The RNA helicase p68 modulates expression and function of the Δ133 isoform(s) of p53, and is inversely associated with Δ133p53 expression in breast cancer

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Abstract

The RNA helicase p68 is a potent co-activator of p53-dependent transcription in response to DNA damage. Previous independent studies have indicated that p68 and the Δ133p53 isoforms, which modulate the function of full-length p53, are aberrantly expressed in breast cancers. Here we identify a striking inverse association of p68 and Δ133p53 expression in primary breast cancers. Consistent with these findings siRNA depletion of p68 in cell lines results in a p53-dependent increase of Δ133p53 in response to DNA damage, suggesting that increased Δ133p53 expression could result from down-regulation of p68 and provide a potential mechanistic explanation for our observations in breast cancer. Δ133p53α, which has been shown to negatively regulate the function of full-length p53, reciprocally inhibits the ability of p68 to stimulate p53-dependent transcription from the *p21* promoter suggesting that Δ133p53α may be competing with p68 to regulate p53 function. This hypothesis is underscored by our observations that p68 interacts with the C-terminal domain of p53, co-immunoprecipitates Δ133p53α from cell extracts and interacts only with p53 molecules that are able to form tetramers. These data suggest that p68, p53 and Δ133p53α may form part of a complex feedback mechanism to regulate the expression of Δ133p53, with consequent modification of p53-mediated transcription, and may modulate the function of p53 in breast and other cancers that harbour wild type p53.

Keywords

Breast cancer; p68 RNA helicase; p53; Δ133p53; gene expression/regulation

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Introduction

The p53 tumour suppressor protein is a latent transcription factor that governs the expression of a network of genes which regulate cell proliferation, DNA repair, growth arrest, and apoptosis (Vogelstein et al., 2000; Vousden and Lu, 2002). Approximately 50% of all human tumours carry inactivating mutations in the p53 gene while in cancers that have wild type (wt) p53 (approximately 75% of all breast cancers) the function of p53 may be compromised through defects in other components of the p53 network. Breast cancer is the most common cancer in women in the western world, with an increasing incidence. Several clinical and pathological factors are important in determining outcome in breast cancer; however p53 function is thought to be critical, with p53 mis-sense mutations associated with poor prognosis (Bertheau et al., 2008; Olivier et al., 2002; Olivier et al., 2006; Petitjean et al., 2007).

Recently, the p53 gene was shown to express at least nine different isoforms (Bourdon et al., 2005). These arise from a combination of (a) alternative splicing in intron 2/internal translation initiation at codon 40, (b) transcription from an internal promoter in intron 4 (giving rise to Δ133p53 that would result in a protein missing the N-terminal 132 amino acids) and (c) alternative splicing in intron 9, which results in the production of three alternatively spliced C-terminal isoforms (α, β and γ) (Bourdon et al., 2005; Courtois et al., 2002; Yin et al., 2002). p53 isoforms are differentially expressed in a variety of cancers, including breast cancers, melanoma and squamous cell carcinomas of the head and neck (Avery-Kiejda et al., 2008; Boldrup et al., 2007; Bourdon et al., 2005). Therefore the expression of p53 isoforms and their potential to affect the function of full-length p53 may be an important factor in determining the response to therapy and prognosis (Bertheau et al., 2008; Bourdon et al., 2005).

Certain p53 isoforms can modulate the action of full-length p53 (p53α). The Δ133p53α isoform inhibits p53-dependent apoptosis in mammalian cell lines (Bourdon et al., 2005) while the Zebrafish orthologue, Δ113p53, is inducible by full-length wt p53 and antagonises p53-dependent apoptosis, suggesting a novel feedback pathway that can modulate the p53 response to stress (Chen et al., 2009). The functional roles of the Δ133p53β and Δ133p53γ isoforms, in terms of their ability to modulate wt full-length p53 function, are still somewhat unclear with separate studies showing conflicting results, possibly reflecting different methodologies (Avery-Kiejda et al., 2008; Bourdon et al., 2005; Graupner et al., 2009). In a recent study Δ133p53α and p53β were shown to regulate p53-mediated replicative senescence with Δ133p53 repressing and p53β inducing senescence (Fujita et al., 2009).

p68 (Ddx5) is a growth- and developmentally-regulated member (Stevenson et al., 1998) of the DEAD box family of RNA helicases that is important for a wide range of cellular processes including pre-mRNA, rRNA and miRNA processing (Bond et al., 2001; Fukuda et al., 2007; Liu, 2002; Salzman et al., 2007) and transcription (reviewed in (Caretti et al., 2007; Fuller-Pace, 2006). There is now a considerable body of evidence indicating that p68 is an important co-activator of transcription factors that are themselves highly regulated, e.g. Estrogen Receptor α (ERα) (Endoh et al., 1999; Watanabe et al., 2001), MyoD (Caretti et al., 2006), Runx2 (Jensen et al., 2008), Androgen Receptor (AR)(Clark et al., 2008) and p53 (Bates et al., 2005). Our previous work, and that of others, has demonstrated that p68 is aberrantly expressed/modified in several types of cancer (Causevic et al., 2001; Clark et al., 2008; Yang et al., 2005), suggesting that p68 may be important in cancer development/ progression perhaps, in part, by regulating the activity of transcription factors such as ERα, AR, or p53.

p68 interacts with, and is a potent co-activator of, p53 (Bates *et al.*, 2005) while p53 isoforms influence the function of full-length p53 (Bourdon *et al.*, 2005; Chen *et al.*, 2009; Fujita *et al.*, 2009). Therefore we reasoned that p68, through association with and/or modulation of expression/function of specific p53 isoforms, may play an important role in the regulation of p53 function; this idea is interesting since both p68 and the p53 isoforms are aberrantly expressed in cancers (Avery-Kiejda *et al.*, 2008; Boldrup *et al.*, 2007; Bourdon *et al.*, 2005; Causevic *et al.*, 2001; Clark *et al.*, 2008; Yang *et al.*, 2005).

Here we show that p68 expression is inversely associated with Δ133p53 expression in breast cancer (i.e. tumours that express low levels of p68 are more likely to express Δ133p53 isoforms) and that p68 siRNA knockdown results in a striking increase in Δ133p53 expression in cell lines in response to DNA damage, consistent with the concept that p68 may repress expression of Δ133p53. The increase in Δ133p53 upon p68 knockdown is dependent on the presence of functional p53, suggesting that p68 modulates the ability of p53 to regulate Δ133p53 expression. Moreover, we show that Δ133p53 inhibits the ability of p68 to co-activate p53-dependent transcription from the p21 promoter indicating that, while p68 negatively regulates Δ133p53 expression, Δ133p53 itself modulates p53 co-activation by p68. Finally, we demonstrate that p68 interacts with the C-terminal domain of p53, co-immunoprecipitates Δ133p53α from cell extracts and interacts only with p53 molecules that are able to form tetramers, implying that p68 may be competing with Δ133p53α to regulate p53 function. These findings suggest that p68, p53 and Δ133p53α may form part of a complex feedback mechanism to regulate the expression of Δ133p53 and its consequent modulation of p53-mediated transcription, and may have implications for the function of p53 in cancers that harbour wild type p53.

Results

p68 expression is inversely associated with Δ133p53 expression in breast cancers

In order to examine potential associations between p68 and p53 isoform expression in breast cancer we examined expression of p68 mRNA and protein, as well as p53 isoform mRNA, from a panel of 147 primary breast cancers. Considerable variation in the levels of p68 mRNA (determined by TaqMan qRT-PCR) and protein, scored using the established 'Quick Score' method (Detre *et al.*, 1995) (Supplementary Figure 1) was observed. The presence or absence of specific p53 isoform mRNAs was determined by RT-PCR using primers that distinguish between p53 molecules that contain the transactivation domain and Δ133p53 species, as well as between α, β and γ, as previously described (Bourdon *et al.*, 2005) (Supplementary Figure 2). Due to the p53 gene structure and the limitations of TaqMan qPCR, it was not possible to obtain quantitative measurements that distinguish between the different p53 isoforms. p68 mRNA expression was found to be inversely associated with the expression of all Δ133p53 isoform mRNAs (Table 1A). For p68 protein there was also an inverse association between detectable p68 protein (score>0) and expression of Δ133p53β mRNA, although the association with expression of Δ133p53α and Δ133p53γ mRNA did not reach statistical significance (Table 1B). p53 isoform protein expression could not be examined by immunohistochemistry due to the lack of isoform-specific antibodies.

p68 siRNA knockdown results in induction of Δ133p53 expression in a p53-dependent manner

Given that p68 expression is inversely associated with Δ133p53 expression in breast cancers and Δ133p53 is itself a p53 target gene (Chen *et al.*, 2009), we considered whether p68 could regulate expression of Δ133p53. While our previous work has demonstrated that p68 stimulates expression of p53 target genes that are normally induced by activated p53 (Bates *et al.*, 2005), we reasoned that, in a different transcriptional context (i.e. the p53 intron 4

promoter), p68 might act to repress p53 transcriptional activity, having previously shown that p68 can, in some contexts, repress transcription (Wilson *et al.*, 2004). We tested whether overexpression, or siRNA knockdown, of p68 had any effect on Δ133p53 expression in the established breast adenocarcinoma cell line model MCF-7 (wt p53) in the presence, or absence, of the DNA damaging agent etoposide, which induces p53 activity. We found that overexpression of p68 had no effect on expression of Δ133p53 RNA (data not shown); however p68 knockdown by siRNA (Figure 1A) resulted in a significant increase in Δ133p53 RNA (Figure 1B) in etoposide-treated cells. Similar results were obtained with a second p68 siRNA (Supplementary Figure 3) and with doxorubicin, another DNA damaging agent (Supplementary Figure 4). As shown previously (Bates *et al.*, 2005), p68 knockdown had no effect on overall p53 levels i.e. p53 species including the transactivation (TA) domain (Figure 1C) but resulted in a failure to induce p21 (Figure 1D). Western blots (Figure 1E) confirmed knockdown of p68, induction of p53 protein by etoposide and inhibition of p21 induction. Consistent with the observed increase in Δ133p53 mRNA upon p68 knockdown, a longer exposure of the p53 western blot performed using the p53-specific polyclonal antibody CM-1 (Bourdon *et al.*, 2005; Fujita *et al.*, 2009) (Figure 1E) showed a modest increase in Δ133p53α protein in response to etoposide in cells treated with the p68siRNA. There was also an increase in a smaller p53 species (Figure 1E*); however, at present it is unclear whether this corresponds to Δ133p53β or Δ133p53γ.

To determine whether the effect of p68 knockdown on Δ133p53 expression was p53-dependent we tested whether Δ133p53 induction was also observed in HCT116 p53−/− (p53 null) cells; these cells are null for full-length p53 (Bunz *et al.*, 1998) but still express Δ133p53 isoforms (Murray-Zmijewski *et al.*, 2006) with wt HCT116 p53 +/+ as controls. As shown in Figure 2A, the induction of Δ133p53 expression by p68 knockdown was only observed in the HCT116 p53 +/+ cells suggesting that it is p53 dependent. (Corresponding western blots are shown in Supplementary Figure 5.) Similarly, there was no induction of Δ133p53α in HCT116 p53 +/+ cells upon simultaneous knockdown of both p68 and p53 (using an siRNA that knocks down full-length p53 and all isoforms containing the 5′ end of the p53 gene (TAp53) but spares Δ133p53 species), confirming the requirement for p53 (Supplementary Figure 6). Moreover, we did not observe any effect of p68 knockdown on Δ133p53 expression in T47D cells, which harbour mutant p53 (Figure 2B), indicating that this effect is dependent on transcriptionally functional p53 and implying that p68 represses the ability of full-length p53 to induce expression of Δ133p53 in response to DNA damage. This is consistent with recent reports showing that Δ113p53 (the Zebrafish Δ133p53 ortholog) is a p53 target gene and is induced by DNA damage (Chen *et al.*, 2009; Marcel *et al.* 2010). Interestingly, in our study, etoposide treatment did not significantly alter Δ133p53 RNA expression in untransfected MCF-7 or cells transfected with a non-specific siRNA (Figure 1B): similar results were obtained with U2OS cells (data not shown). In contrast, in HCT116 cells, etoposide treatment resulted in an increase in Δ133p53 RNA (Figure 2A). However, in all cases, p68 knockdown resulted in a striking increase in Δ133p53 levels upon etoposide treatment indicating that, although there appears to be some cell line dependence in the induction of Δ133p53 RNA by DNA damage itself, p68 knockdown in combination with DNA damage results in a marked induction of Δ133p53 expression in all cell lines tested.

Repression of Δ133p53 expression by p68 is not due to changes in transcription from the p53 intron 4 (Δ133p53) promoter

Given that p68 can repress transcription in a promoter-specific manner (Wilson *et al.*, 2004) and Δ133p53 isoforms are generated through utilisation of the internal promoter in intron 4 of p53 (Bourdon *et al.*, 2005), we speculated that p68 may act to repress transcription from this promoter. Since transcription from the intron 4 promoter is regulated by p53 itself (Chen

et al., 2009; Marcel *et al.*, 2010), we generated a construct in which the p53 intron 4 promoter was fused to a luciferase reporter and measured p53-mediated transcriptional activity from this promoter, in the presence or absence of p68, by luciferase activity assays. H1299 (p53 null) cells in which p68 was knocked down by siRNA were co-transfected with a fixed amount of the intron 4-luciferase plasmid, together with increasing concentrations of a p53 expressing plasmid, and luciferase activity was measured. Cells transfected with a non-silencing siRNA were used as controls and the experiment was performed in the presence and absence of etoposide since the increase in Δ133p53 mRNA upon p68 knockdown was most striking in etoposide treated cells. As shown in Figure 3, p68 depletion did not result in an increase in the ability of p53 to stimulate transcription from the intron 4 promoter regardless of whether the cells were treated with etoposide or not. Instead, in both cases we observed a small reduction of transcriptional activity in the p68 knockdown cells, possibly as a result of pleiotropic effects of the p68 knockdown. Nevertheless, these findings suggest that the increase in Δ133p53 expression resulting from p68 knockdown is not due to p68 repressing transcription from the intron 4 promoter but may instead reflect effects on transcriptional elongation or RNA processing.

Consistent with these findings we did not observe any changes in recruitment of p53 to this promoter by chromatin immunoprecipitation upon p68 knockdown in MCF-7 cells (Supplementary Figure 7) although there was a marked increase in recruitment of p53 to this promoter in response to etoposide treatment, as seen for other p53-responsive promoters.

Δ133p53α inhibits p68 co-activation of p53-dependent p21 induction

p68 is known to stimulate p53-dependent *p21* transcription (Bates *et al.*, 2005) while Δ133p53α has been reported to repress the expression of p21 (Fujita *et al.*, 2009). These findings suggest that, in some contexts, p68 and Δ133p53 may have antagonistic effects on p53-dependent induction of *p21* expression. To investigate this possibility we examined the effect of p68 and Δ133p53α on p53-dependent transcription of a *p21* promoter/luciferase reporter construct. p53 null H1299 cells were transfected with fixed amounts of plasmids expressing Δ133p53α and p68 and increasing amounts of a p53-expressing plasmid, together with a *Renilla*-luciferase reporter as an internal transfection efficiency control. The experiment was performed in the absence and presence of etoposide to determine whether the effects of p68 and Δ133p53α are also influenced by DNA damage. p68 potently stimulated the ability of p53 to induce transcription from the p21 promoter both in the absence and presence of etoposide (Figure 4A, B). Δ133p53α exhibited a modest inhibitory effect on p53-dependent transcription from the *p21* promoter in the absence of etoposide (Figure 4A) and had no obvious effect in the presence of etoposide (Figure 4B). In both cases, however, Δ133p53α inhibited the ability of p68 to co-activate p53-dependent transcription indicating that, at least in the context of transcription from the *p21* promoter, Δ133p53α may be competing with p68 for regulating p53 function. Western blots were performed to confirm expression of the transfected p68, p53 and Δ133p53α proteins (Figure 4C, D). Similarly Δ133p53 inhibited p68 coactivation of p53-dependent transcription of *endogenous* p21 in H1299 cells both in the presence and absence of etoposide (Supplementary Figure 8).

p68 interacts with the C-terminal domain of p53 and co-immunoprecipitates with the Δ133p53α isoform

To explore possible mechanisms by which p68 and Δ133p53α might compete to regulate p53 function we performed a series of GST pull-down experiments to identify the regions/ domains in p53 that interact with p68. We also examined which specific p53 isoforms interact with p68. As Figure 5A shows p68 interacts with full-length p53 (p53α) and isoforms that include the C-terminal region of p53 (See Figure 5D), but does not interact

with the β or γ isoforms. This finding was confirmed by co-immunoprecipitation experiments between endogenous p68 and Δ133p53α, p53β or p53γ from H1299 cell lines stably expressing these isoforms (Figure 5C). Co-immunoprecipitation of endogenous p68 and p53α from U2OS cells served as a control (Bates *et al.*, 2005); it should be noted that in all cases the p53 immunoprecipitating antibody was DO-12, which interacts with the DNA binding domain (Bourdon *et al.*, 2005) and therefore immunoprecipitates all isoforms.

To further characterise the interaction between p68 and the C-terminal domain of p53 we performed GST pull-downs between p68 and several p53 mutants that harbour mutations in, or are lacking parts of, the C-terminal domain. As shown in Figure 5B p68 does not interact with p53 species lacking the C-terminal 30 amino acids. Moreover while mutations of the six C-terminal lysines, which are modified by ubiquitylation, sumoylation, acetylation and methylation (Carter and Vousden, 2009) have no effect on interaction with p68, mutation of isoleucine 332, which has been shown to be important for p53 tetramerisation (Mateu and Fersht, 1998), abolishes interaction with p68 suggesting that p68 interacts with tetrameric p53.

Discussion

Δ133p53 has been shown to modulate both the p53 response to DNA damage, by inhibiting p53-dependent apoptosis (Bourdon *et al.*, 2005; Chen *et al.*, 2009) and p53-dependent replicative senescence (Fujita *et al.*, 2009), and has been implicated in invasion/metastasis of breast cancer cells (Mehta *et al.*, 2007). Therefore deregulation of Δ133p53 expression in the 75% of breast cancers that express wt p53 (Olivier *et al.*, 2002; Olivier *et al.*, 2006) could have important implications for p53 function in tumour development and for response to chemotherapy, particularly since Δ133p53 itself has been reported to be a p53 target gene induced by DNA damage (Chen *et al.*, 2009). In the present study we show that p68 expression is inversely associated with Δ133p53 expression in a panel of 147 breast cancers suggesting that p68 may play a role in regulating Δ133p53 expression in these tumours. In this respect, our finding that depletion of p68 is associated with a striking increase in Δ133p53 mRNA levels in cells treated with the DNA damaging agent etoposide suggests that p68 can repress Δ133p53 expression under conditions of DNA damage. Our data therefore imply that p68 may be part of a feedback mechanism that modulates the induction of Δ133p53 expression after DNA damage thus preventing inhibition of p53 function; this could be critical in tumours expressing wt p53 that are treated with chemotherapy. Moreover, given that activation of DNA damage pathways is a feature of early cancers (Halazonetis *et al.*, 2008), the regulation of Δ133p53 expression by p68 would also have important implications for tumour development.

Δ133p53 is generated by transcription from an internal promoter in intron 4 of the p53 gene (Bourdon *et al.*, 2005). Although, in the context of other p53-responsive promoters (e.g. *p21*), p68 acts to co-activate p53 it was possible that p68 could act as a co-repressor in the context of the intron 4 promoter; p68 has previously been shown to repress transcription from some promoters (Wilson *et al.*, 2004). However our data indicate that p68 does not inhibit the ability of p53 to stimulate transcription from the intron 4 promoter in a luciferase reporter system, nor affect p53 recruitment to this promoter in chromatin immunoprecipitation assays in wt p53 MCF-7 cells. These observations suggest that p68 is not acting as a transcriptional corepressor at the level of transcription initiation, although we cannot exclude the possibility that our luciferase reporter construct is missing elements required for p68 activity. It will be interesting to determine whether p68 is instead acting at post-transcription initiation events, possibly transcriptional elongation or RNA processing. p68 has been reported to be important for pre-mRNA splicing (Liu, 2002) and alternative splicing of certain model genes (Clark *et al.*, 2008; Guil *et al.*, 2003). Our finding that the

increase in Δ133p53 upon siRNA knockdown of p68 is dependent on p53 is particularly interesting in the light of recent reports showing that p53 plays a role in transcriptional elongation (Balakrishnan and Gross, 2008) and that several RNA binding proteins involved in post-transcriptional regulation of p53 affect p53 activity (Zhang and Chen, 2008).

In this study we were unable to determine specifically which Δ133p53 RNA isoforms are induced by p68 siRNA knockdown (i.e. whether Δ133p53α, β or γ), since it was not possible to perform quantitative RT-PCR with specific primers that would distinguish between these isoforms. As new reagents become available for longer-range quantitative PCR or for the detection of each Δ133p53 isoform protein it will be interesting to determine whether loss of p68 is associated with an increase of specific Δ133p53 isoforms. In our study, the association between p68 protein and Δ133p53 expression (in this case only with Δ133p53β) was seen at low levels of p68 protein expression in the breast cancers. This would suggest that the effect is observed in the absence (or with very low levels) of p68 protein and would be consistent with our findings from the cell lines, i.e. that p68 siRNA knockdown has a striking effect on Δ133p53 expression while p68 overexpression has no effect.

Our finding that Δ133p53α inhibits the ability of p68 to stimulate p53-dependent transcription from the *p21* promoter raises the possibility that p68 and Δ133p53α may be competing for interaction with p53 or other factors at the *p21* promoter. The Zebrafish homologue, Δ113p53α, has been shown to form hetero-tetramers with full length p53 (Chen *et al.*, 2009) while our data indicate that p68 interacts with tetrameric p53. Therefore p68 may inhibit the ability of Δ133p53α to form hetero-tetramers with full-length p53 thus favouring formation of homo-tetramers of full length, functional p53. This, together with our data suggesting that p68 represses Δ133p53 expression and reports that Δ133p53 is induced by p53 (Chen *et al.*, 2009) imply that p68, p53 and Δ133p53α form part of a complex feedback mechanism to regulate the expression of Δ133p53 and its consequent modulation of p53 activity, as illustrated in Figure 6. Such feedback loops are characteristic of the p53 pathway (Harris and Levine, 2005).

In summary, our data show that p68 expression is inversely associated with expression of Δ133p53 in breast cancer and that p68 depletion in cells results in a striking increase in Δ133p53 upon DNA damage. Moreover, Δ133p53α appears to inhibit p68 co-activation of p53-dependent transcription from the *p21* promoter, suggesting that p68 and Δ133p53α compete for interaction with and/or modulation of p53 function. Δ133p53α has been shown in several studies to inhibit p53 function and the p53 DNA damage response (Bourdon *et al.*, 2005; Chen *et al.*, 2009; Fujita *et al.*, 2009); therefore, our results support the idea that p68 may not only be important as a co-activator of full-length p53 and a regulator of the p53 DNA damage response (Bates *et al.*, 2005) but may also be modulating p53 function at another level, namely by regulating Δ133p53 expression and, consequently, its effect on p53 activity. Thus the ability of p68 to modulate Δ133p53 expression could have important implications for the majority of breast cancers that harbour wt p53.

Materials and Methods

Cell lines

Cell lines used in this study include wt p53 MCF-7 breast adenocarcinoma and U2OS osteosarcoma, wt p53 (+/+) and p53 null (−/−) HCT116 colon carcinoma cells, p53 mutant T47D breast ductal carcinoma and p53 null H1299 lung carcinoma cells. HCT116 cells were maintained in McCoys medium; other lines were maintained in Dulbecco's Modified Eagles Medium (DMEM); all media were supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100μg/ml streptomycin and 100U/ml penicillin (Invitrogen, Paisley, UK) in

5% CO_2 at 37°C. H1299 cells stably expressing a specific p53 isoform were maintained in DMEM (as above) and 0.5mg/ml G418 (Gibco).

Plasmids

For luciferase assays plasmids expressing p68, full-length p53 and Δ133p53α were in pcDNA3.1(+) (Invitrogen). Luciferase reporter plasmids included the *p21* promoter (el-Deiry *et al.*, 1993) and the p53 intron 4 promoter [bp 11523-13076 (Genbank Accession Number X54156)] cloned in pGL3 (Promega). The *Renilla*-luciferase reporter plasmid was pRL-CMV (Promega). For GST pull-downs, plasmids expressing p53 isoforms were in pSI (Promega) and p53 deletion/mutation plasmids were in pcDNA3.1(+).

Antibodies

The following primary antibodies were used: p68-PAb204 (mouse monoclonal- Millipore), 2907 (rabbit polyclonal) (Bates *et al.*, 2005); p53-DO1 (mouse monoclonal- Santa Cruz Biotechnology), DO-12 (mouse monoclonal) (Bourdon *et al.*, 2005) and CM-1 (rabbit polyclonal) (Bourdon *et al.*, 2005); p21-C19 (rabbit polyclonal- Santa Cruz Biotechnology); actin- A2066 (rabbit polyclonal- Sigma).

RNA extraction

Total RNA was extracted from cells using the RNeasy kit (Qiagen) and from snap-frozen breast cancer samples using the EZ1 RNA Universal Tissue Kit (Qiagen) according to the manufacturer's protocol. 1μg of RNA was reverse-transcribed using M-MLV Reverse Transcriptase and random primers (Invitrogen).

PCR/qPCR

PCR to detect the presence/absence of p53 isoforms in breast cancers was performed as described previously (Bourdon *et al.*, 2005). Quantitative PCR (qPCR) was performed using Applied Biosystems gene expression assays or specifically designed primer/probe sets as described in Supplementary Table 1. TBP was used as a control and fold changes and differences between samples were calculated using the ΔΔCt method in Microsoft Excel.

Immunohistochemistry and scoring of p68 staining in breast cancers

Tissue microarrays were prepared with six 0.6mm cores from each cancer and 4μm sections were used for staining. Antigen retrieval and staining for p68 (with PAb204) was performed as described previously (Clark *et al.*, 2008). Staining was scored using the "QuickScore method" (Detre *et al.*, 1995), calculated by multiplying the percentage of cells staining score (1-6) by the intensity score (0-3) to give a maximum value of 18 (see Supplementary Figure 1). This study fulfilled the Tayside Ethics Review Board's guidelines for the use of stored tissues samples.

Statistical analysis

Association tests for statistical significance between ordinal variables were performed by 2-sided Fisher's exact tests (2FET) and tests for continuous variables used the appropriate choice of 2-sample t-test and Mann-Whitney U-test. All tests were carried out using Mosaic™, an internally developed statistical analysis system (implemented in Matlab, Mathworks Inc.; Version 6.5 Release 13) and verified with Minitab (ver 15.1.0.0, Minitab Inc.). For all analyses a *p* value of < 0.05 was taken to indicate statistical significance.

siRNA transfections

siRNA reverse transfections were performed using Lipofectamine™ RNAiMax (Invitrogen) and siRNA oligonucleotides (30pmols of p68 and 100pmols of TAp53- Dharmacon) (Supplementary Table 3).

Luciferase reporter assays

H1299 cells were transfected using Fugene® 6 (Roche) with plasmids encoding p68, Δ133p53 and full-length p53, all under control of a CMV promoter, and appropriate promoters/luciferase reporters. A *Renilla*-luciferase control plasmid was co-transfected as a control for transfection efficiency. Luciferase activity was measured 24 hours after transfection using the Dual-Glo luciferase kit (Promega).

Nuclear extracts and Co-Immunoprecipitation (Co-IP)

Nuclear extract preparation and immunoprecipitations [using PAb204 (p68) and DO-12 (all p53 isoforms)] were performed as previously described (Bates *et al.*, 2005; Bourdon *et al.*, 2005).

GST-pulldowns

Glutathione *S*-transferase-tagged proteins were expressed and purified as previously described (Bates, 2005). p53 isoforms and p53 deletions/mutations were *in vitro*-translated/^{35}S-labelled using the TNT transcription/translation kit (Promega). GST-pulldowns were performed as described by Hsieh *et al.* (Hsieh *et al.*, 1999).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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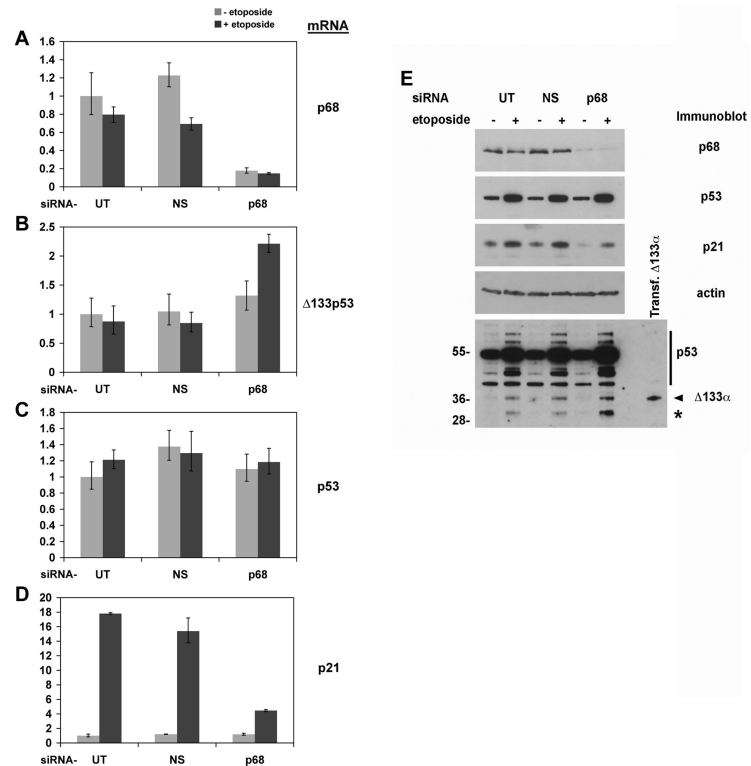
Figure 1. Short interfering RNA (siRNA)-mediated knockdown of p68 results in a striking increase of Δ133p53 mRNA expression in response to DNA damage

MCF-7 cells were transfected with an siRNA targeted against p68 or a non-silencing control (NS). Untransfected (UT) cells served as an additional control. 48hr after siRNA transfection, cells were treated with 100μM of the DNA damaging agent etoposide for 4 hr. qRT-PCR (TaqMan) was performed on RNA extracted from these cells to measure mRNA expression of **A**: p68, **B**: Δ133p53, **C**: p53 and **D**: p21. Expression was normalised to expression of TBP and graphs plotted as fold change from untransfected cells (UT) that had not been treated with etoposide. The average values from 3 independent experiments are shown ± standard error of the mean (s.e.m). **E**; Corresponding western blots showing expression of p68, p53, p21 and Δ133p53 from the p68 siRNA knockdown experiments. A longer exposure of the western blot with the p53-specific antibody (lower panel) to detect p53 isoforms with H1299 transfected with Δ133p53α for comparison. *This protein species is as yet unidentified.

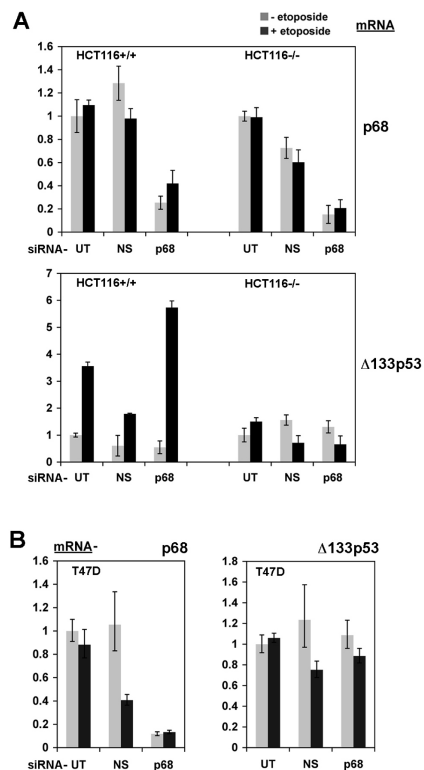**Figure 2. Induction of Δ133p53 mRNA when p68 levels are depleted is p53-dependent**
A: HCT116 p53−/− cells (which still express Δ133p53) and **B:** T47D cells (mutant p53) were treated with a siRNA targeted against p68 or a non-silencing control (NS). After 48hr, cells were treated with 100μM of etoposide for 4 hr and expression of p68 and Δ133p53 was examined by qRT-PCR. Untransfected cells and HCT116 p53+/+ cells served as additional controls. Expression of the gene of interest was normalised to TBP expression and graphs plotted as fold change from untransfected cells (UT) that had not been treated with etoposide. The average values from 3 independent experiments are shown ± s.e.m.
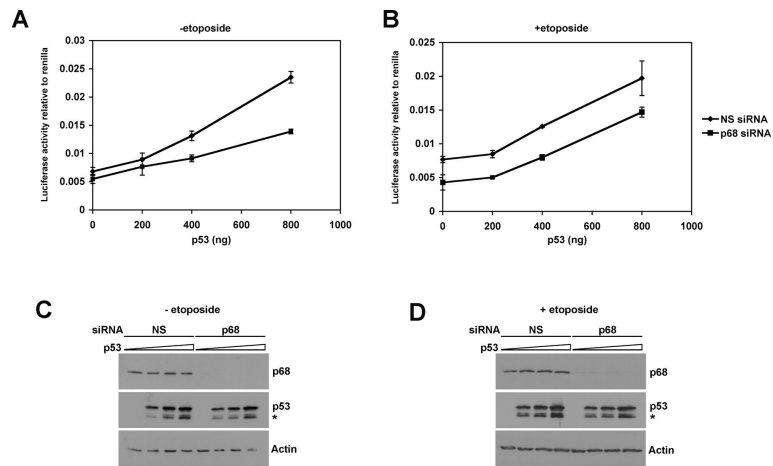
Figure 3. p68 depletion does not increase p53-mediated transcription from the intron 4 promoter in response to etoposide treatment

H1299 cells (p53 null) were treated with a p68 siRNA or a non-silencing control (NS). After 48hr, cells were co-transfected with the intron 4 promoter of p53 fused to the firefly luciferase reporter gene, the *Renilla*-luciferase control plasmid and increasing concentrations of p53 (0, 200, 400 and 800ng). p53 intron 4 promoter activity is shown **A**: in the absence of etoposide, and **B**: in the presence of etoposide (100μM for 4 hr). **C** and **D** show the corresponding western blots to confirm depletion of p68 and expression of p53. Reporter activity was calculated relative to *Renilla* luciferase activity to control for transfection efficiency. The average values from 2 independent experiments are shown ± s.e.m. * denotes a p53 cleavage product resulting from the overexpression.
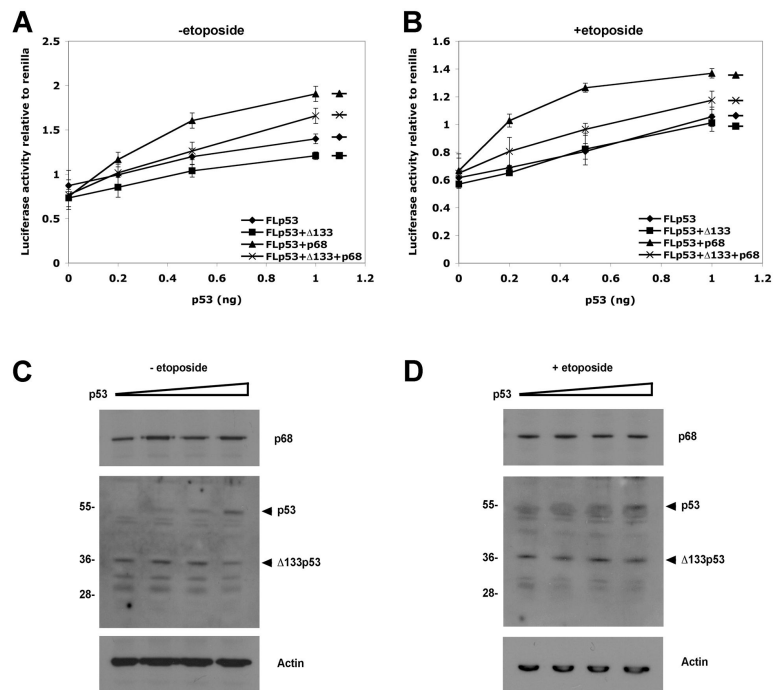
Figure 4. Δ133p53 inhibits p68 co-activation of p53-dependent p21 induction
H1299 cells (p53 null) were cotransfected with the p21 promoter fused to the firefly
luciferase reporter gene, the *Renilla*-luciferase control plasmid, 1μg p68, 1ng Δ133p53 and
increasing concentrations of p53 (0, 0.2, 0.5 and 1ng). p21 promoter activity is shown **A**: in
the absence of etoposide, and **B**: in the presence of etoposide (50μM for 8 hr). **C** and **D**
show the corresponding western blots to confirm expression of p68, p53 and Δ133p53.
Reporter activity was calculated relative to *Renilla* luciferase activity to control for
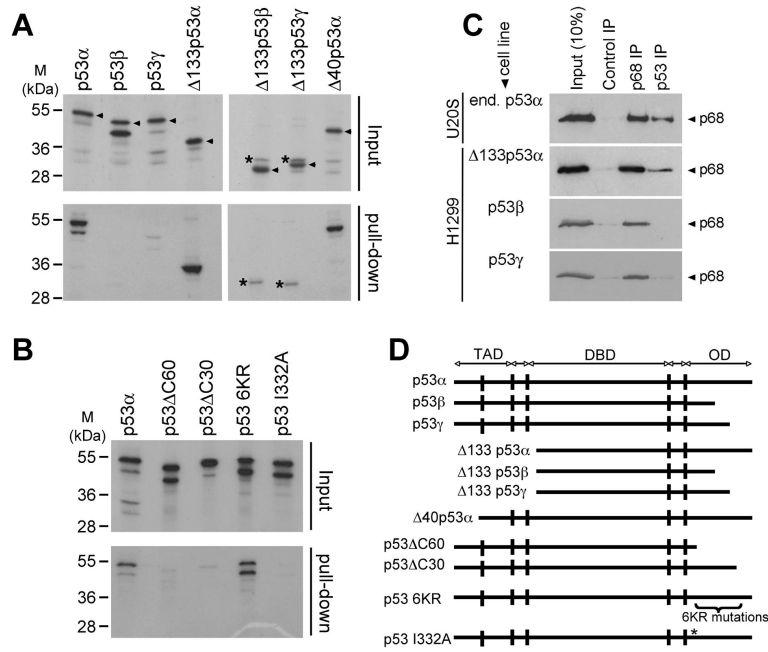transfection efficiency. The average values from 3 independent experiments are shown ±
s.e.m.

Figure 5. p68 interacts with the C-terminal domain of p53 and co-immunoprecipitates with the Δ133p53α isoform; the ability of p53 to tetramerise is important for interaction with p68
GST-pulldowns were performed using GST-tagged p68 and **A:** *in vitro*-translated ^{35}S-labelled p53 isoforms and **B:** p53 deletion/mutation derivatives. In each case the top panel shows *in vitro*-translated/^{35}S-labelled proteins with arrows denoting the bands corresponding to specific p53 isoforms. * indicates non-specific bands. **C:** Co-immunoprecipitation from nuclear extracts of endogenous p68 with endogenous p53 (U2OS cells), or p53 isoforms in H1299 cells stably expressing either Δ133p53α, p53β or p53γ. **D:** Cartoon illustrating the different p53 species referred to in the figure.
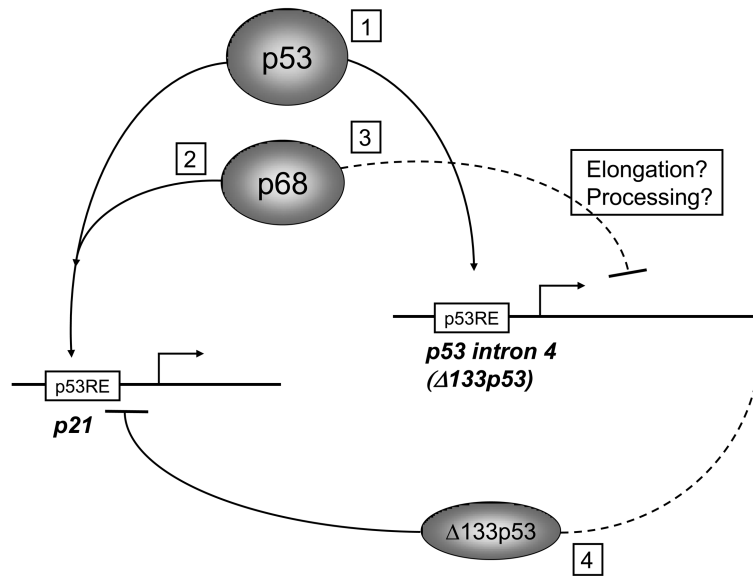
Figure 6. Model illustrating the regulatory interactions between p68, p53 and Δ133p53
p53 transactivates expression of p21 and Δ133p53 (1). p68 alone does not influence expression of these genes but co-activates p53-dependent expression of p21 (2). p68 inhibits p53-dependent expression of Δ133p53 but independently of events at the intron 4 (Δ133p53) promoter (3). Δ133p53 in turn inhibits expression of p21, most likely through direct interaction with p53 and/or p68 (4). Variations in the p68 levels (as observed in tumours) will not only affect p21 expression directly but, additionally, through an indirect mechanism by regulating the levels of an inhibitor of p21 expression (i.e. Δ133p53). Notably, Δ133p53 itself does not appear to influence the levels of p68 (data not shown).

Table 1

Expression of p68 is inversely associated with Δ133p53 expression in breast tumours

Δ133p53 mRNA expression was determined by RT-PCR and was scored as expressed (+) or not expressed (−). p68 mRNA expression was determined by quantitative RT-PCR and expression calculated relative to TBP. p68 protein was scored as 0-18 using the "QuickScore" method. **A**: Associations between p68 mRNA and Δ133p53 isoform mRNA in breast tumours. The appropriate statistical test, either 2-sample t-test* or Mann-Whitney+, was used to calculate associations depending on the distribution of the data. The mean*/median+ and confidence intervals for p68 are shown, with values representing p68 mRNA expression relative to TBP. **B**: Associations between p68 protein and Δ133p53 isoform mRNA in breast tumours. The Fisher's Exact test was used to determine statistical significance. A value of $p < 0.05$ denotes statistical significance.

A p68 mRNA vs Δ133p53 mRNA

	Δ133p53α		Δ133p53β		Δ133p53γ	
Δ133p53 expression	−	+	−	+	−	+
No. of patients	106	41	132	15	122	25
2 sample t-test*/Mann-Whitney+	−ve association* p=0.004		−ve association+ p=0.003		−ve association* p=0.024	
(p68: qRT-PCR) Mean*/Median+	171.479*	101.078*	136.241+	68.60+	163.251*	96.176*
95% CI	(143.251 - 199.708)	(77.774 - 124.383)	(117.936 - 154.546)	(48.663 - 88.543)	(138.019 - 188.482)	(65.460 - 126.893)

B p68 protein vs Δ133p53 mRNA

	Δ133p53α		Δ133p53β		Δ133p53γ	
Δ133p53 expression	−	+	−	+	−	+
(p68=0) No. of patients	2	4	3	3	3	3
(p68=1-18) No. of patients	104	37	129	12	119	22
Fisher's Exact Test	−ve association p=0.051		−ve association p=0.015		−ve association p=0.062	

−ve association = inverse association; CI = Confidence Interval