Identification of *BTG2*, an antiproliferative p53-dependent component of the DNA damage cellular response pathway

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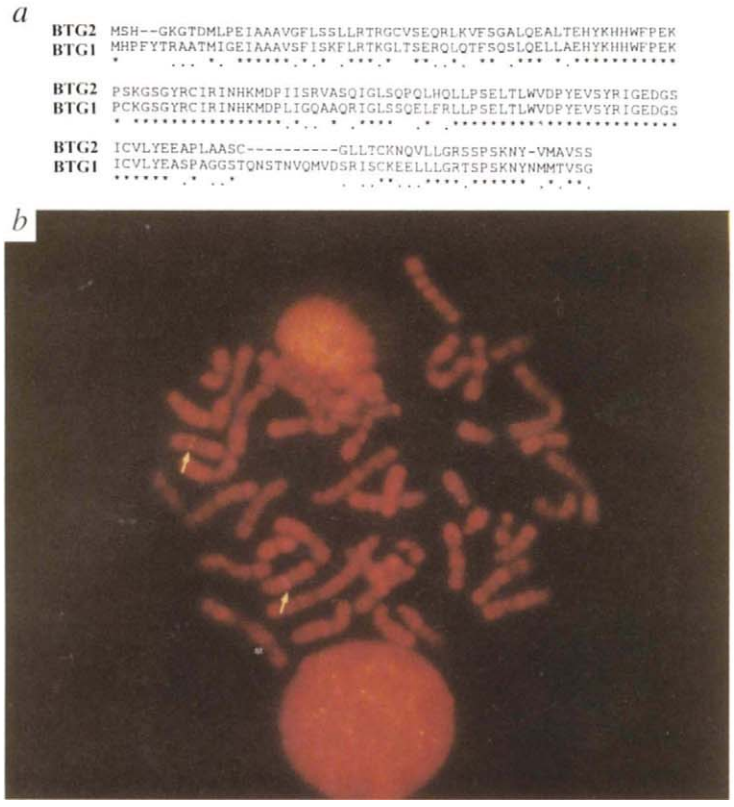
Cell cycle regulation is critical for maintenance of genome integrity. A prominent factor that guarantees genomic stability of cells is p53 (ref. 1). The *P53* gene encodes a transcription factor that has a role as a tumour suppressor[2]. Identification of p53-target genes should provide greater insight into the molecular mechanisms that mediate the tumour suppressor activities of p53. The rodent *Pc3/Tis21* gene was initially described as an immediate early gene induced by tumour promoters and growth factors in PC12 and Swiss 3T3 cells[3,4]. It is expressed in a variety of cell and tissue types and encodes a remarkably labile protein[4,5]. *Pc3/Tis21* has a strong sequence similarity to the human antiproliferative *BTG1* gene cloned from a chromosomal translocation of a B-cell chronic lymphocytic leukaemia[6]. This similarity led us to speculate that *BTG1* and the putative human homologue of *Pc3/Tis21* (named *BTG2*) were members of a new family of genes involved in growth control and/or differentiation. This hypothesis was recently strengthened by the identification of a new antiproliferative protein, named TOB, which shares sequence similarity with BTG1 and PC3/TIS21 (ref. 7). Here, we cloned and localized the human *BTG2* gene. We show that *BTG2* expression is induced through a p53-dependent mechanism and that BTG2 function may be relevant to cell cycle control and cellular response to DNA damage.

We cloned the human *BTG2* cDNA from a lymphoblastoid cell line cDNA library. The sequence of the putative open reading frame predicted a 158 amino acid protein which shares 93.6% identity with the murine TIS21 protein (data not shown) and 66.4% identity with the BTG1 protein (Fig. 1*a*). The only significant difference between BTG1 and BTG2 protein sequences is a 10 amino acid insertion in the C-terminal part of the BTG1 protein. Fluorescence *in situ* hybridization of a *BTG2* specific probe to normal human metaphase chromosomes localized *BTG2* to the 1q32 region (Fig. 1*b*). Furthermore, Southern blot analysis on human–rodent somatic cell hybrid DNA confirmed that *BTG2* is on chromosome 1 (data not shown).

Our assessment of *BTG2* mRNA levels during the growth cycle showed that this gene was preferentially expressed in quiescent cells (Fig. 1*c*), supporting the hypothesis that BTG2 could play a role in the negative control of cell proliferation. Transfections of a *BTG2* mammalian expression vector into NIH3T3 cells revealed that overexpression of this gene caused a decrease in the growth rate and reduced cloning ability of these cells (data not shown), confirming that BTG2 may display a growth suppressive role. To better explore BTG2 function, we generated a *Btg2*-null allele in embryonic stem (ES) cells (Fig. 2*a*). We verified *Btg2* deficiency in homozygous *Btg2/Tis21*$^{-/-}$ ES cells by Southern blot analysis (Fig. 2*b*) and by northern blot analysis (data not shown). *Btg2/Tis21*$^{-/-}$ ES cells were viable and their growth rate was similar to normal ES cells. Because the cell cycle of undifferentiated ES cells cannot be accurately studied by standard FACS analysis procedure, we induced cellular differentiation using retinoic acid[8]. Following differentiation, irrespective of their genotype, all cell lines showed similar cell cycle patterns (see untreated cells, in Fig. 2*c*) and growth rates, as indicated by bromodeoxyuridine incorporation (data not shown). As *Btg2* disruption had no detectable effect on cellular growth of undifferentiated or differentiated ES cells, we tested whether BTG2 was involved in DNA damage-induced growth arrest. The induction of DNA damage by adriamycin or etoposide in parental differentiated ES cells (*Btg2/Tis21*$^{+/+}$ cells) was followed by a delay of cell cycle progression that was detectable as early as 12 hours after treatment (Fig. 2*c*). As described in other cell types[9], growth arrest was transient, most of the cells recovering a normal cell cycle after 72–96 hours. Conversely, as evidenced by the alteration of the G2/M block, cellular response was deficient in differentiated *Btg2/Tis21*$^{-/-}$ clones (Fig. 2*c*). As a control, *erbA*$^{-/-}$ ES cells which were created following the same protocol, and these behaved like parental ES cells in these experiments (data not shown). This confirmed that the alteration of the response of *Btg2/Tis21*$^{-/-}$ cells to DNA damaging agents was a specific consequence of the *Btg2/Tis21* gene disruption and not attributable to manipulations leading to recombinant ES cells. Following 48–72 hours of genotoxic treatment, a greater degree of apoptosis occurred in *Btg2/Tis21* deficient cells than in parental ES cells (Fig. 2*d*). In contrast to *Btg2/Tis21*$^{+/+}$ ES cells, we observed no cell survival of *Btg2/Tis21*$^{-/-}$ clones 5–6 days after treatment, demonstrating an increased sensitivity to the killing effects of adriamycin (Fig. 2*d*). We observed a deficient response to DNA damage using three different *Btg2/Tis21*$^{-/-}$ clones following either adriamycin (0.1 or 0.2 μg/ml) or etoposide (0.5 μg/ml) treatment.

To further study the involvement of BTG2 in the cellular response to DNA damage, we investigated *BTG2* mRNA expression in a series of human cell lines following genotoxic treatments. Basal expression of *BTG2* was low in all exponentially growing cell lines. However, striking induction of *BTG2* expression occurred following adriamycin treatment or ionizing radiation in cell lines with wild-type *P53* (Fig. 3*a*). In these cells, the increase of *BTG2* mRNA levels was concomitant with p53 protein accumulation. We obtained similar results in murine *Btg2/Tis21*$^{+/+}$ ES cells and in wild-type *P53* expressing murine NIH3T3 fibroblasts (data not shown). In contrast, in human cell lines expressing mutant *P53*, *BTG2* induction was weak or undetectable (Fig. 3*a*).

The *P53* tumour suppressor gene product is an important component of the cellular response to DNA damag-



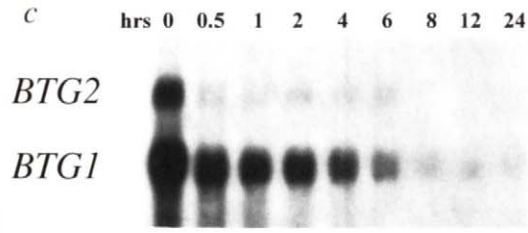
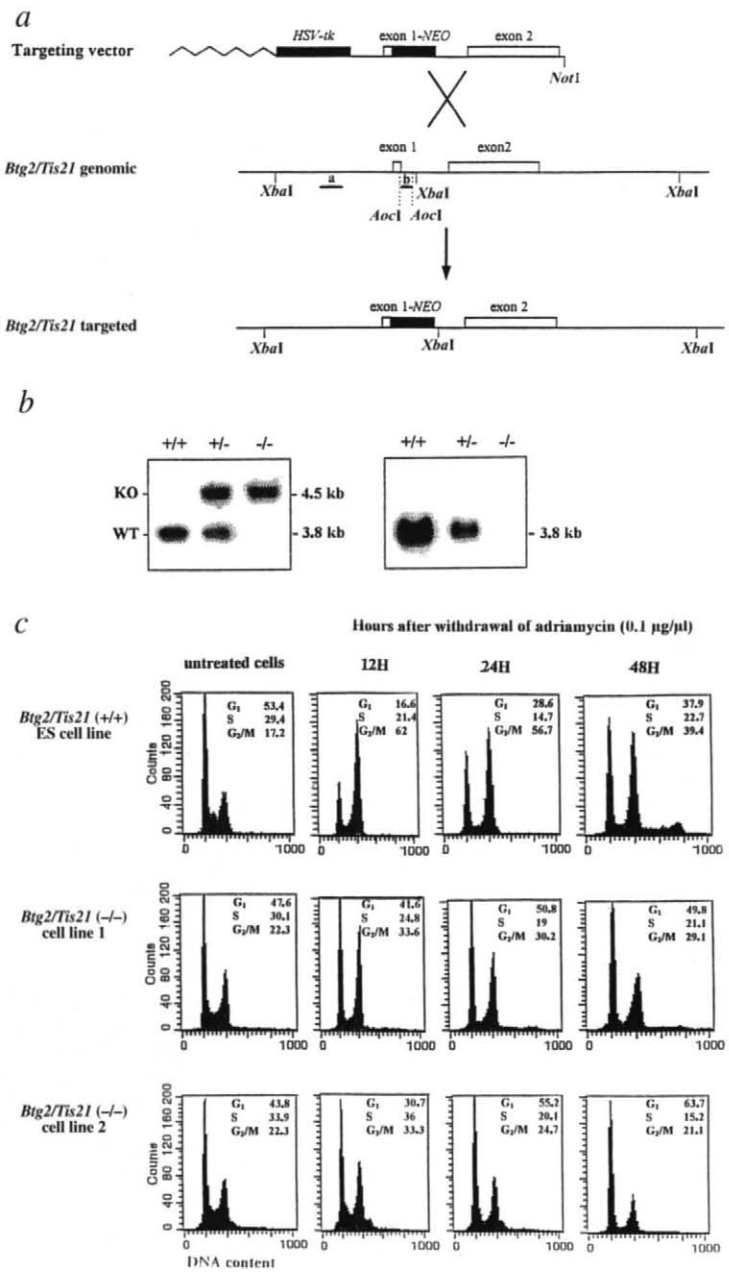


Fig. 1 ***a***, Comparison of the amino-acid sequences of the human BTG1 and BTG2 proteins. The amino-acid sequences are aligned for optimal homology. (*): identical residues. (.): conservative substitution. ***b***, Chromosomal localization of the *BTG2* gene by FISH. Fluorescence *in situ* hybridization (FISH) to normal human metaphase chromosomes using a *BTG2* probe. The arrows indicate the localization of *BTG2* on chromosome 1q32. In 21 of 23 metaphase cells examined, we observed a spot on the two sister chromatids at band 1q32.1 on both chromosomes 1, and only on one chromosome 1 in the remaining two metaphase cells examined. In addition we observed a double spot on only one chromosome in four of these cells: 6q21 and Yq11 in one, 5q32, 11q22 and 1p32 in the others. Thus the chromosomal localization of the *BTG2* probe is at 1q32. ***c***, *BTG2* mRNA expression after PHA (phytohemagglutinin) stimulation of peripheral blood mononuclear cells. *BTG1* gene expression is mainly expressed in the G0/G1 phases of the cell cycle[6].

ing agents. The p53 protein functions as a transcriptional regulator that is able to bind to DNA in a sequence specific manner and to activate transcription of adjacent genes[10–13]. To test directly whether wild-type p53 induced *BTG2* gene expression, we used an experimental model consisting of the human colon carcinoma cell line EB1, containing an inducible wild-type *P53*. EB1 cells are derived from the EB cell line after stable transfection with a construct that contains wild-type *P53* cDNA under the control of the metallothionein MT-1 promoter[14]. EB cells and uninduced EB1 cells, which are deficient in *P53* expression, had no detectable expression of *BTG2* (Fig. 3*b*). Induction of *P53* expression in EB1 cells by metal ions led to rapid *BTG2* overexpression. Conversely, neither the addition of metal ions to the parental EB cells nor ionizing radiation were able to induce *BTG2* expression in the absence of p53. The pattern of expression of $p21^{WAF1/CIP1}$, a well known target of p53 (ref. 15), was identical to that of *BTG2* in all these experimental conditions. These observations strongly suggest that induction of *BTG2* occurred through a p53-dependent mechanism. Consistent with this hypothesis, *Btg2/Tis21* mRNA induction following adriamycin or ionizing radiation was undetectable in *P53*-deficient murine embryonic fibroblasts. Exogenous expression of a murine temperature sensitive mutant p53 (p53val135) in $P53^{-/-}$ embryo fibroblasts resulted in the expression of *Btg2/Tis21* mRNA only at the wild-type permissive temperature (Fig. 3*c*). Similar results were observed using the *P53*-deficient human hepatocarcinoma cell line Hep3B. *BTG2* expression was induced at the permissive temperature in clonal Hep3B-^{135}p53 cells (data not shown).

To investigate the mechanisms by which p53 regulates *BTG2* expression, we sequenced the first 760 bp upstream of the *BTG2* translation initiation site. This GC rich region corresponds to the 5' untranslated region of the gene and contains the presumptive promoter. Whereas the coding sequences of the mouse (*Tis21*) and human *BTG2* genes are highly homologous, the nucleotide sequences immediately upstream of the start codon are more divergent with the exception of a stretch of 42 nucleotides (37/42 identity) located 97 bp 5' of the translation initiation site of the *BTG2* gene. This particular region contains a potential p53 recognition element, matching the four tandem PuPuPuC(A/T) pentamers[16] in 20 of 20 positions. The two 10-bp motifs are separated by a single nucleotide (Fig. 4*a*). It is worth noting that this potential p53-consensus binding site shares 14 of 20 nucleotides with the second p53-binding site of the $p21^{WAF1/CIP1}$ gene recently identified by El-Deiry *et al.*[17]. By using the experimental model of EB/EB1 cells, we found that 5' untranslated sequences of the human *BTG2* gene confer inducibility by p53 when located *cis* to a heterologous promoter (Fig. 4*a*) or upstream of a promoterless reporter gene (data not shown). Furthermore, co-transfection experiments with either wild-type or mutant *p53* expression vectors in a p53-deficient cell line (Hep3B) indicated that this inducibility was specific to wild-type p53 (Fig. 4*b*).

In response to DNA damage, eukaryotic cells delay cell cycle progression at both G1 into S, and G2 into M phases by induction of antiproliferative genes. Arrest in G1 is thought to prevent replication of damaged genetic templates; arrest prior to M allows cells to avoid segregation of defective chromosomes[18]. Loss of the ability to arrest cell cycle can have deleterious consequences, as has been demonstrated in yeast, where inactivation of the *RAD9* gene abrogates the G2 checkpoint control and renders cells more sensitive to genotoxic agents[19]. Although our results do not allow definition of the exact functions of *BTG2*, they suggest that similar mechanisms exist in mammalian cells. *Btg2/Tis21* inactivation in ES cells leads to a striking disruption of DNA damage-induced G2/M arrest and a marked increase in cell death. Additional experiments should help to define whether this results from the absence of a specific G2 block, ongoing apoptosis or loss of other factors.



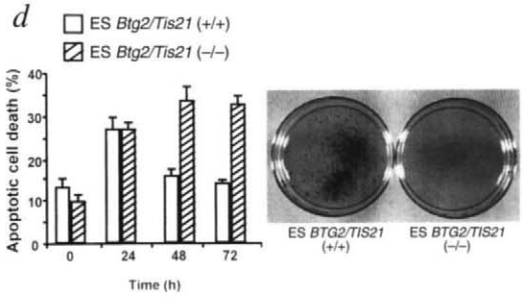


Fig. 2 *BTG2* targeting in ES cells and generation of *Btg2/Tis21*$^{-/-}$ ES cells. ***a***, The targeting construct contains a 4.8-kb *Btg2/Tis21* genomic sequence with a *neo* cassette inserted into the *AocI/AocI* sites. This leads to the deletion of a 0.4-kb fragment containing the 3' end of the *Tis21* exon 1. A thymidine kinase gene (HSVtk) enables the negative selection with Gancyclovir. (abbreviations are a: probe a; b: probe b). ***b***, Left panel, DNA extracted from ES clones was digested with *XbaI* and hybridized with the external probe a. The size of wild-type (WT) and disrupted (KO) alleles are shown. Phenotypes of the ES clones are presented above the figure. Right panel, DNA extracted from ES clones was digested with *XbaI* and hybridized with probe b. Controls including digestions with other restriction enzymes or PCR were used to ascertain these results (data not shown). ***c***, Response of *Btg2/Tis21*$^{+/+}$ and *Btg2/Tis21*$^{-/-}$ cell lines to adriamycin treatment. The percentage of cells in G1, S and G2/M phases of the cell cycle is shown for each histogram. Flow cytometry data, acquired using Becton Dickinson standard acquisition software, excluded cell aggregates and debris and allowed collection of single cell events only. Data were analysed with Cell Quest software (Becton Dickinson Immunocytometry Systems). ***d***, Sensitivity of *Btg2/Tis21*$^{+/+}$ and *Btg2/Tis21*$^{-/-}$ cell lines to adriamycin treatment. Left panel, apoptotic cell death in ES clones 24, 48 and 72 h after adriamycin treatment as measured by the TUNEL method (Boehringer Manheim). Bars represent mean ± s.d. Right panel, cell survival of ES clones 10 days after adriamycin treatment. Cells were stained with 4% Giemsa.

Induction of *BTG2* expression following genotoxic stress through a p53-dependent mechanism suggests that BTG2 mediates p53 functions. Like Rad9p, p53 is not required for normal cell growth and division, but it is a central component of the cellular response to DNA damage and the maintenance of genomic stability, and likely mediates this response by orchestrating a G1 growth arrest. In addition to its role in G1 checkpoint, p53 is implicated in different cellular processes that respond to DNA damage, including apoptosis[20], DNA repair[21] and recently, the control of G2–M transition[22–26]. Studies on *p21*$^{WAF1/CIP1-/-}$ cells, unambiguously established p21$^{WAF1/CIP1}$ as a critical mediator of the p53-dependent G1 arrest[27–29]. However, unlike *P53*$^{-/-}$ animals, *p21*$^{WAF1/CIP1-/-}$ mice do not exhibit a propensity for early tumorigenesis, suggesting that other functions of p53 are responsible for its tumour suppressor role *in vivo*[27,30]. In the cell lines studied, induction of *BTG2* and *p21*$^{WAF1/CIP1}$ genes were closely correlated. Like *p21*$^{WAF1/CIP1}$ (ref. 31), *BTG2* expression was also induced in aging fibroblasts (unpublished data). Human *BTG2* gene maps to the 1q32 region. Interestingly, the human chromosome 1q carries a gene involved in the control of cellular senescence[32]. Structural changes and deletions in this region are also associated with the acquisition of immortality of colorectal adenomas[33], and occur in different human tumours including uterine endometrial carcinomas[34] and breast carcinomas[35]. In the latter tumours, the region 1q23–32 was deleted in 25% of the informative cases. Together with our results, these observations support a role for *BTG2* as a tumour suppressor gene. Detailed analysis of the cell cycle in stable cell lines carrying an inducible *BTG2* transgene along with the use of *BTG2* 'knockout' mice should allow a better understanding of the exact functions of BTG2 and provide new insights into the molecular mechanisms involved in p53-tumour suppressing functions.

Methods

Cloning of the human *BTG2* cDNA. The human *BTG2* cDNA was cloned from a lymphoblastoid cell line cDNA library. Screening was performed using a PCR amplified DNA segment corresponding to nucleotides 2488–2753 of mouse exon 2 *Tis21* (ref. 4). Sequencing was done using the double-stranded DNA sequencing technique (dideoxy chain termination procedure) with sequenase II, as described by the manufacturer (USB).

Chromosomal localization. Fluorescence *in situ* hybridization (FISH) to normal human metaphases was performed using a *BTG2* recombinant phage as a probe as described[36]. The human *BTG2* genomic clone was isolated by screening clones of a human genomic library in λEMBL3 using the human *BTG2* cDNA as a probe. The human–rodent cell hybrid DNA panel

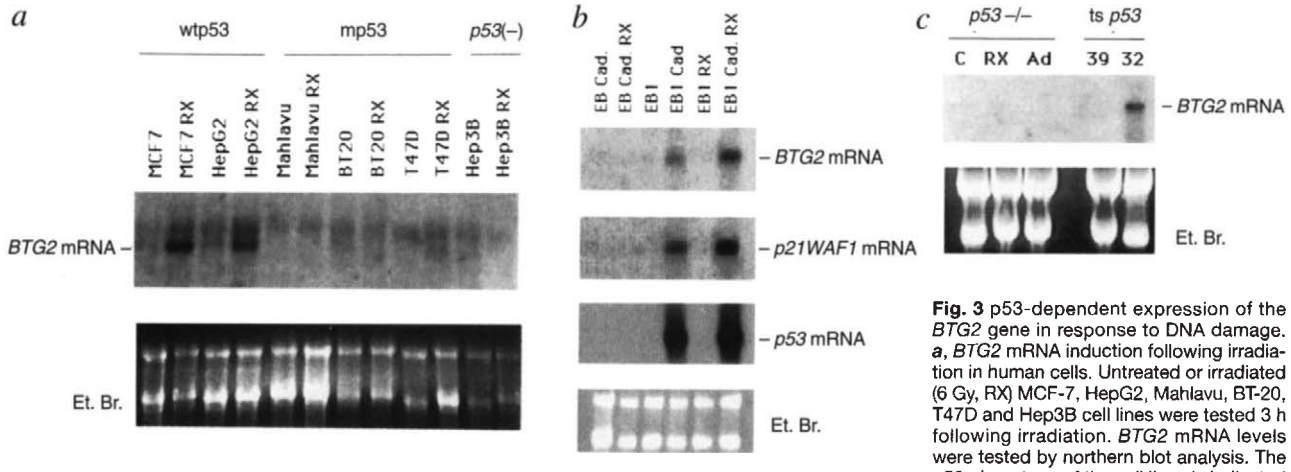


Fig. 3 p53-dependent expression of the *BTG2* gene in response to DNA damage. ***a***, *BTG2* mRNA induction following irradiation in human cells. Untreated or irradiated (6 Gy, RX) MCF-7, HepG2, Mahlavu, BT-20, T47D and Hep3B cell lines were tested 3 h following irradiation. *BTG2* mRNA levels were tested by northern blot analysis. The p53 phenotype of the cell lines is indicated as follows: wtp53: wild-type *P53*, mp53: mutant *P53*; p53(–): both *P53* alleles deleted[40–44]. ***b***, p53-dependent induction of *BTG2* gene in EB1 cells. Parental EB cells were treated with 5 μM cadmium chloride (Cad) for 4 h and were either not irradiated (lane 1) or irradiated (6 Gy; lane 2) and allowed to recover for 3 h. EB1 cells (containing inducible wild-type p53) not treated with cadmium, were either not irradiated (lane 3) or irradiated (lane 5). EB1 cells treated with cadmium (Cad.), were either not irradiated (lane 4) or irradiated (lane 6). Northern blot analysis assessed the levels of *BTG2* mRNA. The blot was probed with *BTG2* cDNA, $p21^{WAF1/CIP1}$ cDNA or *p53* cDNA as indicated. ***c***, Absence of *Btg2/Tis21* induction following genotoxic treatment in *P53*-deficient murine embryo fibroblasts ($P53^{-/-}$). Exogenous expression of a murine temperature sensitive mutant *p53* (ts p53: p53val135) results in *Btg2/Tis21* mRNA expression at the wild-type permissive temperature (32 °C). RX: X-rays (6 Gy) Ad: adriamycin treatment (0.2 μg/ml).

was purchased from Bios Laboratories and hybridized with a *BTG2* specific probe following usual procedures.

Cell lines and treatment. Peripheral blood mononuclear cells (PBL) were obtained from the blood of normal volunteers by centrifugation on Triosil Ficoll. Phytohaemagglutinin was used to stimulate peripheral blood mononuclear cells[6]. CGR8 (ES) cells were cultured as described[8]. They were induced to differentiate by withdrawal of DIA/LIF for 1 d and by treatment with 10^{-6} M retinoic acid for 2 d. EB1 cells are derived from a human colon cell line EB (p53 null) by transfection of wild-type *P53* gene under the control of inducible metallothionein promoter[14]. $p53^{-/-}$ embryo fibroblasts were obtained from L. Donehower and embryo fibroblasts containing a stably integrated murine temperature-sensitive mutant p53 were obtained from M. Oren. EB/EB 1 cells, hepatocarcinoma cell lines (Hep3B, HepG2, Huh7) and breast adenocarcinoma cell lines (MCF-7, BT-20, T47D) were maintained in DMEM supplemented with 10% FCS, glutamine (2 mM) and non-essential amino acids. Cells were treated with either adriamycin (0.1 or 0.2 μg/ml) or etoposide (0.5 μg/ml) for 3 h at 37 °C or exposed to X-ray irradiation at 6 Gy. Then, at indicated times, propidium iodide-stained cells were analysed by FACScan (Becton Dickinson) to determine the cell cycle distribution.

Targeting vector and homologous recombination in ES cells. The *Tis21* gene was isolated by screening a λEMBL3 mouse genomic library derived from CGR8 cells using a *Tis21* specific probe. *Not*I linearized targeting vector was electroporated in CGR8 ES cells. G418 and Gancyclovir double-resistant ES cells were selected and analysed. The $Btg2/Tis21^{+/-}$ ES cell clones were identified by Southern blot analysis as described in the legend of Fig. 2. Four heterozygous clones were obtained. To generate $Btg2/Tis21^{-/-}$ cells, G418 concentration was increased (up to 5 mg/ml) and three independent and correctly targetted null allele clones were selected and further studied.

RNA isolation and northern blot analysis. Total RNA was extracted by guanidium isothiocyanate method. RNA samples (20 μg) were separated by electrophoresis through denaturing formaldehyde agarose gel and transferred to nylon membranes

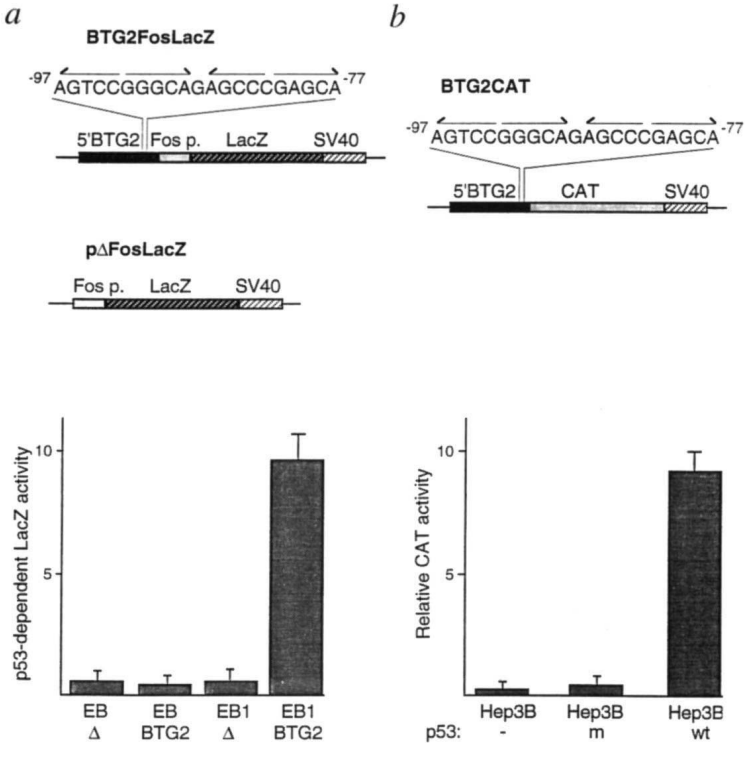


Fig. 4 The 5' untranslated region of the *BTG2* gene confers p53-dependent expression. ***a***, The 5' untranslated region of the *BTG2* gene confers p53-dependent expression upon a heterologous reporter gene. BTG2-FosLacZ has the region of *BTG2* from –760 bp to +20 bp adjacent to a minimal *fos* promoter (Fos p) and a β-galactosidase reporter gene (*lacZ*). Arrows indicate the potential p53-binding sequences. pΔFosLacZ plasmid does not contain the *BTG2* fragment. EB and EB1 cells were transfected with reporter plasmids as indicated and β-galactosidase activity was measured after 48 h in the absence or presence of cadmium chloride (Cad) for the last 12 h. p53-dependent β-gal activity was defined by the ratio of the activity in the presence of cadmium divided by that in the absence of cadmium (mean ± SD, n = 4). ***b***, Wild-type p53-dependent transcriptional activity in Hep3B cells. BTG2-CAT plasmid has the region of *BTG2* from –760 bp to +20 bp adjacent to a promoterless CAT reporter gene. Co-transfections of Hep3B cells were performed using the BTG2-CAT plasmid with pCMV-βgal and either pC53-SN3 (expressing wild-type *P53*), pC53-CX3 (expressing mutant $Ala^{143}P53$) or pCMV-Neo, as indicated. CAT activity was measured 48 h later and normalized relative to β-gal. (mean ± SD, n = 3).

(Hybond-N; Amersham). Membranes were hybridized with labelled *BTG2* cDNA, *p21*$^{WAF1/CIP1}$ cDNA or *P53* cDNA.

Construction of BTG2-FosLacZ and BTG2-CAT plasmids. A 780-bp fragment containing the 5' untranslated region of the *BTG2* gene (–760 to +20) was obtained by PCR amplification using the human genomic clone as template and the primers 5'–CAGAATTCTGTTGGGGGAGGTGGA–3' and 5'–GAATTCCCTTCCCGTGGCTCAT–3'. Products from two independent PCR reactions displayed similar sequences. The 780-bp PCR fragment was cloned upstream of a deletion mutant of the murine *fos* promoter and a β-galactosidase reporter gene into pBluescript II SK(+) (Stratagene) or upstream of a CAT reporter gene (CAT basic vector, Promega) to yield plasmid BTG2-FosLacZ and plasmid BTG2-CAT respectively. The plasmid pΔFosLacZ (gift of T. Frebourg) lacks the *BTG2* fragment.

Transfections. Transient transfections of EB/EB1 cells (at 70% confluence) were performed using 20 μg of lipofectin (Gibco BRL) and 10 μg of either BTG2-FosLacZ or pΔFosLacZ constructs. Each transfected plate was split in 2 after 24 h. Cells were allowed to recover for 12 h and 1 plate was treated with 5 μM cadmium chloride. The following day, cell lysates were assayed for measurements of β-gal activity as previously described[37]. Co-transfections of Hep3B cells were carried out using a total of 20 μg of plasmids: BTG2-CAT plasmid with pCMV-βgal and either pC53-SN3 (expressing wild type *P53*) (ref. 38), pC53-CX3 (expressing mutant Ala^{143}p53) or pCMV-Neo. Cell lysates were assayed for measurements of CAT (Boehringer Mannheim) and β-gal activity after 48 h. Previous studies showed that p53 does not influence the activity of pCMV-βgal plasmid[39].

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