p53 is a tumor suppressor that prevents the emergence of transformed cells by inducing apoptosis or senescence, among other responses. Its functions are regulated tightly by posttranslational modifications. Here we show that Bruton’s tyrosine kinase (BTK) is a novel modulator of p53. We found that BTK is induced in response to DNA damage and p53 activation. BTK induction leads to p53 phosphorylation, which constitutes a positive feedback loop that increases p53 protein levels and enhances the transactivation of its target genes in response to stress. Inhibiting BTK reduced both p53-dependent senescence and apoptosis. Further, BTK expression also upregulated DNA damage signals and apoptosis. We conclude that despite being involved in oncogenic signals in blood malignancies, BTK has antineoplastic properties in other contexts, such as the enhancement of p53’s tumor suppressor responses. Along with evidence that BTK expression correlates with good prognosis in some epithelial tumors, our findings may encourage a reevaluation of the clinical uses of BTK inhibitors in cancer therapy.

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be expressed in response to damage and contributes to the tumor suppressor mechanisms regulated by p53.

Materials and Methods

Cell culture and chemicals
Human diploid dermal fibroblasts (HDF), HCT116 and HT1080, were maintained in DMEM supplemented with 10% FBS (Gibco) and penicillin–streptomycin (50 U/mL). Elp53 were maintained as described (27). Elp53 were a gift from Dr. S. Aaronson (Mount Sinai School of Medicine, New York, 2008), HCT116 from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, 2008), and HT1080 from Dr I. Roninson (South Carolina College of Pharmacy, Charleston, 2008). HDF were obtained from ATCC (2014). All cells were expanded and stored in liquid nitrogen when received and original vials were thawed for these experiments. No further validation or authentication was performed. Chemicals: Ibrutinib (Sellechem, PCI-32765), CGI-1746 (Axon Medchem, 2018), tert-butylhydroperoxide (Merck), doxorubicin (Sigma-Aldrich), sorastaurin (Sellechem), and dactolisib (Sellechem). Peripheral blood samples were obtained from patients with CLL after informed consent and approval from the Local Research Ethics Committee. The patients were treatment naive and had a cell count ≥50 × 10^9/l. Peripheral blood mononuclear cells (PBMC) were processed as previously described (28). An adenovirus containing p53 (Adp53), a gift of B. Vogelstein (Johns Hopkins University, Baltimore, MD), or LacZ (AdLacZ) were amplified as previously described (29). Cells were exposed to 10 μL of the appropriately diluted virus stock.

PPISURV analysis
PPISURV (30) was used to correlate survival rates in cancer patients to the expression level of BTK. In each data set, samples were grouped with respect to expression rank of the gene, which reflects relative mRNA expression level and introduces no normalization bias. The “low expression” and “high expression” groups are those where expression rank of the gene is less or more than average expression rank across the data set, respectively. This separation of patients into ”low” and ”high” groups in the data set along with survival information is next used to find statistical differences in survival outcome. The R statistical package was used to perform survival analyses and to draw KM plots. Unadjusted P-values were generated using standard survival analysis packages.

Growth curves
A total of 1 × 10⁴ cells were plated in 6 cm plates a day before chemicals were added. Cells were counted every 4 days using a BIO-RAD TC20 automated cell counter. After which fresh media and chemicals were added to the cultures.

Immunoblot analysis
Western blots were performed as described (31). Primary antibodies used: anti-phospho-Histone 2A (ser 139, Millipore 05-636), anti-phosphoATM (S1918, Abcam ab81292), anti-phospho-p53 (ser 15, Cell Signaling 9284), anti-p53 (DO-1, Santa Cruz, sc-129), anti-BTK (D3H, Cell Signaling Technology 8547), anti-p16 (Abcam ab54210), anti-β-actin (Abcam ab8227) and anti-caspase 3 (Cell Signaling Technology 96625), anti-PARP-1 (Cell Signaling Technology 9542). An ECL detection system (Thermo Scientific) was used to visualize the results. Alternatively, an Odyssey Clx Infrared Imaging System (Li-COR) was used. For Ponceau staining, 1× Ponceau staining (Sigma; P 3504) was added to the membrane for 5 minutes with shaking.

Immunofluorescence
Cells were processed as previously described (26). Coverslips were incubated overnight at 4°C with 1: 50 anti-BTK (D3H5, Cell Signaling Technology 8547) or 1:100 anti-p53-FTC (Santa Cruz sc-126). Secondary anti-rabbit (1:500) antibody (Alexa Fluor 594; Invitrogen) and DAPI (Invitrogen) were used. Slides were analyzed using a Nikon TE300 microscope.

Senescence-associated-β-galactosidase staining
Cells were stained as previously described (32).

Colonie formation assay
Five hundred cells were split into 60 mm plates in triplicates and assays were performed as described (31).

BTK overexpression
Transfection was performed using Lipofectamine 2000 (Invitrogen) following manufacturer's protocols. Eight micrograms of empty plasmid (Mission pLKO.1 Empty vector control plasmid DNA; Sigma Aldrich SHC001) or a BTK plasmid (OriGene RG211582) were used. Medium was changed after 5 hours and cells were left for 18 to 24 hours.

BTK silencing
shRNA against BTK (Santa Cruz sc-29841-sh) or luciferase (control, Sigma Aldrich SHC007, Mission pLKO.1puro Luciferase) were transfected into Elp53 and HCT116 using Lipofectamine 2000 following manufacturer's protocols using 1 μg of shRNA. Two μg/mL puromycin was added for 2 weeks to select for transfected cells.

Quantitative real-time PCR

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were performed as previously described (34). A total of 3 × 10⁶ cells per sample were used. PCR was performed with 1 μL of DNA. The p53 (Ab-6, Gallibichem®OP43) antibody was used. Primers used: PUMA, GCGAGACTTGCGCTGCTGT (FWD), CGTTCGAGGTCCGCTTCTCT (REV); p21, GTTCCGCAATGGGTGGTCT (FWD), TCAGGCTAGCTGAGGTTCTAG (REV); BTK: TCCGACAGCGGCGCTTCTAG (FWD), CGCAGCTCACTTAAAGCGCT (REV).
Role of BTK in the p53 Pathway

Figure 1. p53 induction increases BTK. A, Western blot analysis showing BTK and p53 expression in lysates of EJp53 in the absence of p53 (−) or 6 days after removal of tet to upregulate p53 (+). B, Western blot analysis of lysates of HCT116 24 hours after being treated with 400 μmol/L of tert-butyl hydroperoxide (tBH) for 2 hours. C, Western blot analysis of lysates of HCT116 p53+/− and p53−/− treated with 1.5 μmol/L doxorubicin for 20 hours. D, real-time PCR showing changes in BTK mRNA expression in HCT116 cells treated with 1.5 μmol/L doxorubicin for 20 hours. **, P = 0.0016 (t test). Results are the mean of three experiments. Error bars, SD.

Comet assay
A total of 40,000 cells were processed as described (35). Briefly, 170 μL of a 0.6% low melting point agarose was added to the cell pellet. Eighty microliters of the mixture was placed onto the precoated slides with normal melting point agarose and covered by a coverslip. After the gel became solid, cover slips were removed and slides kept in lysis buffer (2.5 M NaCl, 0.1 M Na3EDTA, 10 mMol/L Tris-HCl, 1% TritonX-100 at pH 10) overnight. Slides were then washed for 20 minutes with ice cold ddH2O, protected from light, and exposed to ice cold alkaline buffer (pH > 13) for 20 minutes, protected from light. This step allows the unwinding of the DNA before applying the current at 30 v/300 mA for further 20 minutes. Slides were washed twice for 5 minutes with ddH2O, and placed at 37°C overnight. Slides were rehydrated with ice cold ddH2O for 30 minutes at room temperature and stained with propidium iodide (PI, 2.5 μg/mL) for further 20 minutes, then washed with fresh ddH2O for 20 minutes and placed in an oven before being scored with a fluorescence microscope. Comets were visualized in the fluorescence microscope at a magnification of ×200. Images were captured and analyzed with the Komet Analysis software version 5.5 (Andor Bioimaging, Nottingham, UK). Hundred cells were analyzed per sample, 50 per duplicate slide. The percentage of DNA in the tail of the comet was calculated for each cell by the software.
Statistical analysis

All error bars represent the SD. Statistical significance (not significant, ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$) was calculated using two-tailed unpaired $t$ tests with Prism 6 (GraphPad) software.

Results

BTK is induced in p53-mediated DNA damage responses

In a proteomics screen (26), we identified BTK as a protein induced in cells undergoing senescence. BTK, a nonreceptor kinase involved in BCR signaling (16), is highly expressed in different types of leukemia and lymphomas (36) and this has led to BTK inhibitors being used to treat B-cell malignancies (25). To better understand the role of BTK in tumor suppressor pathways, we used Ejp53, a p53-null bladder cancer cell line with a tetracycline (tet)-regulatable p53 expression system (27). We found that BTK protein levels were elevated after inducing p53 expression in these cells (Fig. 1A). Moreover, the colon cancer cell line HCT116, which has wild-type p53, also showed a p53-dependent BTK induction after being treated with DNA damaging agents (the oxidant tBH and doxorubicin) for 20 hours, 24 hours after being transfected with an empty vector (Control) or a plasmid expressing BTK. Results show the mean of three experiments. Error bars, SD. C, Western blot analysis showing BTK and p53 protein levels in lysates of Ejp53 cultured in the absence of p53 (−) or 6 days after tet removal to induce p53 expression (+), treated with DMSO (−) or 0.5 μmol/L Ibrutinib (+) from the beginning of the experiment, compared with Ejp53 stably expressing a shRNA against BTK. D, Western blot analysis showing p53 protein levels in lysates of Ejp53 cultured in the presence or absence of tet to induce p53 for the specified time and treated with 0.5 μmol/L Ibrutinib or 2 μmol/L CGI-1746 since the beginning of the experiment. E, in vitro phosphorylation assay using recombinant BTK and GST-p53, either full length (FL) or fragments (comprising amino acids 1-80, 100-300 or 300-393). F, Western blot analysis of lysates of the same cells in B, showing protein levels of BTK and Ser15 phosphorylated p53 (P-p53).

Figure 2.

BTK phosphorylates p53 and stabilizes its protein levels. A, Western blot analysis of Ejp53 in the absence of p53 (−), 6 days after removal of tet to upregulate p53 (+), or transfected with a plasmid containing BTK (+) or an empty vector control (−). Transfection was performed 24 hours before tet removal and lysates were collected 2 days after transfection. B, real-time PCR showing changes in p53 mRNA expression in HCT116 cells treated with 1.5 μmol/L doxorubicin for 20 hours, 24 hours after being transfected with an empty vector (Control) or a plasmid expressing BTK. Results show the mean of three experiments. Error bars, SD. C, Western blot analysis showing BTK and p53 protein levels in lysates of Ejp53 cultured in the absence of p53 (−) or 6 days after tet removal to induce p53 expression (+), treated with DMSO (−) or 0.5 μmol/L Ibrutinib (+) from the beginning of the experiment, compared with Ejp53 stably expressing a shRNA against BTK. D, Western blot analysis showing p53 protein levels in lysates of Ejp53 cultured in the presence or absence of tet to induce p53 for the specified time and treated with 0.5 μmol/L Ibrutinib or 2 μmol/L CGI-1746 since the beginning of the experiment. E, in vitro phosphorylation assay using recombinant BTK and GST-p53, either full length (FL) or fragments (comprising amino acids 1-80, 100-300 or 300-393). F, Western blot analysis of lysates of the same cells in B, showing protein levels of BTK and Ser15 phosphorylated p53 (P-p53).
after damage. Moreover, it did not increase the levels of p53 mRNA (Fig. 2B), which indicated a potential involvement of BTK in the posttranscriptional modifications of p53. To test this possibility, we used Ibrutinib, a BTK inhibitor recently approved to treat different forms of leukemia (23), CGI-1746, a more specific BTK inhibitor (25), and an EJp53 stably expressing a shRNA against BTK (Supplementary Fig. S1B). Blocking BTK by any of these methods severely reduced the levels of p53 induced by tet removal in EJp53, both after short- and long-term inhibition (Fig. 2C and D). Reduction in p53 protein levels in the absence of BTK was also observed in EJp53 and HCT116 infected with an adenovirus containing p53 (Supplementary Fig. S1C), as well as primary malignant B cells stimulated with CD154 and IL4, a culture protocol that we observed induces p53 expression (Supplementary Fig. S1D). This shows that the effects of BTK on p53 levels can be seen in different models of p53 induction and confirms that BTK is involved in the stabilization of p53 protein levels.

We reasoned that the positive effects of BTK on p53 accumulation could be mediated by phosphorylation, the most common mechanism of p53 regulation (5). As shown in Fig. 2E, we found that BTK was able to phosphorylate p53 in vitro and, using recombinant peptides (Supplementary Fig. S2A), we predicted that this phosphorylation takes place mainly in residues 1 to 80 of p53. This region of p53 contains serine 15 (S15), which is important in the MDM2-mediated regulation of p53 protein levels (5). Using a phospho-specific antibody, we confirmed that BTK increases S15 phosphorylation in cells in the presence or absence of DNA damage (Fig. 2F). Moreover, a S15A p53 mutant showed reduced phosphorylation by BTK in vitro (Supplementary Fig. S2B). p53 is known to be phosphorylated by ATM, ATR, DNA-PK, and PKC (37), among other kinases. Using specific inhibitors, such as daclotisib (38) and sotrastaurin (39), we found that the effects of BTK on p53 activity were independent of all these kinases (Supplementary Fig. S2C), suggesting a direct phosphorylation. This data together indicates that BTK increases the phosphorylation of p53 at S15, among other residues at the N terminus, which would result in the disruption of MDM2-p53 interactions and thus provide a mechanism for the increase in p53 stability.
Figure 4.
BTK contributes to p53-induced senescence. A, growth curves of EJp53 in the absence of p53 (Control) or after induction, treated with DMSO (Control and p53), 0.5 μmol/L Ibrutinib (left plot), or 2 μmol/L CGI-1746 (middle plot). Fresh media and drugs were added every time cells were counted. Right plot shows growth of EJp53 stable expressing a control shRNA (shLuci) or a specific one against BTK (shBTK) in the absence of p53 or after induction. Graphs represent mean of a triplicate experiment; error bars, SD. ***, P < 0.0001. (Continued on the following page.)
BTK enhances the transactivation of p53 target genes

Our data shows that BTK is important for the phosphorylation and stabilization of p53. This suggests that it could play a role in enhancing p53 activity. We explored this hypothesis by measuring the effect of BTK on the expression of p53 target genes. As shown in Fig. 3A, BTK increased the upregulation of the mRNA of both p21 (also known as Cdkn1a) and PUMA after treatment with doxorubicin. This was confirmed by a higher activity of the luciferase reporters in the presence of BTK (Fig. 3B). Finally, a ChIP analysis showed increased binding of p53 to the p21 and PUMA promoters after DNA damage when BTK was expressed (Fig. 3C).

In these experiments, BTK did not significantly increase the activity of p53 in the absence of damage, which is consistent with the fact that although p53 can be induced by BTK expression alone, it remains in the cytosol and it is thus inactive (Supplementary Fig. S1A). Importantly, when a BTK lacking the kinase domain (40) was expressed, the increase in luciferase activity of the p21 reporter was not observed (Fig. 3D and E), indicating that the positive effects on p53 transactivation are mediated by the kinase activity of BTK.

BTK inhibition blocks p53-induced senescence

We next studied the involvement of BTK in the cellular responses to p53. As shown in Fig. 4A, inhibition of BTK by chemical or genetic approaches increased cell proliferation in EJp53 induced to senesce. The inhibition of the permanent growth arrest was confirmed by the fact that the morphological changes associated with senescence were not as evident in cells in which BTK had been blocked (Fig. 4B) and that these cells were able to form colonies (Fig. 4C). EJp53 are a representative model of stress-induced premature senescence (41). To expand our observations, we also investigated whether BTK plays a role in replicative-induced senescence by serially passaging normal human fibroblasts in the presence of BTK inhibitors. As shown in Fig. 4D, Ibrutinib was able to delay the growth arrest of fibroblasts as they entered senescence. Moreover, expression of p16 (a well-known marker of senescence; ref. 42) was reduced in late passage cells when BTK was inhibited, similarly to what happened to the levels of p53 (Fig. 4E). This suggests that replicative senescence was also delayed in the absence of BTK. Indeed, the percentage of cells positive for senescence associated (SA)-β-gal, a widely used marker of senescence (32), was significantly lower when BTK was inhibited, and less cells showed the morphological changes typical of senescence (Fig. 4F). Collectively, these results indicate that BTK has a critical role in inducing and/or maintaining both p53-dependent stress-induced and replicative senescence.

BTK expression induces DNA damage signals and apoptotic responses

Our results suggest a new role for BTK in tumor suppressor responses in epithelial cells. We further explored this by studying the role of BTK in DNA damage responses. We first transfected BTK into HCT116 (Fig. 5A) and measured the activation of elements of the DNA damage pathways. We found that BTK expression leads to the phosphorylation of ATM and H2AX (Fig. 5B). BTK induced ATM in normal fibroblasts as well (Supplementary Fig. S3A). This correlated with increased damage to DNA, as measured by a comet assay (Fig. 5C). Together with this, we observed that BTK expression was able to induce apoptosis in HCT116 cells, as measured by PI and Annexin V stainings (Fig. 5D) as well as cleavage of caspase 3 and PARP (Fig. 5E). Moreover, this was also observed in Elp53 cells in the absence of p53 expression (Supplementary Fig. S3B), which suggests that it is at least in part p53-independent. BTK prevented Elp53 growth (Supplementary Fig. S3C) and colony formation (Supplementary Fig. S3D), similarly to the expression of p53. Finally, inhibition of BTK by Ibrutinib in HCT116 cells treated with doxorubicin significantly reduced the amount of cell death induced by the drug (Fig. 5F), thus confirming the involvement of BTK in the response to DNA damage in these cells. These data together suggest a proapoptotic and antiproliferative role of BTK that relies on its participation in DNA damage pathways and may not depend completely on its effects on p53.

Discussion

Our study reveals that BTK is a novel modulator of p53 activity and is involved in the cellular response to DNA damage. Although BTK has been implicated in oncogenic signaling in blood malignancies (16), here we show that it can also participate in tumor suppressor pathways. This is consistent with previous reports that also uncovered proapoptotic and anti-neoplastic properties of BTK (43–45). This suggests that BTK expression has radically different functions depending on the cellular context, which would include being part of prosurvival pathways in B cells and enhancing arrest/death signals in damaged epithelial cells. This paradox is supported by our bioinformatics analysis, which shows an opposed prognostic value of BTK levels of expression in blood malignancies and solid tumors (Fig. 1E).

Our experiments also allowed us to propose a mechanism by which BTK impacts on cell fate responses to damage. We found that following BTK expression, there is an increase in p53 phosphorylation at S15, among other residues, which would explain the BTK-mediated stabilization of p53 protein levels (5). This is independent of the activity of the kinases that most commonly phosphorylate p53 in response to damage, such as ATM, ATR, DNA-PK, or PKC. Although it would have to be further confirmed, this suggests that BTK could be directly phosphorylating p53 to activate it. Of note, despite being originally described as a tyrosine kinase, BTK is known to be able to phosphorylate serines as well.
which would fit this hypothesis. Nevertheless, we found that BTK can increase ATM and γH2AX, consistent with a role in enhancing of DNA damage signals. We also confirmed that the kinase function of BTK is essential for its effects on p53 accumulation. However, p53 still needs to be activated by other signals in the DNA damage pathway in order to relocate to the nucleus. All our data indicate that BTK is not sufficient to activate p53 on its own, but that it can increase its activity in the context of a DNA damage response. Indeed, we have observed that BTK expression increases the binding of p53 to the promoters of its target genes after genotoxic stress, which results in higher levels of expression of both proapoptotic and proarrest signals.

The importance of BTK in the different cellular responses to p53 is underscored by the consequences of its inhibition, because using both chemical inhibitors and RNAi we showed that p53 accumulation is compromised. The potential neoplastic effects of inhibiting BTK in clinical settings should need to be carefully considered. Large cohorts of patients are currently being given BTK inhibitors for the treatment of leukemia, which our results suggest could potentially affect p53 activity. So far there have been no reports of increased incidence of secondary malignancies but our data suggests that it is a possibility that should be studied. Inhibition of BTK results in the partial blocking of p53-induced senescence, which shows that BTK is a novel regulator of this tumor suppressor pathway. This is especially relevant because, despite the considerable knowledge accumulated in the 50 years since Leonard Hayflick first described cellular senescence (47), the molecular pathways involved in this phenotype are still incompletely characterized. Our results show that in the absence of BTK, p53-dependent senescence can be partially avoided and cells are allowed to continue growing, probably as a direct consequence of p53 protein levels being strongly reduced. Moreover, replicative senescence, which also relies on other triggers, such as p16 (42), was also delayed in the absence of BTK, which suggests that BTK could also be contributing to this phenotype in a p53-independent manner. These results provide a rationale to use BTK inhibitors to block senescence in those situations in which it

Figure 5.
Induction of the DNA damage and apoptotic pathways by BTK. A, Western blot analysis showing total BTK levels in lysates of HCT116 24 hours after being transfected with an empty vector (C) or a BTK vector (BTK). B, Western blot analysis of the same cells showing levels of BTK, ATM, and γH2AX. C, Comet assay performed in HCT116 cells untreated (C), treated with 50 μmol/L H2O2 for 30 minutes on ice (a positive control of DNA damage), or 24 hours after being transfected with an empty vector (V) or a vector expressing BTK. A total of 200 cells were scored for each condition. Error bars, SEM. ***, P < 0.0001 (unpaired t test). D, representative FACS plots of HCT116 transfected with an empty vector (Control) or BTK (BTK), then stained with PI (top) or Annexin V (bottom). Numbers represent percentage of sub-G1 events (dead cells, top) or Annexin V positive cells (apoptotic cells, bottom). E, Western blot analysis showing caspase 3 (full length, 32 kDa; cleaved forms, 17 and 12 kDa) and PARP (full length, 116 kDa; top cleaved form, 89 kDa) cleavage in lysates of the same cells shown in A, F, left, representative FACS analysis of PI-stained HCT116 treated with DMSO (Control), 1.5 μmol/L doxorubicin or doxorubicin and 0.3 μmol/L ibrutinib for 48 hours. Numbers indicate the percentage of events in the sub-G1 phase of the cell cycle (dead cells). Right, plots showing percentage of events in the sub-G1 in these cells. Results represent average of two independent experiments (done in triplicate or duplicate) and error bars show SD. **, P = 0.0092.
has a negative effect on the organism. For instance, they could have a therapeutic effect on diseases in which senescent cell accumulation is thought to be important, such as diabetes, osteoporosis, COPD, or neurodegenerative disorders (48). The accumulation of senescent cells strongly contributes to the progression of breast cancers (49) and this could also be avoided with the adequate regime of BTK inhibitors.

We observed that blocking BTK reduces p53-induced apoptosis as well. We show that BTK can have proapoptotic effects when overexpressed, which suggests that it could contribute to apoptotic signals in certain contexts, even in the absence of p53. Because we have shown that DNA damage signals are activated when BTK is expressed, it would be important to characterize other effects of BTK in these pathways, directly or through indirect mechanisms. We propose that the role of BTK on enhancing p53-dependent cell death is likely to be at least at two different levels: it would be important to stabilize p53 through phosphorylation and it would also contribute apoptotic signals independently.

In summary, our data presents a new side of BTK as a key modulator of p53 functions, being involved in a positive feedback loop that links its induction after p53 upregulation to its stabilization of p53. Thus, BTK emerges as a proapoptotic kinase and an important regulator of p53-mediated senescence and apoptosis. This underscores the complexity of BTK functions, which can sometimes be antagonistic and are likely to be determined by cellular context.

Disclosure of Potential Conflicts of Interest
Salvador Macip has ownership interest (including patents) in a patent application. No potential conflicts of interest were disclosed by the other authors.

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