Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth

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Summary

***p53*-dependent apoptosis is a major determinant of its tumor suppressor activity and can be triggered by hypoxia. No p53 target is known to be induced by p53 or to mediate p53-dependent apoptosis during hypoxia. We report that p53 can directly upregulate expression of *Bnip3L*, a cell death inducer. During hypoxia, *Bnip3L* is highly induced in wild-type p53-expressing cells, in part due to increased recruitment of p53 and CBP to *Bnip3L*. Apoptosis is reduced in hypoxia-exposed cells with functional p53 following *Bnip3L* knockdown. In vivo, *Bnip3L* knockdown promotes tumorigenicity of wild-type versus mutant p53-expressing tumors. Thus, *Bnip3L*, capable of attenuating tumorigenicity, mediates p53-dependent apoptosis under hypoxia, which provides a novel understanding of p53 in tumor suppression.**

Introduction

Solid tumors are poorly oxygenated as compared with normal tissues and possess regions of hypoxia (Vaupel et al., 1991). Apoptosis induced by hypoxia is a mechanism for elimination of stressed cells (Shimizu et al., 1996). In response to hypoxia, hypoxia-inducible factor 1 (HIF-1) activates genes involved in angiogenesis, anaerobic metabolism, and iron homeostasis (Semenza, 2000), which contribute to adaptive survival. HIF-1 also activates proapoptotic members of the *Bcl-2* family, including *Bnip3L* (Denko et al., 2003; Piret et al., 2002), which induces cell death, and plays an essential role in cardiac cell death during hypoxia (Kubasiak et al., 2002; Yussman et al., 2002). Under severe hypoxia, p53 protein is stabilized probably through HIF-1-dependent (An et al., 1998; Carmeliet et al., 1998; Piret et al., 2002; Hansson et al., 2002) and/or -independent mechanisms (Wenger et al., 1998; Pan et al., 2004). Wild-type p53-expressing tumor cells are targeted for apoptosis during hypoxia (Graeber et al., 1996). Thus, p53 is a key regulator of proliferation or survival during hypoxia associated with tumor progression (Schmaltz et al., 1998).

p53 is frequently mutated in human cancer and is considered a "guardian of the genome" in preventing cancer (Chan et al., 2000; Lane, 1992; Levine, 1997). Abundant evidence (Symonds et al., 1994; Attardi and Jacks, 1999; Aurelio et al., 2000; Bardeesy et al., 1995; Eischen et al., 2001; Meijerink et al., 1998; Schmitt et al., 2002; Soengas et al., 2001) indicates that the apoptotic activity of p53 is essential for tumor suppression. During tumor growth, hypoxia is a major stress that cells encounter (Achison and Hupp, 2003; Vaupel et al., 1991). To date, a number of p53 targets have been identified and proposed to mediate p53-dependent cell death in response to genotoxic stresses (Fei et al., 2002; Nakano and Vousden, 2001; Oda et al., 2000a, 2000b; Villunger et al., 2003; Jeffers et al., 2003). However, none of them has been shown to mediate p53-dependent cell death under hypoxia. In fact, p53 has been shown to be incapable of upregulating its known transcriptional targets during hypoxia, due to a failure to recruit coactivator proteins such as CBP or p300 to their promoters (Koumenis et al., 2001). We report here that *Bnip3L* is a proapoptotic transcriptional target of p53. Its induction by p53 under hypoxia appears to occur through the recruitment of coactivator CBP to *Bnip3L*, leading to a greater magnitude of Bnip3L induction in wild-type as compared to p53-deficient cells. Silencing of *Bnip3L* significantly blocks the apoptotic advantage in wild-type p53-expressing cells under hypoxia. *Bnip3L* knockdown promotes tumorigenicity in human tumor xenograft models if tumor cells contain wild-type but not mutant p53. In particular, nontumorigenic U2OS osteosarcoma cells were converted into a tumorigenic state following injection of nude mice with U2OS cells harboring stable *Bnip3L* knockdown. Our results suggest that *Bnip3L* can suppress tumor xenograft growth in vivo and is a mediator of p53-dependent apoptosis under hypoxia. The regulation of *Bnip3L* by p53 provides a novel mechanism by which p53 acts as a tumor suppressor in vivo.

SIGNIFICANCE

The p53 gene is important in tumor suppression during hypoxia, but none of its known proapoptotic targets is transactivated by p53 under hypoxic conditions. We show here that p53 can transactivate proapoptotic *Bnip3L* during hypoxia and that CBP and p53 are recruited to *Bnip3L* in vivo. Although *Bnip3L* is a HIF target, its silencing only significantly protects cells in culture from apoptosis induced by hypoxia if cells contain wild-type p53. Silencing *Bnip3L* promotes tumor growth in vivo through reduced sensitivity to hypoxia and increased proliferation. This work provides insights into hypoxic death of tumors and possible strategies for therapeutic restoration of tumor sensitivity during hypoxia.

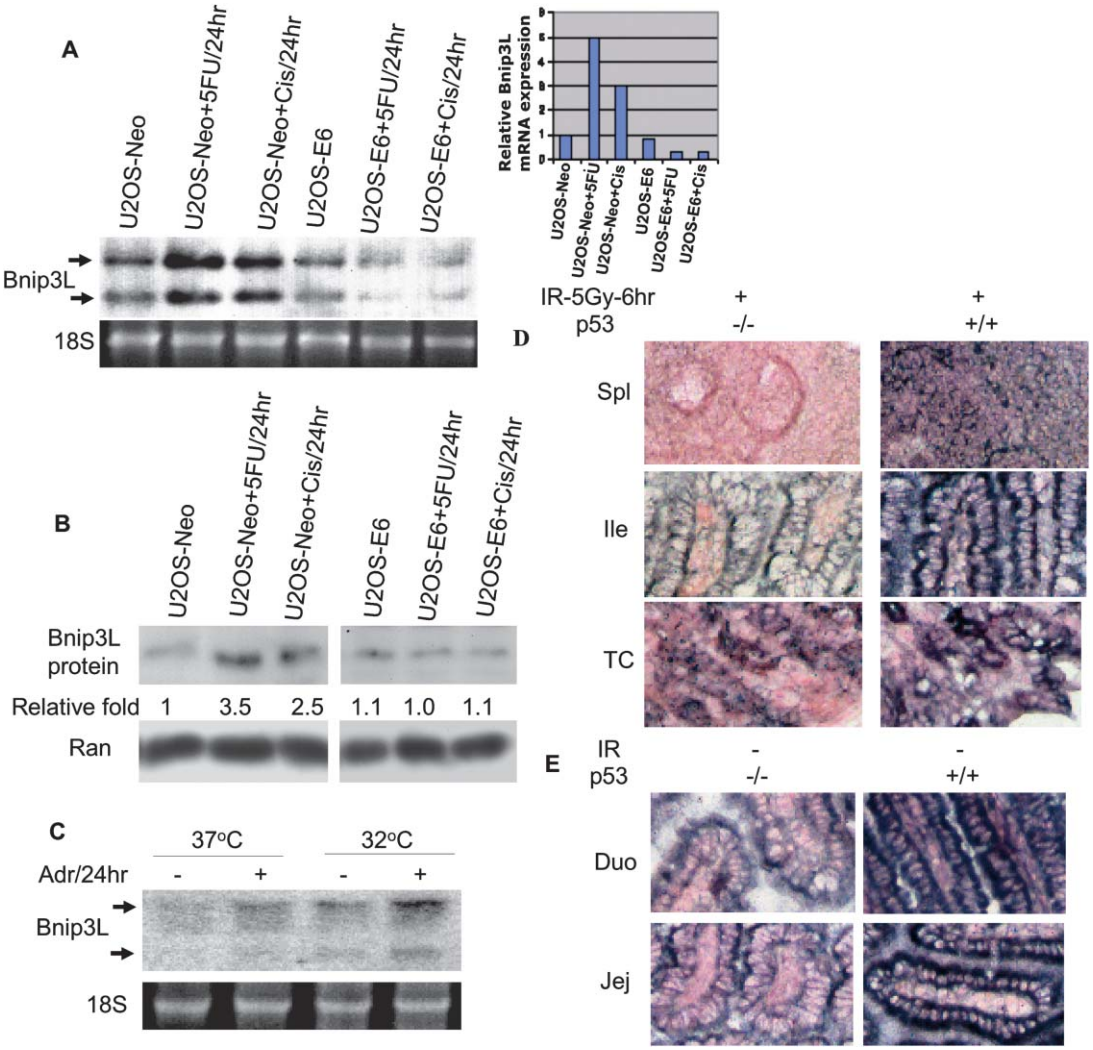

Figure 1. *Bnip3L* is a p53-regulated gene

A and B: *Bnip3L* induction at the mRNA level (**A**) or protein level (**B**) depends on wild-type p53 status following 5-FU and cisplatin (*Cis*) exposure of U2OS cells. The arrows indicate the two human Bnip3L transcripts (3.9 and 1.6 kb). Ethidium staining shows the 18S rRNA in the lower panel as a loading control. The quantification of *Bnip3L* induction by a phosphorimager is provided.

C: Bnip3L mRNA induction in MEFs carrying temperature-sensitive p53.

D and E: In vivo dependence of *Bnip3L* expression on wild-type p53 or γ irradiation-induced DNA damage in mice by in situ hybridization analysis. Spl, spleen; Ile, ileum; DC, descending colon; Duo, duodenum; Jej, jejunum.

Results

Bnip3L is a p53-regulated gene

Among the known activities of p53, sequence-specific DNA binding and transactivation explain the majority of its effects (El-Deiry, 1998). We hypothesized that the apoptotic effect of p53 under hypoxia may be mediated through transcriptional control, which prompted us to analyze candidate apoptotic target(s) that might mediate p53-dependent cell death during hypoxia. Using a microarray screening approach (Sax et al., 2002), we found that *Bnip3L* is upregulated 2.8-fold by wild-type p53. Bnip3L is a known apoptotic mediator (Piret et al., 2002) that is ubiquitously expressed as 1.6 and 3.9 kb mRNA transcripts (Yasuda et al., 1999). To confirm that *Bnip3L* is a p53-regulated gene, we examined *Bnip3L* induction in human tumor cells with varying p53 status before and after exposure to chemotherapeutic agents (Figures 1A and 1B). A higher level of Bnip3L mRNA expression was observed in wild-type p53-expressing U2OS cells after treatment with 5-fluorouracil (5-FU) (5-fold) or cisplatin (3-fold), as compared to untreated U2OS cells or treatment of U2OS cells carrying human papillomavirus E6 that targets p53 for degradation (Figure 1A). A similar pattern of Bnip3L protein induction was observed in chemotherapy-treated U2OS-Neo cells but not U2OS-E6 cells (Figure 1B). We further confirmed the regulation of *Bnip3L* by p53 in VM10 murine embryonic fibroblasts carrying temperature-sensitive p53 (Sax et al., 2002) and in $p53^{+/+}$ versus $p53^{-/-}$ mice. Bnip3L mRNA levels increased in VM10 cells when wild-type p53 function was restored (32°C), and a further increase occurred upon adriamycin exposure (Figure 1C). Using in situ hybridization, we found that Bnip3L expression (Figure 1D) increased in irradiated spleen, ileum, and transverse colon of wild-type but not p53-deficient mice. We also noted a higher basal expression of *Bnip3L* in

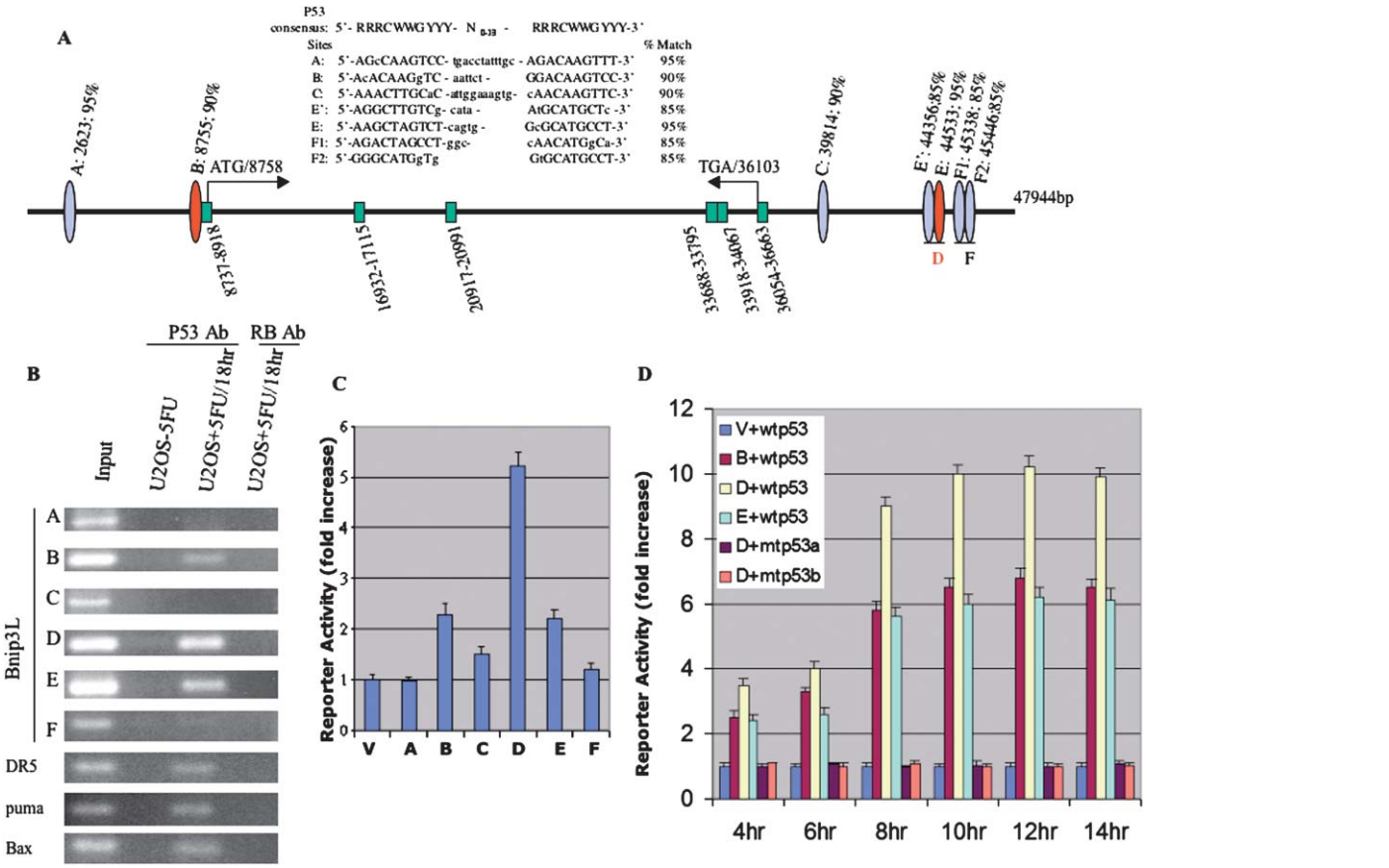

Figure 2. *Bnip3L* is a direct target of p53

A: The human *Bnip3L* gene, located on chr. 8, contains six exons (green rectangles) and five introns. Putative p53 binding sites (A, B, C, E, E′, F, and F′; oval shapes) with 85% or greater homology to the p53 binding consensus sequence are shown along with their sequences (nucleotides in uppercase indicate homology, whereas lowercase indicates a mismatch with the consensus). Fragments D and F contain two putative p53 binding sites each (E′ and E or F1 and F2, respectively). The red color indicates the fragments (B, D, and E) that could mediate p53-dependent transactivation. The first nucleotide is 8727 bp upstream of the first exon, based on the gene prediction method "BestRefseq" using accession number NT-023666. The relative locations for exons and putative p53 sites are labeled.

B: P53 binds to human *Bnip3L* in vivo. U2OS cells were incubated with (+) or without (−) 2 μg/ml 5-FU for 18 hr, and this was followed by a ChIP assay. Anti-Rb antibody was used as a negative control. *DR5*, *Puma*, and *Bax* were used as positive controls.

C and D: p53 transactivates reporters containing *Bnip3L* fragments. Reporter constructs (labeled in the figure as A, B, C, D, E, or F) and the original pGL-3 plasmid vector (V) were mixed with a renilla luciferase-expressing plasmid at a ratio of 10:1 as a transfection efficiency control. Plasmids were transfected into U2OS cells by Lipofectamine 2000 (Invitrogen). In **D**, reporter constructs with fragments B, D, or E or the pGL-3 vector (V) were transfected with pcDNA-wtp53 in Saos-2 cells. The reporter construct containing fragment D was cotransfected with mtp53a (R248G) or mtp53b (R275H) in Saos-2 cells. Transfected U2OS cells were plated in triplicate for 24 hr after transfection and treated with 2 μg/ml 5-FU for 20 hr. The relative fold-luciferase activity was standardized to the renilla luciferase activity. The DNA fragments B, D, and E increased reporter activity by 2-, 5-, and 3-fold in U2OS cells, and up to 6-, 10-, and 6-fold high at 10 hr after transfection in Saos-2 cells, respectively, as compared to the basal reporter luciferase expression.

duodenum and jejunum of wild-type versus *p53* null mice (Figure 1E). Taken together, *Bnip3L* expression is induced by p53 following DNA damage in vitro and in vivo.

Bnip3L is a direct target of p53

To gain insight into whether p53 might regulate *Bnip3L* directly, we searched the NCBI database and found that human *Bnip3L* contains seven putative p53 DNA binding sites with 85% or greater identity to the p53 consensus DNA binding sequence (El-Deiry et al., 1992) (Figure 2A). To determine whether p53 can bind to candidate p53 sites in human *Bnip3L*, a chromatin immunoprecipitation (ChIP) assay was performed using lysates from U2OS cells with or without 5-FU treatment. Precipitation with an anti-p53 antibody of 200–300 bp DNA fragments corresponding to sites B, D, or E was observed in 5-FU-treated U2OS cells (Figure 2B). In contrast, no DNA was recovered from precipitates of untreated cells or cells immunoprecipitated with an anti-RB control antibody. Thus, following 5-FU exposure, p53 localizes to human *Bnip3L* in vivo. To confirm that these p53 binding DNA segments can confer p53-dependent transcriptional activity, the six DNA fragments containing the above sites (Figure 2A) were cloned individually upstream of the minimal SV40 promoter in a reporter plasmid. We found that the D-reporter was activated 5-fold in 5-FU-treated U2OS cells (Figure 2C) and 10-fold in Saos-2 cells (Figure 2D) cotransfected with a wild-type p53-expressing plasmid. The D-reporter was not induced by either of two different mutant p53-expressing plasmids (plasmid "a" contains mutant p53 with an R248G substitution, whereas plasmid "b" contains mutant p53 with an R275H substitution). The B- and E-reporters were also induced

by p53 to similar levels (over 2-fold in U2OS cells and 6-fold in Saos-2 cells) as compared to the basal promoter vector (Figures 2C and 2D). The expression levels of the transfected p53 proteins in Saos-2 cells and the original images of the luciferase assays are provided in Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/. These results are consistent with the ChIP assay results (Figure 2B). Site A- and C-reporters were weakly transactivated by p53 (Figure 2C), possibly because they contain a spacer region between the p53 DNA binding half-site decamers (Tokino et al., 1994). It is possible that site E′ may have contributed to induction of luciferase activity in the D-reporter versus the E-reporter (Figures 2C and 2D). In summary, *Bnip3L* appears to be a target of p53, which can be directly regulated by p53 through sequence-specific DNA binding and transcriptional activation.

Bnip3L, unlike other proapoptotic targets of p53, remains inducible by p53 under hypoxia

In response to hypoxia, Bnip3L and its close family member Bnip3 have been identified as apoptotic mediators (Denko et al., 2003; Sowter et al., 2001). Wild-type p53 accumulates in tumor cells under hypoxic conditions (Figure 3A) and can exert an apoptotic function (Graeber et al., 1996; Graeber et al., 1994). However, none of the known apoptotic targets of p53 appear to be induced by p53 or to mediate p53-dependent apoptosis under hypoxic conditions. Thus, we explored the possibility that *Bnip3L* may be regulated by p53 during hypoxia. We found that Bnip3L mRNA is induced to significantly higher levels during hypoxia in wild-type p53-expressing U2OS-Neo, PA1-Neo, or parental U2OS cells as compared to the respective p53-deficient U2OS-E6, PA1-E6, or Saos-2 cells (Figures 3B–3D). Similarly, Bnip3L protein is elevated to much higher levels in wild-type p53-expressing cells as compared to p53-deficient control cells during hypoxia (Figures 3E and 3F). To investigate the uniqueness of *Bnip3L* regulation by p53 under hypoxia, expression of the *Bnip3L*-related *Bnip3*, or the other p53 targets *Puma*, *DR5*, *Bax*, or $p21^{WAF1}$ was analyzed. Unlike *Bnip3L*, the induction of hypoxia-inducible *Bnip3* was essentially identical among cells with or without functional p53 (Figure 3G). No transcriptional induction of *Puma*, *DR5*, *Bax*, or *p21* was observed under hypoxia (Figure 3G). In fact, *Bax* expression appeared to be reduced after hypoxia (Figure 3G), which is in agreement with recent findings that *Bax* and *Bid* are repressed under hypoxia (Erler et al., 2004). These results indicate that *Bnip3L*, unlike other known transcriptionally upregulated targets of p53, is upregulated by p53 under hypoxia. Moreover, the regulation by p53 during hypoxia is specific to *Bnip3L* and not to the closely related *Bnip3*.

The contrast in induction patterns under hypoxia between *Bnip3L* and other targets of p53 prompted us to determine whether interactions of p53 with its DNA binding sites in chromatin may vary among its target genes. ChIP assays revealed that genomic regions of *Bnip3L* bound by p53 in response to 5-FU treatment (Figure 2B) were the same fragments bound by p53 under hypoxia (Figure 4A). In contrast, p53 did not localize to the p53 binding regions of *Bax*, *Puma*, or *DR5* under hypoxia (Figure 4A), although p53 was observed to bind to these genes following exposure to genotoxic stresses (Figure 2B; Kaeser and Iggo, 2002). Thus, *Bnip3L* is directly bound by p53 under hypoxia and appears to be unique among known proapoptotic p53 targets in recruiting p53 protein to its genomic locus under hypoxia.

The work of Giaccia and colleagues indicated that p53 targets induced by DNA damage are not upregulated during hypoxia, because p53 fails to recruit CBP or p300 to their DNA binding sites (Koumenis et al., 2001). Here, we show that CBP is efficiently recruited to the p53 DNA binding regions of *Bnip3L* (regions B, D, and E), but not to *Bax*, *Puma*, or *DR5* (Figure 4B). This provides a mechanistic insight into the difference in the regulation of *Bnip3L* versus other known p53 targets under hypoxic conditions. Because HIF-1 is known to regulate *Bnip3L* (Piret et al., 2002) and HIF-1 function relies on the coactivators CBP and p300 (Arany et al., 1996), we hypothesized that HIF-1 may recruit CBP to the *Bnip3L* locus regardless of p53. We found that five of six DNA fragments containing putative p53 DNA binding sites within *Bnip3L* were coimmunoprecipitated with anti-CBP antibody under hypoxic conditions either in wild-type U2OS-Neo or p53-deficient U2OS-E6 cells (Figure 4B). However, not all of the fragments immunoprecipitated by the anti-CBP antibody were bound by p53 (Figures 2B and 4A). Because CBP was recruited to *Bnip3L* in hypoxia-exposed p53-deficient U2OS-E6 cells, the results argue that CBP recruitment is independent of p53 but may facilitate coactivation in cells with wild-type p53. Thus, p53 can enhance induction of *Bnip3L*, in concert with HIF-1 and/or other factors under hypoxic conditions.

To further investigate the kinetics and magnitude of *Bnip3L* induction in wild-type versus p53-deficient cells under hypoxic conditions, we performed a detailed time course to unravel the relationships between Bnip3L mRNA, Bnip3L protein, and p53 protein (Figure 4C). We found that Bnip3L mRNA induction occurs to higher levels in wild-type p53-expressing U2OS-Neo cells (16-fold) as compared to the observed 5-fold induction in the U2OS-E6 cells (Figure 4C). The induction of Bnip3L mRNA and protein peaked at 24 hr after p53 stabilization and was detected by 18 hr in the hypoxia-exposed U2OS-Neo cells (Figure 4C). It appears that increases in Bnip3L protein levels occur to a much greater extent in wild-type p53-containing cells as compared to the p53-deficient cells exposed to severe hypoxia (Figure 4C). Thus, a clear difference in the magnitude of Bnip3L mRNA and protein induction depends on p53 status in hypoxia-exposed cells. Bnip3L mRNA was induced 16-fold at 24 hr in U2OS-Neo cells but only 5-fold in the U2OS-E6 cells at 24 hr. Despite the 5-fold increase in Bnip3L mRNA expression by 24 hr after hypoxia, no increase in Bnip3L protein expression was observed in the U2OS-E6 cells exposed to severe hypoxia for 30 hr. This is in contrast to the 5-fold increase in Bnip3L protein expression detected by 24 hr in hypoxia-exposed U2OS-Neo cells (Figure 4C). Thus, one aspect of the importance of the p53-dependent regulation of *Bnip3L* involves the magnitude of Bnip3L mRNA and protein induction under conditions of hypoxia.

Bnip3L mediates p53-dependent apoptosis during hypoxia

In response to hypoxia, p53 contributes to a higher apoptotic activity in wild-type p53-containing cells (Schmaltz et al., 1998) (Figures 5A and 5B). *Bnip3L* was highly induced in cells with wild-type p53 (Figures 3 and 4C) as compared to the E6-expressing cells under hypoxia. The kinetics of cell death in hypoxia-exposed U2OS-Neo and U2OS-E6 cells (Figure 5B)

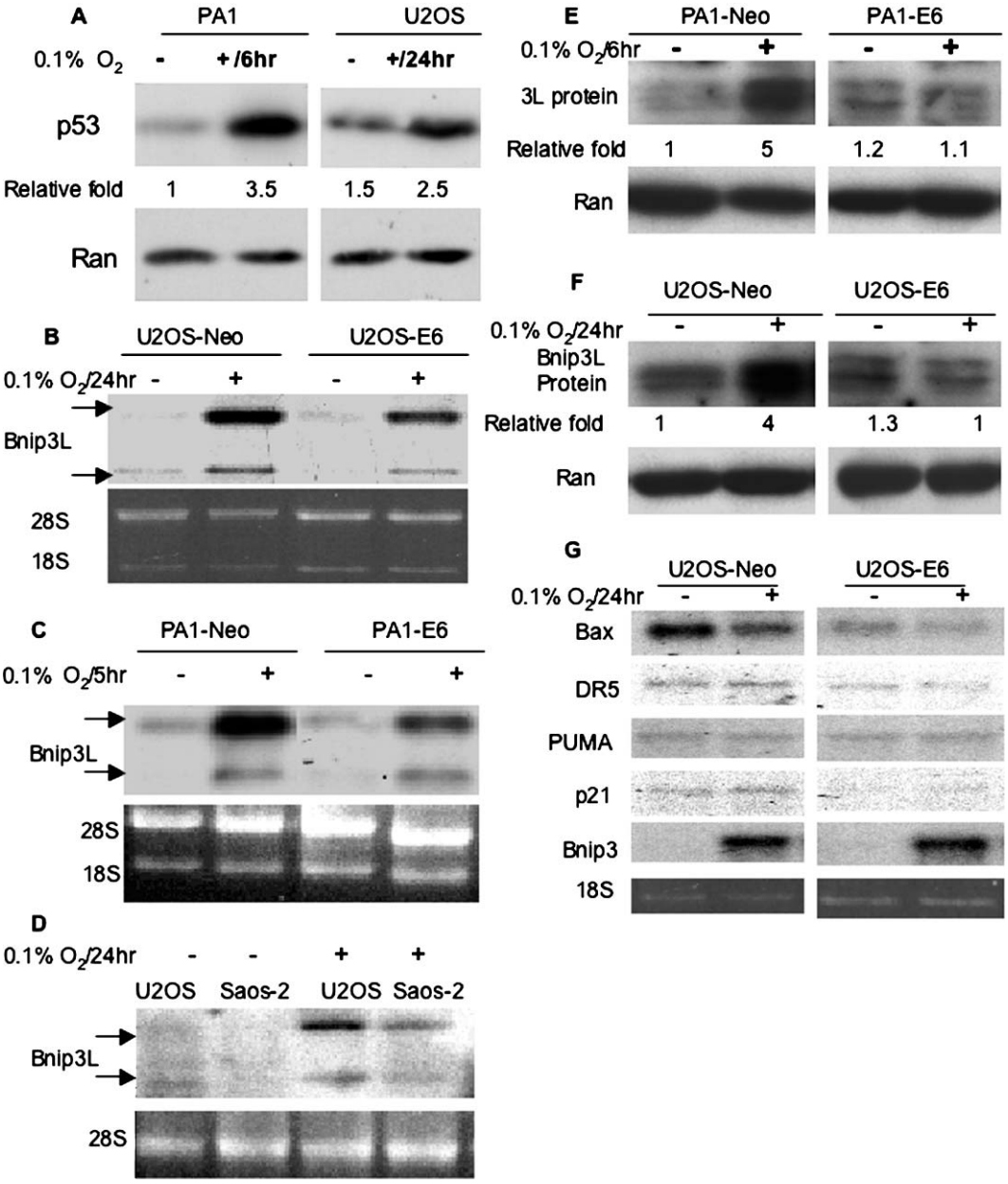

Figure 3. *Bnip3L* is inducible by p53 under hypoxia

A: P53 protein level is elevated in response to severe hypoxia.
B–F: A higher Bnip3L mRNA (**B–D**) or protein (**E and F**) induction occurs during severe hypoxia in wild-type as compared to p53-deficient cells.
G: Wild-type p53 status does not predict expression levels of other proapoptotic targets, or the close family member of *Bnip3L*, *Bnip3*, under hypoxic conditions. "−" indicates normoxia in experiments; "+" refers to 0.1% O_2.

correlated well with the kinetics of Bnip3L mRNA and protein induction (Figure 4C). To further address the relevance of the observed greater magnitude of induction of *Bnip3L* when cells contain wild-type p53, we generated Tet-inducible clones of *Bnip3L* in a wild-type p53-deficient background (Figure 5C). Apoptosis was observed in the Tet-inducible clones of *Bnip3L* following Bnip3L mRNA and protein induction in a manner independent of p53 regulation (Figure 5C). These results demonstrate a dosage effect of Bnip3L mRNA and protein expression with regard to apoptosis and further support the idea that *Bnip3L* induction is sufficient to induce apoptosis when conditionally expressed (and therefore may be sufficient to promote cell death when induced by p53 under hypoxia).

We investigated the role of *Bnip3L* in p53-dependent cell death under hypoxia through siRNA-mediated knockdown. siRNA oligonucleotides directed against *Bnip3L* in PA1-neo and U2OS-Neo cells reduced Bnip3L mRNA by 80% (Figure 5D). We found a 60% reduction in Bnip3L protein in the transfected hypoxia-exposed U2OS-Neo cells (Figure 5E). Forced downregulation of *Bnip3L* expression (Figures 5D and 5E) during hypoxia was correlated with significant blockade of apoptotic death in wild-type p53-containing PA1-Neo and U2OS-Neo cells (Fig-

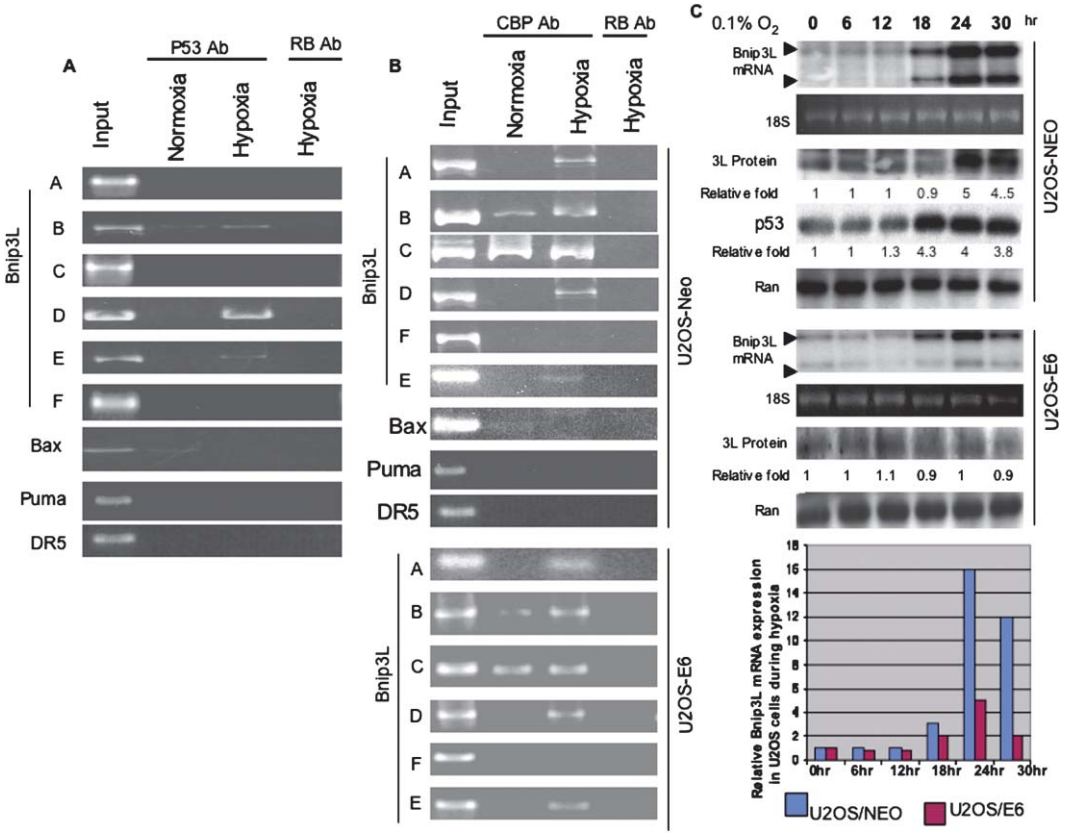

Figure 4. *Bnip3L* remains as a direct target of p53 under hypoxia

A: P53 binds to the human *Bnip3L* locus but not to *Bax*, *Puma*, or *DR5* during hypoxia in vivo. A ChIP assay was performed with anti-Rb antibody as a negative control. For 22 hr, 0.1% O_2 was used as the hypoxia condition.

B: CBP is recruited to the p53 binding region of *Bnip3L* but not to the p53 binding regions of *Bax*, *Puma*, or *DR5*. A ChIP assay was performed with anti-Rb antibody as a negative control.

C: Kinetics and magnitude of induction of Bnip3L mRNA, Bnip3L protein, and p53 protein under severe hypoxia in U2OS cells. The quantitative information for Bnip3L mRNA expression, analyzed using a phosphorimager, is provided below the blots.

ures 5F and 5G, upper panels). In contrast, the p53-deficient E6-expressing cell lines were not affected (PA1-E6; Figure 5F) or became only slightly more resistant to cell death after *Bnip3L* knockdown (U2OS-E6; Figure 5G). As additional evidence for these effects, we tested different *Bnip3L* RNAi sequences (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/). *Bnip3L* knockdown protected PA1-Neo and U2OS-Neo cells from the apoptotic effects of severe hypoxia (Supplemental Figure S2). The results in Figure 5 and Supplemental Figure S2 suggest that *Bnip3L* contributes to apoptotic death in wild-type p53-expressing cells exposed to hypoxia. The induction of *Bnip3L* by hypoxia in E6-expressing cells appears to have little effect in inducing cell death as noted by the lack of substantial change in percent of cells undergoing apoptosis following *Bnip3L* knockdown. The kinetics of the observed death and lack of significant effect following *Bnip3L* knockdown correlate well with the observation that Bnip3L protein expression in E6-expressing cells was not detectably elevated under the conditions studied here (Figure 4C). These results support the conclusion that *Bnip3L* appears to mediate in part p53-dependent apoptosis under hypoxia. These studies suggested a possibility that we further tested, that loss of *Bnip3L* regulation in part through p53 dysfunction may afford a selective growth advantage to tumors under hypoxic conditions.

Knockdown of *Bnip3L* promotes human tumor xenograft growth in vivo

Tumors must endure a certain level of hypoxia during their development (Hammond et al., 2002; Vaupel et al., 1991). Wild-type p53-expressing tumor cells, which possess a higher apoptotic activity, are naturally eliminated during tumor formation (Graeber et al., 1996). The high apoptotic activity of wild-type p53-expressing cells under hypoxia correlates well with the high frequency of p53 mutations in human tumors, which provide a selective growth advantage under hypoxic stress during tumor evolution and progression. Because *Bnip3L* is a proapoptotic target of p53 functioning under hypoxic conditions, a role in suppressing tumor formation may provide a mechanism by which p53 mediates tumor suppression, particularly under hypoxic conditions. Thus, in order to elucidate the importance of *Bnip3L* in tumor formation, we generated stable cell lines U-11 and U-17 (derived from U2OS) that constitutively express siRNA directed against human *Bnip3L*, or pooled U2OS cells infected with *Bnip3L* RNAi-expressing retrovirus (U2OS p3LKD cells) (Supplemental Figure S3A at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/). At 10 days after injection of 10 million cells (U-11, U-17, U2OS p3LKD, or selected control parental cells U2OS-s; control and *Bnip3L* knockdown injections on op-

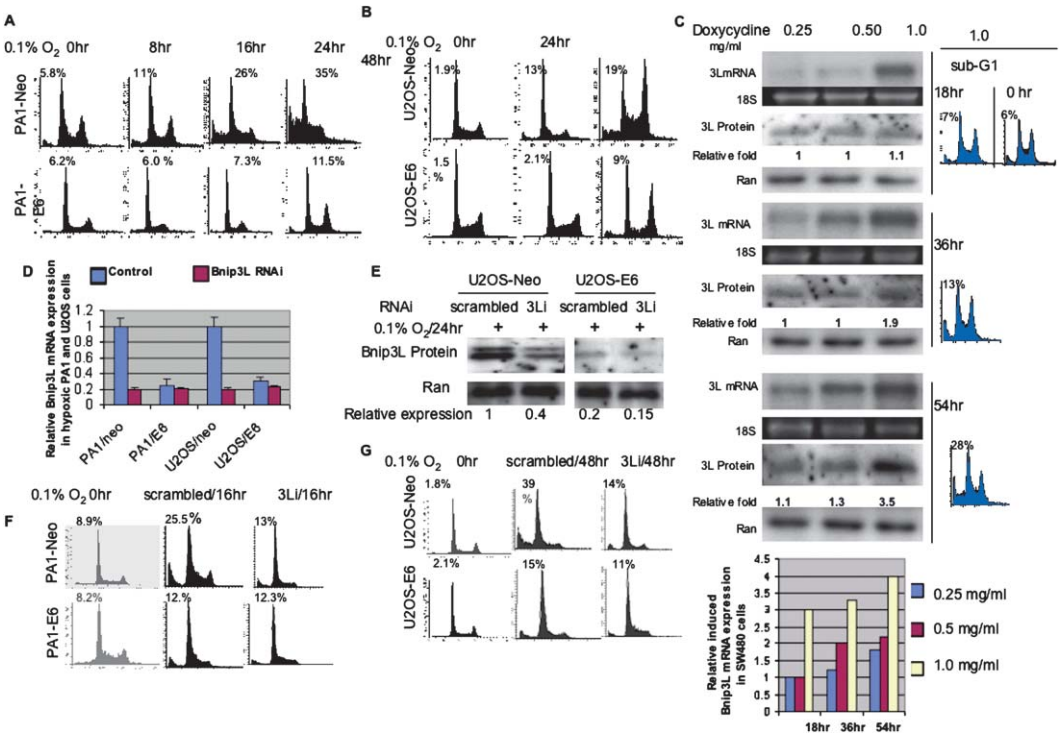

Figure 5. *Bnip3L* is a mediator of p53-dependent cell death under hypoxia

A and B: Higher apoptotic activity is observed in wild-type p53-expressing PA1-Neo and U2OS-Neo cells as compared to PA1-E6 and U2OS-E6 cells exposed to severe hypoxia.

C: Induction of exogenous Bnip3L mRNA and protein expression promotes apoptosis in mutant p53-expressing SW480 tumor cells following use of the Tet-on inducible system to restore regulated expression of *Bnip3L*. The level of Bnip3L mRNA progressively increased according to the dose of doxycycline. Bnip3L protein was clearly increased at the highest dose of doxycycline (see 54 hr time point). The percentage of cell death correlated with the increased level of Bnip3L protein. No increase in cell death was observed in parental SW480 cells treated with 1 μg/ml doxycycline for 54 hr (data not shown). The quantitative information for Bnip3L mRNA expression is provided below the blots.

D and E: Both levels of Bnip3L mRNA (**D**) and protein (**E**) were analyzed to determine the efficiency of RNAi by real-time quantitative RT-PCR (**D**) or Western blotting under hypoxia (**E**). A similar result to that in Figure 5E was noted in Western blotting the RNAi-transfected PA1 cells (data not shown).

F and G: Knockdown of *Bnip3L* diminishes the apoptotic death in both PA1-Neo and U2OS-Neo cells. "3Li" refers to *Bnip3L* RNAi-transfected cells.

posite flanks as indicated in Supplemental Figure S3B), the U-11, U-17, and pooled U2OS p3LKD xenografted cells formed tumors. In contrast, the U2OS-s xenograft disappeared by 5 days after injection, consistent with prior reports that U2OS cells do not form tumors in nude mice (American Type Culture Corporation; Anderson et al., 2000). In subsequent experiments, a constitutively expressing firefly luciferase gene cloned in a retrovirus vector was introduced into the U2OS-s, U-11, U-17, and p3LKD cells to monitor the dynamic course of tumor formation in nude mice. Bioluminescence intensities (Figure 6) of firefly luciferase-expressing U-11, U-17, or p3LKD and U2OS-s, were roughly equal, with perhaps more signal from the parental U2OS-s injection sites at the beginning of the experiment (day 0). On subsequent days, the bioluminescence emitted from the U2OS-s cells decreased as compared to the U-11, U-17, or p3LKD xenografts, and gradually the control bioluminescence from the U2OS-s xenografts disappeared (compare days 0, 3, and 6 in Figure 6). In contrast, the bioluminescence emitted by the U-11, U-17, or p3LKD xenografts initially increased in intensity and persisted through day 21 (Figure 6) and subsequently decreased (data not shown). A higher intensity of bioluminescence was also observed in *Bnip3L* knockdown PA1 xenografts (Supplemental Figure S3C) as compared to the control PA1 xenograft (PA1-s) over time, and a slight growth advantage was also observed in the mutant p53-expressing SW480 xenografts with *Bnip3L* knockdown (Supplemental Figure S4). These data suggest that *Bnip3L* suppresses tumor growth as a tumor suppressor, particularly in tumors that contain wild-type p53. We speculate that the ultimate reduction of bioluminescence in the *Bnip3L*-silenced U2OS derivatives (data not shown beyond 21 days) may reflect the action of other p53 targets and/or other death inducing protein(s), which may contribute to tumor suppression. Moreover, host immunity resulting from NK cell activity and/or other factors may contribute to the elimination of U2OS-derivative xenografts and the eventual suppression of the *Bnip3L*-silenced tumors.

Bnip3L is a mediator of p53-dependent radiosensitivity

Wild-type p53 plays a critical role in radiosensitivity (Fei et al., 2002; Fei and El-Deiry, 2003). Tumors harboring mutations in p53 generally carry a poor prognosis following radiotherapy, as has been observed, for example, in breast cancer (Marchetti et al., 2003). However, the efficacy of ionizing radiation directly relies on adequate oxygen tensions (Weinmann et al., 2003). To probe a potential role of *Bnip3L* in p53-dependent radiosensitivity, mice were irradiated with 5 Gy on day 4 after injection

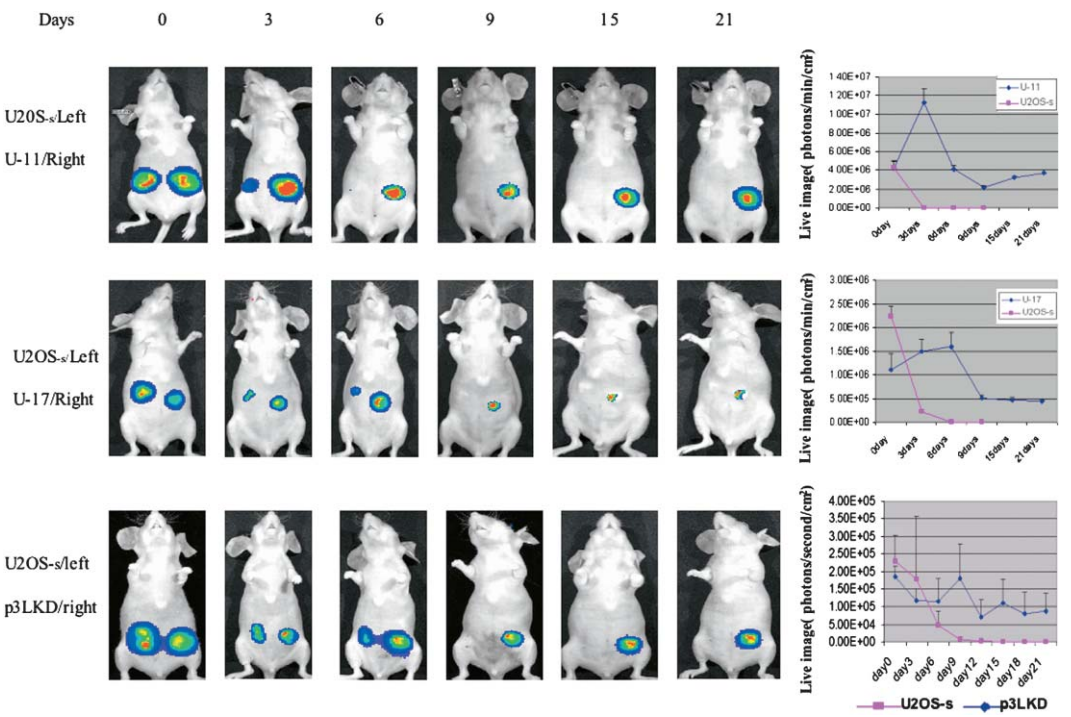

Figure 6. Knockdown of *Bnip3L* promotes tumorigenicity

U-11, U-17, pooled U2OS 3L knockdown (p3LKD; *Bnip3L* silencing was mediated through retrovirus infection of RNAi-expressing cDNA), or U2OS-s cells were infected with firefly luciferase-expressing retroviruses. Ten million cells of each were injected into mice. On day 0, xenografts of U2OS-s/U-11 (upper), U2OS-s/U-17 (middle), and U2OSs/p3LKD (bottom) emitted similar levels of bioluminescence, or there was a slightly higher emission from the U2OS-s xenografts. Bioluminescence was imaged over time and quantified. The bioluminescence from U2OS-s xenografts disappeared by day 6, whereas U-11, U-17, and p3LKD xenografts continued to emit light detectable up to day 21 shown here. A typical mouse image (the same mouse is shown over the time course) is presented from a total of four mice for U-11/U-17 xenografts or from a total of eight mice for p3LKD xenografts. Quantification with error bars of the photon counts per unit time per cm^2 from the wild-type (U2OS-s) as compared to the *Bnip3L*-silenced tumor xenografts appears to the right.

of 10 million U2OS-s versus either U-11 or p3LKD cells (Figure 7A and Supplemental Figure S5A at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/). On day 4, the U2OS-s xenografts were still alive and were excised at 6 hr after irradiation and compared with the nonirradiated controls (Figure 7A and Supplemental Figure S5). Active caspase-3 appeared to be significantly increased in irradiated U2OS-s xenografts as compared to the nonirradiated U2OS-s and either the irradiated or untreated U-11 or p3LKD xenografts (Figures 7B and 7C; Supplemental Figures S5B and S5C). There was more active caspase-3 in irradiated U-11 or p3LKD versus unirradiated U-11/p3LKD xenografts, respectively, consistent with the hypothesis that *Bnip3L* may be involved in but is likely not the only mediator of apoptosis following irradiation. Nonetheless, silencing of *Bnip3L* appears to reduce apoptotic activity initiated in irradiated wild-type p53-containing xenografts, suggesting an important role for *Bnip3L* among the apoptotic targets of p53.

Decreased sensitivity to hypoxia in vivo contributes to tumor growth following *Bnip3L* silencing in U2OS xenografts

We further examined the U2OS-s control, U-11, and pooled U2OS (p3LKD) tumors with *Bnip3L* silencing to gain insights into whether the growth advantage and greater proliferation in U-11 or p3LKD may be due to reduced sensitivity to hypoxia. U2OS-s, U-11, or p3LKD xenografts were excised from tumor-bearing mice on day 4, prior to regression of the U2OS-s tumors (Figure 7D and Supplemental Figure S5D at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/). Immunostaining for the Ki67 antigen confirmed the proliferative advantage in the U-11 and p3LKD tumors in which *Bnip3L* was silenced (compare Figure 7D and Supplemental Figure S5D), and staining for active caspase 3 confirmed a lower level of apoptosis in U-11 versus U2OS-s and p3LKD versus U2OS-s (Figure 7D and Supplemental Figure S5D). However, examination of either Glut1 expression (Figure 7D) or pimonidazole adducts (Supplemental Figure S5D) showed comparable staining between U2OS-s, U-11, or U2OS p3LKD tumors, suggesting similar degrees of hypoxia. These results suggest that silencing of *Bnip3L* does not reduce the hypoxic gene induction response leading to increased Glut1 expression; rather, despite the hypoxia, there is reduced apoptosis and increased proliferation in the U-11 or p3LKD versus U2OS-s tumors. One interpretation is that silencing of *Bnip3L* results in reduced sensitivity to hypoxia and increased cell survival that is permissive for tumor proliferation and the observed growth advantage of U-11 or p3LKD tumors under hypoxia.

Discussion

A high frequency of *p53* mutations is associated with a growth advantage in developing tumors (Vogelstein et al., 2000). Numerous studies have documented (Symonds et al., 1994; Attardi and Jacks, 1999; Aurelio et al., 2000; Bardeesy et al., 1995; Eischen et al., 2001; Meijerink et al., 1998; Schmitt et al., 2002;

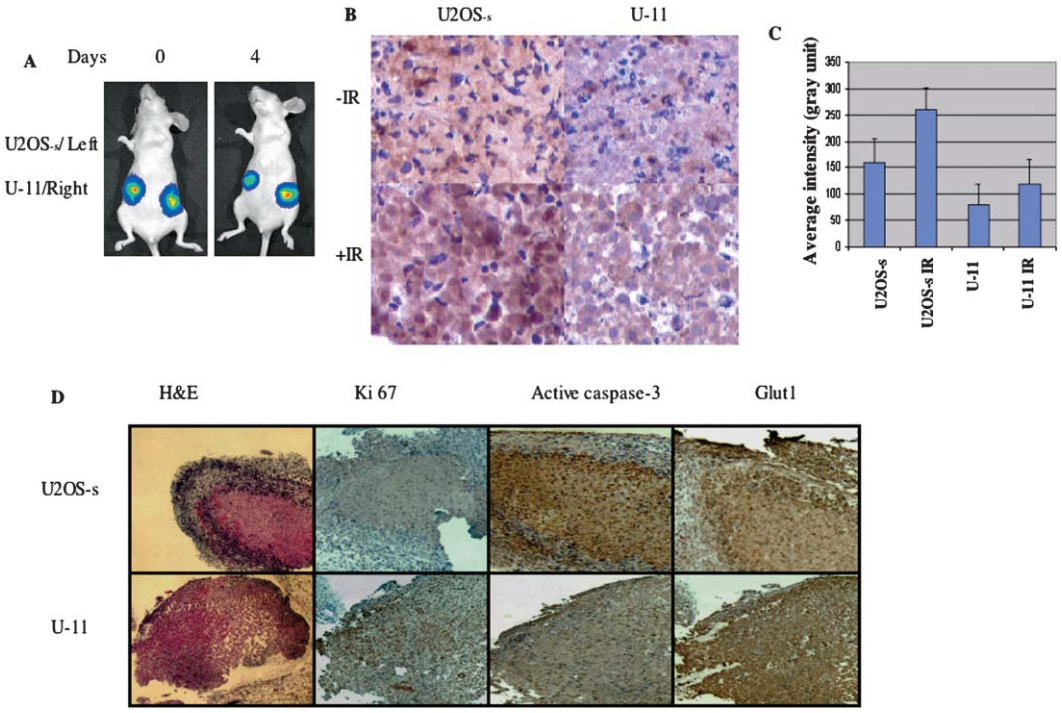

Figure 7. Knockdown of *Bnip3L* is accompanied by reduced apoptosis in irradiated or unirradiated xenografts, and the reduced sensitivity to hypoxia-mediated cell death is accompanied by increased proliferation of tumors

A–C: Two mice were injected with 10 million cells of U2OS-s or U-11 cells. On day 4 (images in **A**), mice were either untreated or treated with 5 Gy ionizing radiation, and the xenografts were excised 6 hr later. The image shown was obtained from one of the two mice (the same mouse) at the two different time points shown. The active caspase-3 signal (brown color in cytoplasm) (**B**) was highest in the irradiated (+IR) U2OS-s xenograft, moderate in the nonirradiated (−IR) U2OS-s xenograft, low in the +IR U-11 xenograft, and lowest in the unirradiated U-11 xenograft. The relative amount of active caspase-3 expression is shown (**C**) by using NIH ImageJ software.

D: Day 4 xenografts were harvested, and the nonirradiated U2OS-s or U-11 xenografts revealed similar levels of Glut1. In *Bnip3L*-silenced xenografts, active caspase-3 levels were lower, whereas Ki67 expression was higher as compared to the control xenograft.

Soengas et al., 2001) that the apoptotic effect of p53 plays a critical role in tumor suppression. To date, several proapoptotic targets of p53 have been suggested to mediate p53-dependent apoptosis following exposure to genotoxic stresses. However, none of the known p53-activated proapoptotic genes has been shown to mediate p53-dependent apoptosis in response to hypoxia, as it occurs during tumor development and progression. Here, we demonstrate that *Bnip3L* is a recognized target of p53 and is a strong candidate mediator of p53-dependent apoptosis during hypoxia. Importantly, *Bnip3L* appears to inhibit tumorigenicity such that silencing of *Bnip3L* allows conversion of nontumorigenic U2OS-s cells to tumorigenic xenografts. Silencing *Bnip3L* reduces sensitivity to apoptosis under hypoxic conditions, thereby permitting a higher degree of proliferation (Figure 7D and Supplemental Figure S6A). These studies suggest that loss of *Bnip3L* regulation by p53 under hypoxia may be a pivotal event in tumor development, which contributes to decreased apoptosis, and cells with lowered apoptotic potential (Graeber et al., 1996) grow into tumors. Taken together, the work shown here provides a mechanism regarding how p53 induces apoptosis and suppresses tumors under hypoxic conditions. Moreover, this work provides a mechanistic insight into the selection pressure that occurs under hypoxia and an apoptotic mediator that needs to be suppressed to permit tumor growth.

P53 induces *Bnip3L* under hypoxia

Bnip3L, unlike its family member *Bnip3*, not only responds to HIF-1 (Sowter et al., 2001; Bruick, 2000) but also responds to wild-type p53 (Figures 1–3). Other known proapoptotic targets of p53 are either uninduced (Koumenis et al., 2001), repressed (Figure 3G; Erler et al., 2004), or induced without a preference to p53 status in hypoxic cells (Kim et al., 2004). To date, *Bnip3L* is the only apoptotic target of p53 identified that appears to be upregulated by wild-type p53 in response to hypoxia. Here, we show that p53 localizes to the p53 DNA binding sites of *Bnip3L* but not to *Bax*, *Puma*, or *DR5* during hypoxia (Figure 4A), although these targets can be induced and p53 localizes to their genomic loci following DNA damage. A failure of p53 to transactivate its known targets under hypoxia has been previously reported and was attributed to a lack of recruitment of CBP or p300 to their genomic loci (Koumenis et al., 2001). We confirmed the lack of induction of *Bax*, *Puma*, or *DR5* during hypoxic exposure of wild-type p53-expressing human tumor cell lines (Figure 3G). We confirmed that CBP is not recruited to the p53 DNA binding regions of *Bax*, *Puma*, or *DR5* (Figure 4B) consistent with the Koumenis et al. (2001) model. However, CBP was efficiently localized to the p53 DNA binding regions of *Bnip3L* (Figure 4B). Two regions that bound CBP, including p53 DNA binding site-containing fragments A and C, were not shown to be bound by p53 (Figures 2B and 4A), which suggests

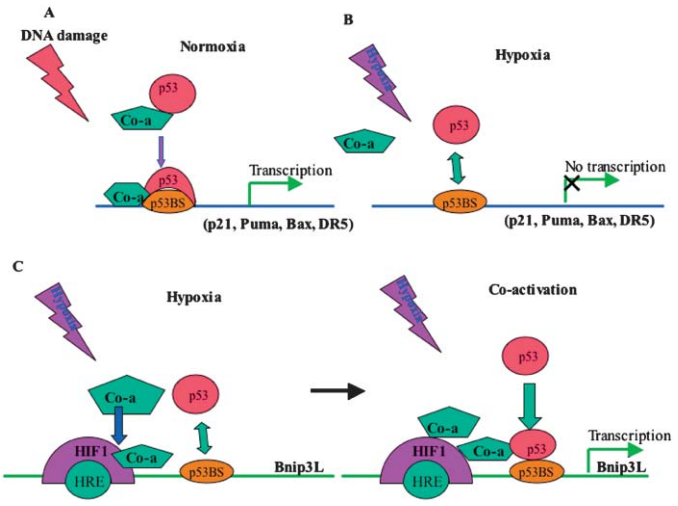

Figure 8. Model of *Bnip3L* regulation by p53 under hypoxia

A: Under normoxia, p53 recruits coactivators (Co-a) CBP or p300 to its consensus binding sequence (CBS) and transactivates its target genes in response to DNA damage. During hypoxia, p53 fails (**B**) to recruit coactivators to its binding sequence and does not transactivate its known proapoptotic targets or p21. *Bnip3L*, through HIF-1 (**C**), recruits coactivator CBP close to p53 binding regions under hypoxia. p53 protein interacts with CBP while interacting with its binding sites. A cooperative interaction involving HIF-1, CBP, and p53 is proposed to lead to higher levels of Bnip3L in cells containing wild-type p53. In such cells, Bnip3L appears to be a major mediator of apoptosis when the cells are exposed to severe hypoxia.

that other *cis* elements within *Bnip3L* may play an essential role in the recruitment of CBP to *Bnip3L* under hypoxia. We propose a model (Figure 8) aiming to explain how p53 may regulate *Bnip3L* under hypoxia. Under hypoxic conditions, p53 fails to interact with CBP (Koumenis et al., 2001) and interacts with its p53 DNA binding sites weakly and transiently (Figure 8B). However, p53 may interact with CBP and bind firmly to putative p53 DNA binding sites when the local level of CBP is stimulated by HIF-1, which primes the putative p53 DNA binding sites in the vicinity of HIF-1 response elements (HRE). We speculate that CBP promotes p53 recruitment by altering chromatin structure to allow access to DNA binding sites for p53 or by physical proximity to allow interaction between CBP and p53 (Figure 8C), and this will not occur in p53-deficient cells, although CBP may be present (Figure 4B). In our studies, p53 protein is absent in E6-expressing cells under hypoxia, although HIF-1 protein is present (Supplemental Figure S6A at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/). Thus, in cells lacking p53, our model predicts a failure to optimally induce *Bnip3L* to sufficient levels, thereby leading to resistance to the apoptotic effect of severe hypoxia.

We note that the genomic locus of *Bnip3L* contains p53 DNA binding sites upstream of the first coding exon and downstream of the last coding exon (Figure 2A). It is not uncommon for p53-regulated genes to contain multiple p53 response elements or to have sites located within downstream introns. It is not clear if sites downstream of coding exons are significantly different in function from sites located within introns in terms of transactivation by p53. Nonetheless, the genomic region containing site B is a good candidate mediator of the observed p53-dependent *Bnip3L* transactivation under hypoxia because of its location near the first coding exon of *Bnip3L* (Figure 2A), its ability to bind p53 (Figure 2B) including under hypoxia (Figure 4A), and its potential to mediate p53-dependent transactivation of promoter-reporter plasmids (Figures 2C and 2D). Moreover, a candidate HIF site (Supplemental Figure S6B at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/) is located within 850 base pairs of the p53 site B. Thus, it is possible that CBP is recruited to the genomic region of site B through HIF, and then p53 can be recruited to the region through its DNA binding response element. We note that the mouse *Bnip3L* locus contains candidate p53 binding sites, with 80% homology to the consensus sequence, both upstream of the first coding exon and downstream of the last coding exon (Supplemental Figure S6C). We also note that there are two candidate p53 binding sites with 75% homology to the p53 consensus in intron 1 of mouse *Bnip3L* (Supplemental Figure S6C) and that there is a 75% homology site in intron 1 of human *Bnip3L* (data not shown). Thus, regulation of *Bnip3L* by p53 appears to be conserved between human and mouse cells, as evidenced by structural similarity between the two loci (Figure 2A and Supplemental Figure S6C) and evidence for p53-dependent *Bnip3L* induction in both species (Figures 1–3). In the future, more detailed studies will examine the contribution of specific p53 sites to *Bnip3L* regulation under various conditions including DNA damage and hypoxia, with detailed analysis of the spatial and temporal interactions between HIF-1, CBP, and p53 proteins at the genomic regulatory region of *Bnip3L*. To date, an analysis documenting the importance of any specific genomic DNA response element for p53 has not been performed for any gene, including those that contain multiple p53 sites. Such studies on the mechanism(s) by which p53, CBP, and HIF coordinately regulate *Bnip3L* may provide a greater understanding of *Bnip3L* regulation during p53-dependent tumor suppression under hypoxia and may help to find ways to maintain or restore apoptotic target gene expression and prevent tumor progression.

Bnip3L is an apoptotic mediator of p53 under hypoxic conditions

We have shown a clear difference in the magnitude of Bnip3L mRNA and protein induction depending on p53 status in hypoxia-exposed cells (Figure 4C). p53 protein stabilization occurred before the induction of Bnip3L mRNA and protein, and a much greater extent of induction of *Bnip3L* was found in wild-type p53-containing cells as compared to p53-deficient cells following exposure to hypoxia. Thus, p53 appears to play a critical role in the magnitude of *Bnip3L* induction under hypoxia. We believe that the magnitude of *Bnip3L* induction in wild-type p53-expressing hypoxic cells is a major determinant of subsequent cell death. To further explore the possible importance of the greater magnitude of induction of *Bnip3L* in wild-type p53-containing cells exposed to hypoxia, we established Tet-inducible clones of *Bnip3L* in mutant p53-expressing SW480 cells. This design was intended to (1) uncouple p53 regulation from the level of *Bnip3L* induction and (2) determine whether *Bnip3L* induction to high levels (not normally achieved in the absence of wild-type p53) would be sufficient to induce apoptosis in p53-deficient cells. The Tet-inducible *Bnip3L* SW480 cells demonstrated that there is a dosage effect of Bnip3L mRNA and protein expression with regard to the observed apoptosis (Figure 5C). This *Bnip3L*-dependent apoptosis in a p53-deficient background required high levels of Bnip3L

mRNA and protein, and correlated well with what we described regarding *Bnip3L* regulation under hypoxia in wild-type versus p53-deficient U2OS cells (Figure 4C). Cells deficient in p53 function showed only modest increases in *Bnip3L* mRNA expression that we believe may not result in efficient cell death induction under hypoxia (Figure 4C). Moreover, the studies using Tet-inducible *Bnip3L* in p53-deficient SW480 cells confirm that *Bnip3L* is sufficient to induce apoptosis when conditionally expressed and therefore may be sufficient when induced by p53 under hypoxia. This is important because p53 may induce other targets that may be involved in cell death under hypoxia, but induction of *Bnip3L* is sufficient once a certain level of induction is reached.

To further support our conclusion that p53-dependent regulation of *Bnip3L* under hypoxia is relevant to the observed apoptosis and tumor suppression, we investigated the effects of *Bnip3L* silencing in p53-deficient cells. The p53-deficient cells were already more resistant to hypoxia-induced apoptosis (Figures 5A and 5B), and the magnitude of Bnip3L mRNA and protein induction by hypoxia was low (Figure 4C). Silencing of *Bnip3L* in p53-deficient U2OS-E6 cells only slightly reduced the observed apoptosis following exposure to hypoxia (Figure 5G) as compared to U2OS-Neo cells, where a dramatic protection was observed (Figure 5G). Taken together with the results of the Tet-inducible *Bnip3L* experiments (Figure 5C), these findings reinforce two conclusions: (1) p53-deficient human tumor cells die inefficiently when exposed to severe hypoxia, despite the fact that *Bnip3L* and other hypoxia-inducible targets are induced, and (2) blockade of *Bnip3L* has much more significant effects on hypoxia-induced cell death in wild-type p53-expressing cells because of its greater magnitude of induction. We believe the greater magnitude of *Bnip3L* induction represents the contribution of p53 to hypoxia-induced cell death and further conclude that hypoxic death is inefficient in p53-deficient cells, possibly because sufficient levels of Bnip3L are not achieved (Figure 4C). When such levels are artificially created in cells lacking wild-type p53, cell death is observed (Figure 5C). We conclude that the greater sensitivity to hypoxia in wild-type p53-containing cells results from *Bnip3L* regulation by p53, but we cannot rule out other mediators of p53, direct effects of p53, and/or other factors that may also contribute to the cell death triggered by hypoxia.

Bnip3L appears to play a crucial role in tumor suppression as documented by our observations that silencing of *Bnip3L* lowers sensitivity to cell death induced by hypoxia (Figure 7D and Supplemental Figure S5D at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/) in vivo. Moreover, *Bnip3L* knockdown converts nontumorigenic U2OS cells into tumorigenic xenografts. This was demonstrated by two approaches: (1) stable clonal cells and (2) retrovirus-infected pooled cells to eliminate potential artifacts due to selection during cloning procedures (Figure 6). These results are consistent with the concept that a lower apoptotic potential promotes tumor growth (Graeber et al., 1996), and this may be compromised when p53 is mutated. In our studies, the blockade of tumor suppression due to *Bnip3L* silencing occurred in U2OS xenografts, despite the fact that these cells contain wild-type p53 and maintain a modest apoptotic response to ionizing irradiation (Figure 7B and Supplemental Figure 5B). We propose that p53-dependent upregulation of *Bnip3L* during hypoxia provides a mechanism by which the p53 tumor suppressor functions in vivo in inhibiting tumor development.

Altered *Bnip3L* regulation by p53 in cancer

Deficient *Bnip3L* upregulation may occur in several ways in tumors. These include the loss of functional p53 through p53 mutation, or loss of Bnip3L function. Some mutations of *Bnip3L* have been reported in breast cancer and ovarian cancer; its expression is low or absent in both lung cancer cell lines and human lung cancers (Lai et al., 2003; Sun et al., 2004). This is consistent with the present demonstration that silencing of *Bnip3L* promotes tumorigenicity (Figure 6 and Supplemental Figures S3 and S4 at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/). In addition, there is a possibility that *Bnip3L* regulation by p53 could be impaired during tumor formation even in some tumors that retain wild-type p53, possibly through changes in selectivity of target gene activation that promote tumor growth. For example, we observed no *Bnip3L* upregulation by p53 in HCT116 or H460 tumor cell lines following exposure to DNA damaging agents or hypoxia (data not shown). A recent report demonstrated, among other changes, downregulation of *Bnip3L* in HCT116 cells selected for resistance to 5-FU (De Angelis et al., 2004). Another study reported hypermethylation of *Bnip3* in pancreatic cancer (Okami et al., 2004). Thus, a number of genetic or epigenetic changes, which may occur during tumor progression, may lead to the loss of *Bnip3L* regulation by p53 when both are present, thereby contributing to decreased sensitivity to hypoxia and tumor progression.

Bnip3L and *Bnip3* are potent apoptotic mediators during hypoxia (Kubasiak et al., 2002; Piret et al., 2002; Yussman et al., 2002); however, *Bnip3* is not upregulated by p53. Interestingly, we found that the kinetics of Bnip3 mRNA and protein induction were slower than those of Bnip3L mRNA and protein induction in hypoxia-exposed SW480 cells (Supplemental Figure S4C at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/), and in vivo knockdown of *Bnip3* in the SW480 cells had no apparent effect on xenograft growth (Supplemental Figures S4A and S4B). Taken together, without upregulation by p53, *Bnip3L* or *Bnip3* may play a role in hypoxia-triggered cell death with a suggestion that *Bnip3L* may be induced earlier and contribute slightly more to the cell death. However, these effects are weak in terms of their tumor suppressive potential in cells lacking wild-type p53, and such p53-deficient human cells appear to have little difficulty in surviving and forming tumors in vivo, regardless of the silencing of either *Bnip3* or *Bnip3L*. *Bnip3L* appears to play a crucial role in tumor suppression that is compromised when p53 is mutated or *Bnip3L* is silenced, and its induction provides a mechanism by which p53 functions in vivo in inhibiting tumor growth. In tumor progression, cells with lower expression of *Bnip3L* have a lower apoptotic potential, and this may facilitate their growth and expansion despite hypoxia (Figures 6 and Supplemental Figures S3 and S4). In tumor therapy, a lower expression of *Bnip3L* is expected to contribute to radioresistance (Figure 7B and Supplemental Figure S5B). We suggest that *Bnip3L* regulation by p53 may contribute to hypoxia-induced cell death and tumor suppression. The restoration of *Bnip3L* induction in cancer treatment therefore offers a strategy for promoting increased sensitivity to hypoxia and increased apoptosis in response to therapy of hypoxic tumors.

Experimental procedures

Reporter plasmids, transfection, and reporter imaging assays

Six genomic DNA fragments containing sites A, B, C, D, E, and F (in Figure 2A) were cloned into the pGL3 promoter-reporter (Promega). Transfection was performed using Lipofectamine 2000 (Invitrogen). The bioluminescent images were obtained using a Xenogen IVIS as previously described (Wang and El-Deiry, 2003, 2004).

Bnip3L siRNA oligonucleotide transfection

siRNA oligonucleotides directed against human *Bnip3L* included 5′-AA-CAG UUCCUGGGUGGAGCUA-3′ for Supplemental Figure S3 (http://www.cancercell.org/cgi/content/full/6/6/597/DC1/) and 5′-AA-CACGUACCAUCC UCAUCCU-3′ for Figure 4, and scrambled oligonucleotides were purchased from Dharmacon Research (Lafayette, CO). The transfection was performed as suggested by Dharmacon.

Xenograft formation

Nude mice (4–6 weeks old) from Charles River laboratory were injected with 300 μl Matrigel (Invitrogen) flurry (prepared at a 1:1 ratio with 1× PBS) containing 10 million cells with or without constitutively expressed luciferase. The luciferase activity was measured by intraperitoneal injection of 50 mg/mouse D-luciferin into anesthetized mice, followed by detection of live images using the Xenogen IVIS as described (Wang and El-Deiry, 2003, 2004).

Establishment of stable siRNA-expressing cell lines and luciferase-expressing retrovirus

Bnip3L RNAi-expressing cDNA (see 64 nt sequences provided in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/) was cloned into pSuper or Super-retro plasmid, and firefly luciferase cDNA was cloned into pBabe-puro plasmid. The retroviruses made from Phoenix cells were spinoculated into cells as described (Bunnell et al., 1995; Bahnson et al., 1995; Burns and El-Deiry, 2001). The cell line selection was performed as described previously (Brummelkamp et al., 2002).

Inducible *Bnip3L* expression using the Tet-on system

These procedures were performed according to the protocol provided by the manufacturer of the Tet-on system (Clontech). The *Bnip3L* cDNA was subcloned into the pRevTRE vector at the BamH1 and the HindIII sites.

Supplemental data

A more detailed description of the Experimental Procedures is provided in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/6/597/DC1/.

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