycle arrest and apoptosis experiments we obtained similar results using a second p68 siRNA directed against a different region in the p68 gene (Supplementary Figures S1B, S3B). Importantly, treatment of both MCF-7 and U2OS cells with other DNA damaging agents and Nutlin-3 (which induces p53 activity in the absence of DNA damage) gave similar results (Supplementary Figures S5, S6).

Taken together, our data show that p68 is not required for the induction of pro-apoptotic genes or apoptosis by DNA damage and suggest that, at least under certain conditions, it may protect against apoptosis. In contrast, p68 is critical for induction of *p21* and the G1/S

checkpoint, suggesting that p68 levels may play an important role in influencing the decision between cell cycle arrest and apoptosis in response to DNA damage.

In a previous study (8) we observed that p68 siRNA knockdown also inhibited induction of *Fas* and *PIG3* upon etoposide treatment. However, with the siRNA transfection reagents available at that time, the earlier experiments required two sequential transfections to achieve efficient p68 knockdown. These may have resulted in additional transfection-induced stress, which could explain the different results. In the current study (using a single siRNA transfection and different transfection reagents) all DNA damaging agents and all doses/times (including 100 $\mu$ M etoposide for 4 hr as in the previous study) gave similar results (Supplementary Figures S5, S6 and S7), i.e. p68 was required for *p21* but not pro-apoptotic gene induction, indicating that these are not dependent on the DNA damage or dose used.

### **p68 is important for recruitment of p53 and RNA polymerase II to the p21 promoter but has minor effects on recruitment to the Bax or PUMA promoters**

We had previously shown that p68 is recruited to the *p21* promoter in a p53 and DNA damage-dependent manner (8). By chromatin immunoprecipitation, using a p68-specific antibody, we confirmed that p68 is recruited to the *p21* as well as the *Bax* and *PUMA* promoters and that its recruitment to the p21 and PUMA promoters is enhanced by DNA damage (Figure 3A). Since p68 has been shown to be important for recruitment of other transcriptional regulators to certain promoters (11) we reasoned that one mechanism by which p68 could selectively modulate induction of certain p53-target genes in response to DNA damage might be through differentially regulating the recruitment of p53, and/or of other components of the transcriptional machinery, to specific promoters. We therefore compared the recruitment of p53 and RNA polymerase II (RNA Pol II) to the *p21*, *Bax* and *PUMA* promoters in MCF-7 cells, under conditions of p68 siRNA knockdown, in the presence and absence of etoposide treatment (5 $\mu$ M for 16 hr-Figure 3B, C). While p68 depletion had little effect on the baseline recruitment (in the absence of DNA damage) it resulted in a striking reduction (>50%) in the recruitment of both p53 and RNA Pol II to the *p21* promoter following DNA damage. In contrast, there was only a minor reduction in recruitment of p53 or RNA Pol II to the *Bax* and *PUMA* promoters upon p68 knockdown. Similar results were obtained after treatment with 100 $\mu$ M etoposide for 2 hr (Supplementary Figure S8). These findings demonstrate that p68 is crucial for recruitment of p53 and RNA Pol II to the *p21* promoter, but not to the *Bax* and *PUMA* promoters, following genotoxic stress thus providing a way in which p68 may *selectively* regulate p53- and DNA damage-dependent induction of *p21*.

### **p68 is critical for $\gamma$ -irradiation-induced p21 expression in some but not all tissues in a conditional p68 knockout mouse model**

Given our findings in cell lines, we wished to investigate whether p68 also influences the decision between cell cycle arrest and apoptosis *in vivo*. Since constitutive p68 knockout results in embryonic lethality (~E11.5) (12) we generated a conditional, tamoxifen-inducible (*Cre-ERT2*), p68 knockout (p68KO) mouse, to allow us to inducibly knock out p68 expression in adult mice, and investigated the p53 response to  $\gamma$ -irradiation. As shown in Supplementary Figure S9, a significant Cre-dependent knockout of p68 was achieved in a wide range of tissues. Within specific tissues we observed a strong bias in the extent of p68 depletion towards certain sub-populations of cells. For example, in liver there was a complete p68 knockout in hepatocytes but apparently normal p68 expression in Kupffer cells while, in the spleen, p68 was depleted in most haemopoietic cells with a significant proportion of stromal cells retaining p68 expression.

In order to investigate the importance of p68 in the p53 response to DNA damage *in vivo*, p68KO (Cre<sup>+</sup>) and Cre<sup>-</sup> controls were treated with tamoxifen to induce Cre and then subjected to 2 Gy  $\gamma$ -irradiation (a standard dose for patients receiving radiotherapy). Expression of p53, and of p21 and cleaved caspase-3 (as indicators of cell cycle arrest and apoptosis respectively), was examined as described previously (13, 14) in spleen, liver, bone marrow and large intestine (Figures 4, 5), tissues in which the p53 response to irradiation has been established previously (14-17). Tissues from mice that had not been irradiated were similarly stained as controls and showed no significant p53, p21 or caspase-3 staining (Figure 4A, C).

In the irradiated spleen (Figure 4B), p53 staining was observed in the nuclei of cells in both the red pulp and white pulp areas; the proportion of cells staining positive for p53 was similar in the control and p68KO mice, confirming that depletion of p68 does not affect p53 stabilisation. However, there was a striking reduction in the proportion of cells staining for p21 in the p68KO mice, as compared with the control mice (Figure 4A, B) indicating that, as observed in our cell line models (Figures 1, 2), p68 is important for the induction of p21. In contrast, the induction of cleaved caspase-3 was similar in the control and p68KO mice, consistent with our finding that p68 siRNA knockdown does not affect the induction of pro-apoptotic genes in cell lines (Supplementary Figures S1, S3, S5, S6). In the liver, p53 was barely detectable in hepatocytes after irradiation (Figure 4D), as described previously (15, 16). Nevertheless, in the control (Cre) mice there was a significant increase of p21 in hepatocytes following irradiation, indicating activation of p53. High p21 induction in the context of low levels of p53 in the liver has been described previously (17); moreover, we confirmed that irradiation-induced p21 expression in hepatocytes is p53-dependent by demonstrating that there is no p21 induction in p53-null mice (Supplementary Figure S10A). Strikingly, in the p68KO mice, there was no induction of p21 in the hepatocytes (from which p68 was completely depleted) while induction in the Kupffer cells (which retain p68) appeared to be normal. In both control and p68KO mice there was little induction of cleaved caspase-3; this is consistent with earlier findings showing little apoptosis induction in the liver by irradiation (15-17). Our data thus demonstrate that, in the spleen and liver, p68 depletion results in the abrogation of DNA damage-induced p21 expression but has no measurable effect on induction of apoptosis.

The data from bone marrow and large intestine are more complex, suggesting tissue specificity in p68 function. A significant, p68 knockout is obtained; however p68KO results in hypoplastic bone marrow and alterations in tissue organisation in the large intestine, even in the absence of irradiation as shown by histologic examination (Figure 5). Moreover, there was significant expression of p53 and p21 in the p68KO mice in the *absence* of radiation (Figure 5A, C); this however, was not enhanced upon irradiation (Figure 5B, D), suggesting that the p53/p21 expression is due to other, DNA-damage independent, cellular stresses induced by lack of p68 in these tissues. In contrast in the control mice there was no p53 or p21 staining in the non-irradiated mice (Figure 5A, C) with a significant induction upon irradiation (Figure 5B, D). In bone marrow, although p68 knockout resulted in an increase in caspase-3 independently of irradiation (Figure 5A), there was a further enhancement upon irradiation (Figure 5B) suggesting that, in this tissue, lack of p68 favours the induction of apoptosis. No significant caspase-3 staining was observed in large intestine (Figure 5C, D). As expected, p21 and caspase-3 induction in bone marrow was p53-dependent since no expression was observed in p53-null mice (Supplementary Figure S10B).

### The effect of p68 status on radiosensitivity of haemopoietic stem cells

In order to examine the effect of p68 depletion on the sensitivity of bone marrow haemopoietic stem cells to  $\gamma$ -irradiation, suspensions of femoral bone marrow obtained from individual mice were  $\gamma$ -irradiated with doses of 1, 2 or 3 Gy (or sham-irradiated) and

immediately plated in the established clonogenic CFU-A assay (18), which produces mature cells derived from members of the haemopoietic stem cell compartment (short term repopulating haemopoietic stem cells) and is a well-established technique for assessing effects on the survival of irradiated cells. p68 knockout in these cells was confirmed by immunocytochemistry (Supplementary Figure S11). As shown in Figure 6 and Supplementary Table S1, haemopoietic cells from the p68KO mice were significantly more sensitive to  $\gamma$ -irradiation than their control counterparts, consistent with our observation that in the bone marrow of  $\gamma$ -irradiated p68KO mice there was a slightly increased number of cells staining positive for cleaved caspase-3 (Figure 5A, B), as compared with control mice, and would be indicative of increased apoptosis. This idea is also supported by our findings in cell lines, which demonstrated that DNA damage in the context of p68 siRNA knockdown resulted in an enhanced induction of several pro-apoptotic genes (Supplementary Figures 2, 4). It is established that radiosensitivity of haemopoietic cells is p53-dependent (19, 20); therefore our data are consistent with lack of p68 favouring the induction of p53-dependent apoptosis in this context, possibly through loss of protection by p21.

## Discussion

The biological outcome of p53 induction is not only dependent on the particular stress but also on the cell/tissue environment and the many factors that act as co-regulators of p53 function (1, 3). Our data demonstrate that p68 plays an important role in the p53 response to DNA damage both in cell lines and *in vivo*. In cell lines, our siRNA knockdown experiments demonstrate that p68 is crucial for both basal and DNA damage-induced expression of p21. Interestingly, in the absence of DNA damage there was no obvious difference in the cell cycle profiles between control cells and those in which p68 or p21 had been depleted by siRNA. However, in response to etoposide treatment, there was a striking reduction in the G1 population of cells lacking p68 under conditions of DNA damage, suggesting a failure of the G1/S checkpoint. Moreover, the effect of p68 siRNA knockdown on the response to DNA damage was similar to that observed with a p21 siRNA, indicating that the defect observed upon depletion of p68 may be due to the lack of p21 induction.

In contrast, p68 appears to be dispensible for expression of pro-apoptotic genes; indeed p68 siRNA knockdown results in an increased expression of several pro-apoptotic genes. These observations are supported by our data showing that p68 is not required for the induction of apoptosis in a cell line model and that bone marrow cells from p68KO mice are more sensitive to irradiation than their control counterparts. These findings suggest that p68 is required for p53-dependent p21 expression and that, following genotoxic stress, p68 favours cell survival.

Other factors have been found to modulate p53 transactivation to favour the induction of cell cycle arrest. iASPP (21) and YB1 selectively inhibit the ability of p53 to induce expression of pro-apoptotic genes but have no effect on *p21* transactivation. In contrast, Brn-3a (22, 23) and Hzf (24) repress the induction of pro-apoptotic genes while enhancing induction of cell cycle arrest genes. p68 appears to have a somewhat different modulatory effect on p53 function: in our cell line models p68 siRNA results in the abrogation of *p21* expression while the enhancement of induction of pro-apoptotic genes appears to exhibit cell- and/or DNA damage-specific effects, suggesting that p68 might repress the expression of pro-apoptotic genes only in certain contexts.

Our chromatin immunoprecipitation data provide a possible mechanism by which the selective effect of p68 depletion on *p21* induction could be achieved. Although p68 is recruited to the *p21*, *Bax* and *PUMA* promoters, p68 siRNA knockdown results in a striking reduction in the recruitment of p53 and RNA Pol II to the *p21* promoter upon DNA damage,

but has only minor effects on their recruitment to the *Bax* and *PUMA* promoters, suggesting that p68 may be important for the DNA damage response at *selected* p53-dependent promoters. Similarly Hzf and Brn-3a selectively enhance recruitment of p53 to the *p21* promoter (22, 24). One possibility for this selectivity is that promoter-specific sequence or structural elements, partner proteins present in certain cell types or indeed p53 post-translational modifications (25), may influence the requirement for p68 at these promoters and may explain the tissue dependent effects we observe *in vivo*. In this respect the previously reported differences in the mechanism of activation and the composition of transcriptional initiation complexes at the *p21* and pro-apoptotic *Fas* promoters are particularly relevant (26, 27). Interestingly, the *p21* promoter has been shown to be 'pre-loaded' with p53 and paused RNA Pol II in the absence of stress (26), but to be intrinsically inefficient for reinitiation (27). Our data show that p68 depletion had a negligible effect on p53 and RNA Pol II recruitment to the p21 promoter in the absence of DNA damage but a striking effect on their recruitment after DNA damage, suggesting that p68 may play an additional role in the enhancement of transcriptional initiation in response to stress and providing a possible explanation for the observed effect of p68 depletion on cell cycle profiles only in the presence of DNA damage.

The results from our p68 knockout mouse support our model from the cell line data but demonstrate tissue specificity in the requirement of p68 for p53-dependent p21 induction. In the liver and spleen p68 is required for p21 induction in response to irradiation. Although there was little p53 induction in the liver there was a strong p53-dependent p21 induction in the control mice, which was absent in the mice lacking p68, underscoring the requirement for p68 and indicating that only low levels of p53 induction are required to induce significant levels of p21. High p21 induction by relatively low p53 levels has been previously reported (28, 29). The results from the bone marrow and large intestine are more difficult to interpret since organisation/cell populations are altered in these tissues in the p68KO mice and basal levels of p53 and p21 are high in the absence of DNA damage and were not induced upon irradiation, suggesting that lack of p68 induces other, DNA damage-independent, cellular stresses. This is perhaps not surprising given the demonstrated multiple functions of p68 (5). Interestingly, however, although there was a higher level of caspase-3 staining in non-irradiated p68KO mice compared with controls, this was further increased upon irradiation, consistent with our finding that bone marrow cells from these mice are more sensitive to irradiation. Taken together our data indicate that the requirement of p68 for p53-dependent p21 expression is tissue- and context-dependent. Interestingly, the p53 response itself shows tissue and context specificity in terms of the induction of cell cycle arrest or pro-apoptotic genes by irradiation (14, 17, 30).

Our findings thus demonstrate that p68 is a *selective* regulator of the p53 DNA damage response and that it can modulate the decision between cell cycle arrest and apoptosis in a tissue- and context-dependent manner. This is particularly interesting since several studies have shown that p68 is aberrantly expressed and/or modified in a wide range of cancers (7, 31-33). p68 levels and function in cancer tissues could therefore modulate p53-dependent tissue responses to radiotherapy or chemotherapy by determining whether cells survive or die. This in turn could be important in the development of better therapeutic strategies for cancer treatment, or choice of treatment depending on p53 and p68 status, that strike the correct balance between cell cycle arrest/DNA repair and apoptosis to achieve the optimal therapeutic effect and minimise deleterious side effects.

## Materials and Methods

### Antibodies

p68: PAb204 (Millipore, Temecula, USA) and 2907 (polyclonal against the C-terminal 15 residues of p68); p53: DO1 (Santa Cruz Biotechnology, Santa Cruz, USA) and CM1 (polyclonal-recognises human and mouse p53), p21: H164 (Santa Cruz Biotechnology-western blotting) and Ab-5 (Oncogene Science, Cambridge, USA-immunohistochemistry); Bax: N-20 (Santa Cruz Biotechnology); RNA Pol II: Ab817 (Abcam, Cambridge, USA); cleaved caspase-3: 9664 (Cell Signalling, Hitchin, UK); actin: A2066 (Sigma-Aldrich, Poole, UK). Vectastain ABC kits (Vector Labs) were used for immunohistochemistry.

### Transfections/siRNA transfections

siRNA transfections were performed using Lipofectamine<sup>TM</sup> RNAiMax (MCF-7) and Lipofectamine<sup>TM</sup> 2000 (U2OS) (Invitrogen) and 30pmols of p68, p21 or non-silencing siRNAs [Dharmacon (Lafayette, USA)-Supplementary Table S2A].

### RNA extraction and qRT-PCR

Total cell RNA was extracted using the RNeasy kit (Qiagen, Crawley, UK). 1µg of RNA was reverse-transcribed using M-MLV Reverse Transcriptase and random primers (Invitrogen). Quantitative PCR was performed using TaqMan Universal PCR Master Mix and gene expression assays (Applied Biosystems, Foster City, USA) or specifically-designed primers (Eurofins, Ebersberg, Germany)-Supplementary Table S2B-C.  $\beta$ -actin was used as control; fold changes were calculated using the  $\Delta\Delta C_t$  method in Microsoft Excel.

### Chromatin Immunoprecipitation

p53-, p68- or RNA Pol II-bound chromatin was immunoprecipitated using 3µg of DO-1, PAb204 or Ab817 antibody respectively, as described previously (8). qPCR was performed using SYBR Green QuantiTect Mastermix (Qiagen, Crawley, UK). Promoter occupancy was calculated as % of input DNA using the  $\Delta\Delta C_t$  method in Microsoft Excel. Primer sequences and PCR cycles are in Supplementary Table S2D.

### Cell cycle arrest and apoptosis analysis-Flow cytometry

Analysis of cells for cell cycle arrest was performed after BrdU treatment and propidium iodide staining as described previously (34). Apoptotic cells were detected by AnnexinV-FITC (Cambridge Bioscience, Cambridge, UK).

### Induction of p68 knockout in mice and $\gamma$ -irradiation

The gene-targeting strategy (Supplementary Figure S12A), and generation of founder mice was performed by TaconicArtemis (Koln, Germany). Induction of Cre and irradiation were as described in Supplementary Figure S8 and correct Cre-mediated excision of the p68 gene was confirmed by PCR- see Supplementary Figure S12B). All experiments on mice were performed in accordance with Home Office guidelines under project licence PPL60/2841.

### Preparation of mouse tissues for western blotting and immunohistochemistry

For western blotting, tissues were ground into powder under liquid nitrogen and homogenized in lysis buffer containing 50mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 3.575M  $\beta$ -mercaptoethanol, 1mM EDTA and 0.5mM DTT. For immunohistochemistry, tissues were fixed by immersion in 10% buffered formalin and processed to paraffin wax. 4µm sections were stained as described previously (7).



## Irradiation of bone marrow cells and Clonogenic CFU-A assay

Suspensions of femoral bone marrow obtained from individual mice were  $\gamma$ -irradiated (or sham-irradiated) using a Bio International 637 Cesium irradiator. Immediately after irradiation cells were plated in 45-mm Petri dishes containing 2ml  $\alpha$ -MEM supplemented with 20% pre-tested horse serum (Invitrogen/Life Technologies) and conditioned media from the AF1, 19T and L929 cell lines batch tested to produce maximal colony stimulating activities as previously described (18).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

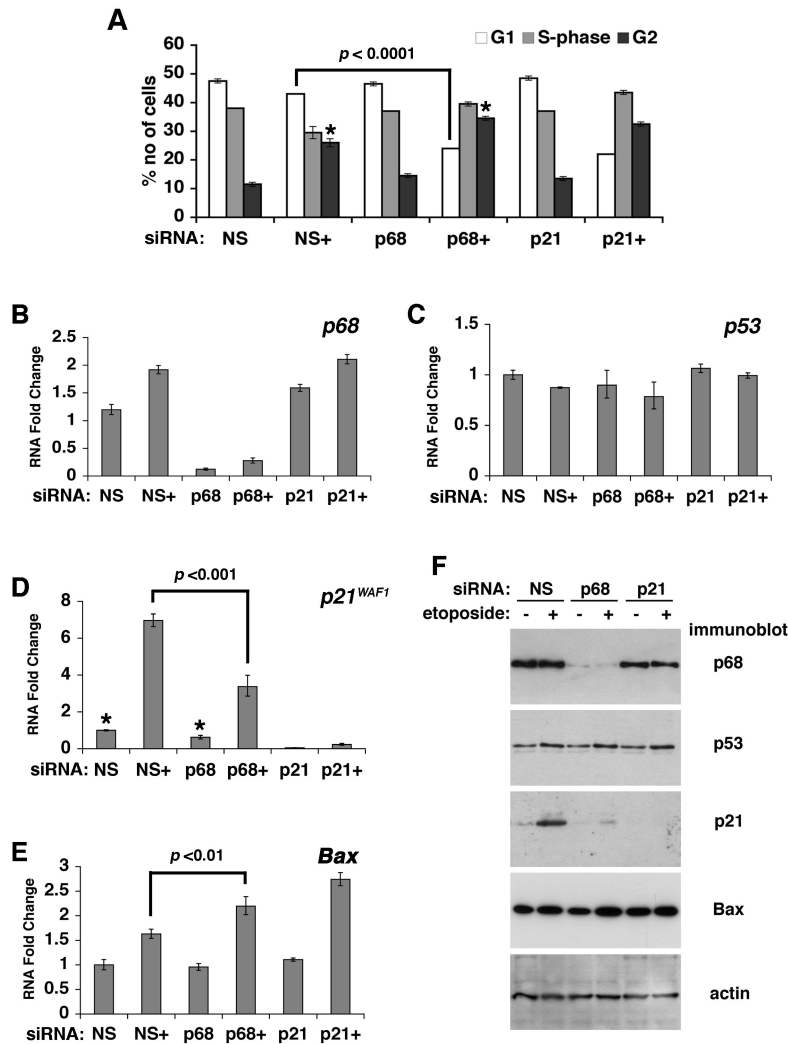
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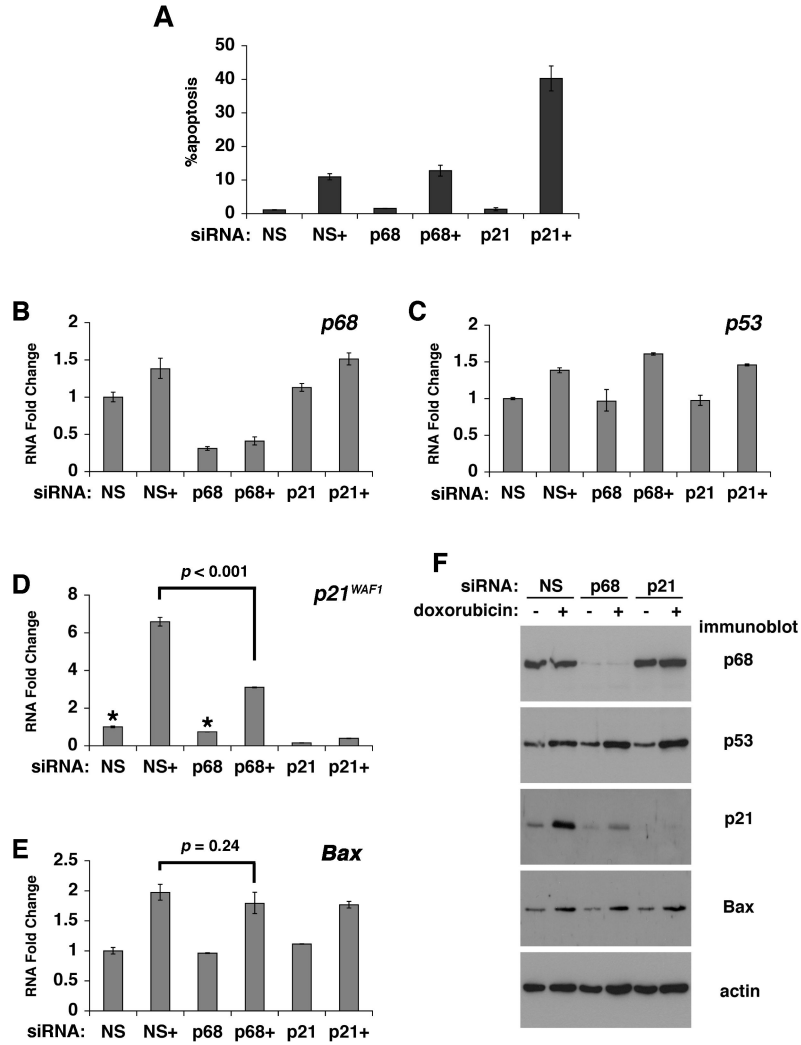
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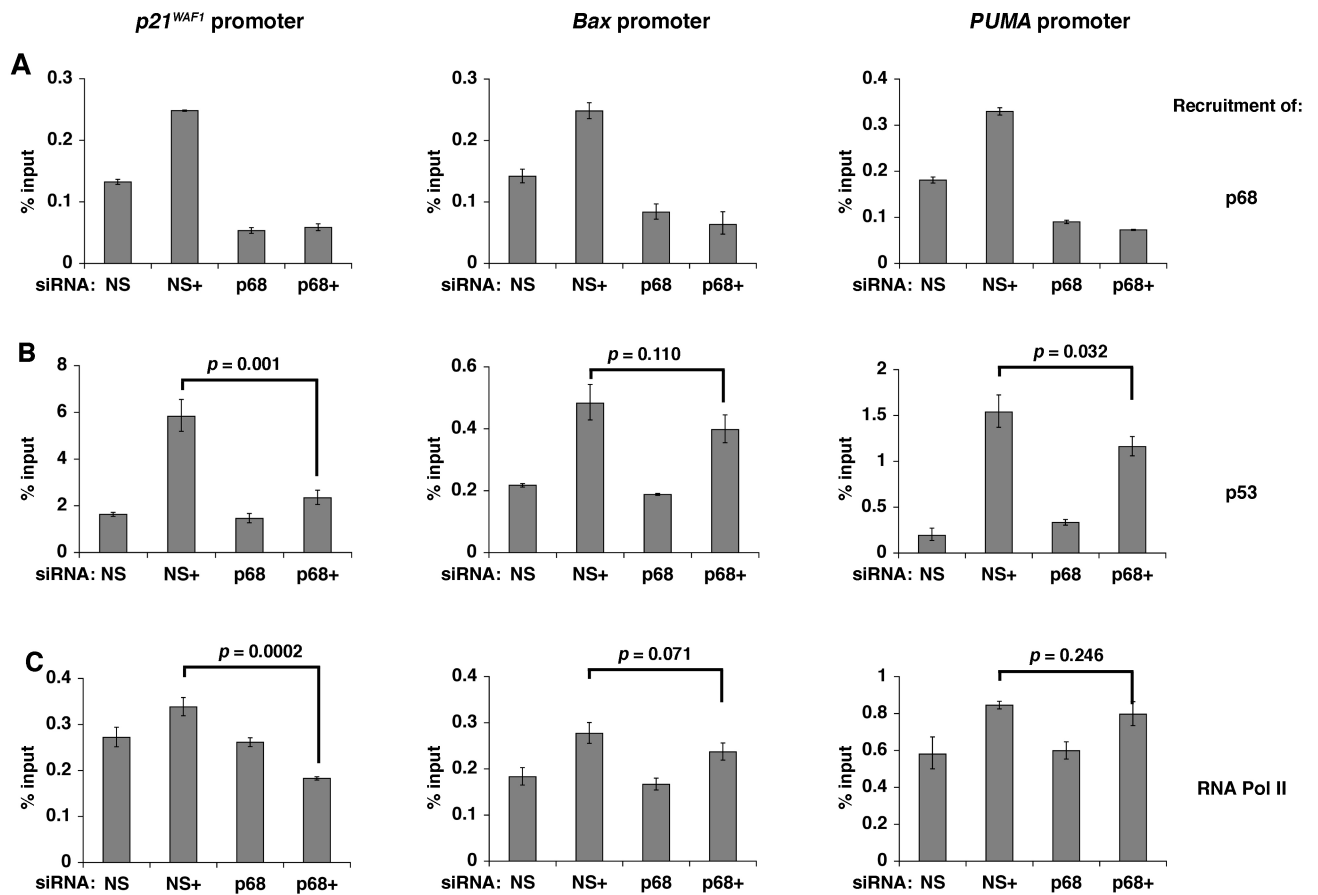
**Figure 1. p68 is critical for DNA-damage induced G1/S cell cycle arrest and p21 expression but is not required for induction of pro-apoptotic genes**

MCF-7 cells were transfected with non-specific (NS), p68 or p21 siRNAs and treated with etoposide prior to analysis by flow cytometry or RNA extraction for qRT-PCR (TaqMan). Untreated cells served as controls. +: cells treated with 5 $\mu$ M etoposide for 16 h. A: Percentage of cells in G1, S and G2 as determined by flow cytometry after BrdU treatment and propidium iodide staining. B-E: Levels of mRNAs as indicated shown relative to respective mRNA cells transfected with a non-specific siRNA and not treated with etoposide. In each case this is shown as 1. F: corresponding western blots.

In all graphs the values shown are the averages from 3 independent experiments  $\pm$  s.e.m. and statistically significant differences are indicated. Where graphs are marked by \* instead of bars for clarity, p values were  $<0.01$ .



**Figure 2. p68 is not required for DNA damage-induced apoptosis or the induction of Bax**  
 U2OS cells were transfected with non-specific (NS), p68 or p21 siRNAs and treated with doxorubicin prior to analysis by flow cytometry or RNA extraction for qRT-PCR (TaqMan). Untreated cells served as controls. +: cells treated with 1.5µM doxorubicin.  
 A: Percentage of apoptotic cells as determined by Annexin V staining and flow cytometry.  
 B-E: Levels of mRNAs as indicated shown relative to respective mRNA cells transfected with a non-specific siRNA and not treated with doxorubicin. In each case this is shown as 1.  
 F: corresponding western blots.  
 In all graphs the values shown are the averages from 3 independent experiments +/- s.e.m. and statistically significant differences ( $p < 0.05$ ) are indicated.

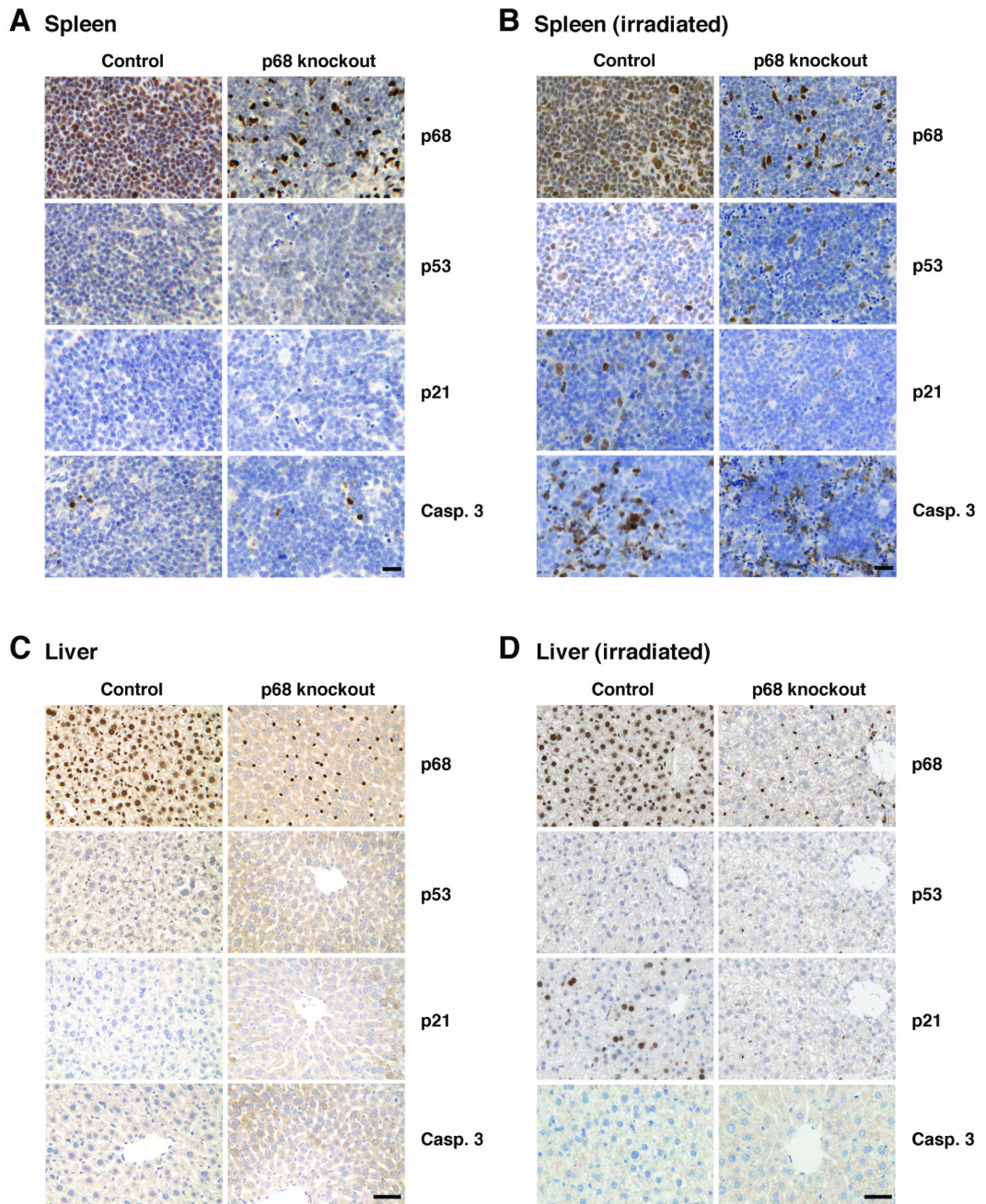


**Figure 3. p68 is required for recruitment of p53 and RNA Pol II to the p21 promoter but not to the Bax and PUMA promoters**

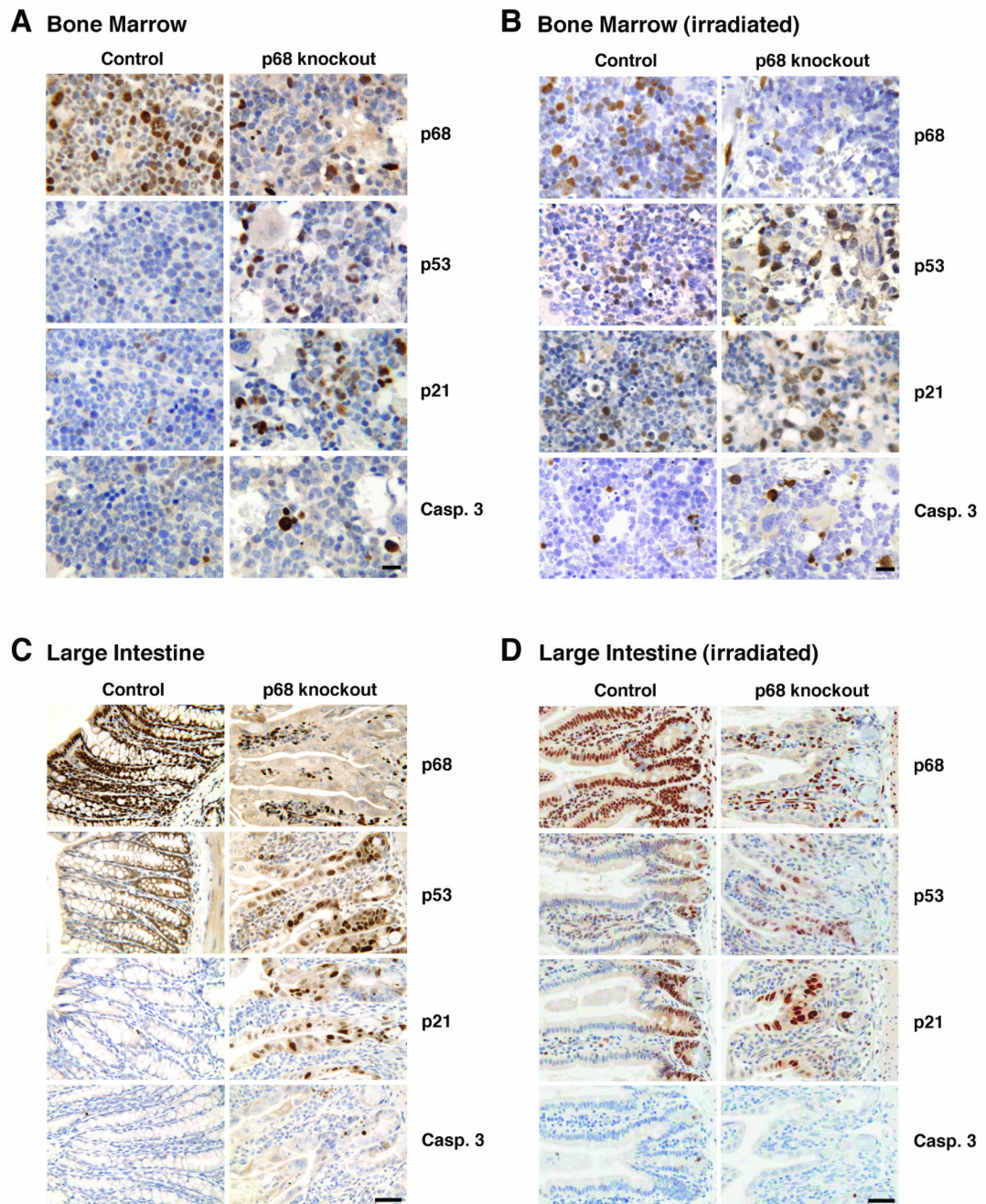
MCF-7 cells were transfected with non-specific (NS) or p68 siRNAs and treated with etoposide prior to chromatin immunoprecipitation and qPCR (SYBR Green). Untreated cells served as controls. +: cells treated with 5 $\mu$ M etoposide for 16 h.

Recruitment of (A) p68, (B) p53 and (C) RNA Pol II to the *p21*, *Bax* and *PUMA* promoters is shown as percentage relative to input DNA.

In all graphs the values shown are the averages from 3 independent experiments  $\pm$  s.e.m. and statistically significant differences ( $p < 0.05$ ) are indicated.



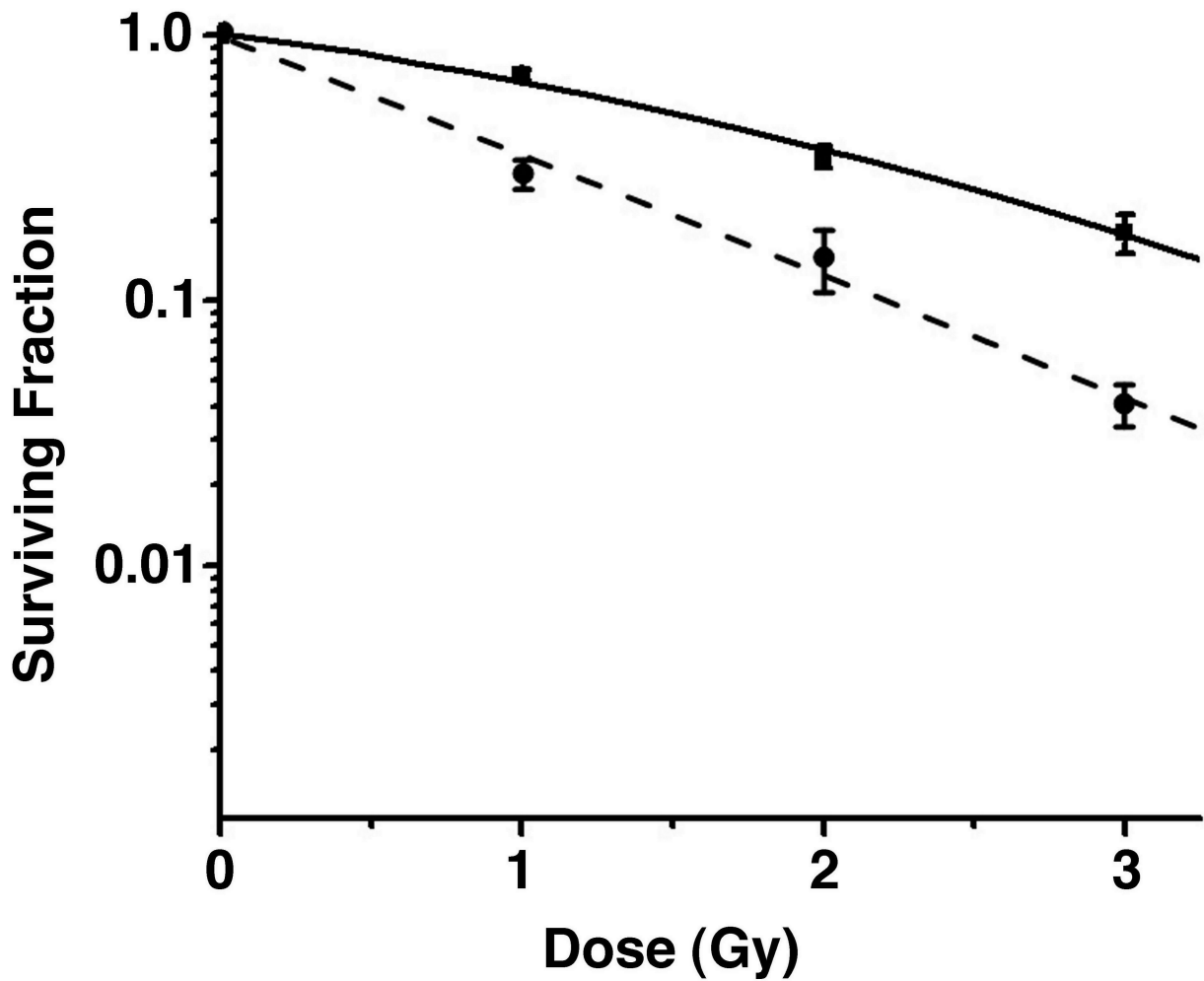
**Figure 4. The p53 DNA damage response in spleen and liver from p68 knockout mice** Immunohistochemistry showing p68, p53, p21 and caspase-3 expression in: spleen (A, B) and liver (C, D) from untreated and  $\gamma$ -irradiated control and p68 knockout mice. Tissues were harvested 2 h after irradiation. Images are representative of 6 control and 6 p68KO mice. Bar= 50 $\mu$ m.



**Figure 5. The p53 DNA damage response in bone marrow and large intestine from p68 knockout mice**

Immunohistochemistry showing p68, p53, p21 and caspase-3 expression in: bone marrow (A, B) and large intestine (C, D) from untreated and  $\gamma$ -irradiated control and p68 knockout mice. Tissues were harvested 2 h after irradiation. Images are representative of 6 control and 6 p68KO mice. Bar= 50 $\mu$ m.





**Figure 6. Bone marrow cells from p68 knockout mice are more sensitive to  $\gamma$ -irradiation than those from control mice**

Survival of multipotential haemopoietic cells (from clonogenic CFU-A assays) after exposure to 1-3 Gy  $\gamma$ -irradiation. Bone marrow cells were obtained from 4 individual control mice (■-solid line) and 8 individual p68KO mice (●-dashed line).