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Requirement of evading apoptosis for HIF-1α-induced malignant progression in mouse cells

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Key words: apoptosis, autophagy, genetic alteration, hypoxia, tumor progression

Abbreviations: BMK, baby mouse kidney; CFS, common fragile site; EMT, epithelial-mesenchymal transition; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; HIF-1α, hypoxia-inducible factor 1α; PAS, Per-ARNT-Sim; PAS1B, HIF-1α PAS-B

Tumor hypoxia is correlated with genetic alteration and malignant progression. Our previous studies indicated that the hypoxia-inducible transcription factor, HIF-1α, is responsible for hypoxic suppression of DNA repair in tumor cells by a non-canonical mode of action that requires the HIF-1α PAS-B subdomain. The involvement of HIF-1α in genetic alteration has raised an intriguing question as to whether normal cells would respond to hypoxic stress differently to avert genetic alteration. In this study, we chose several mouse cell types ranging from benign to malignant, apoptosis-proficient to apoptosis-deficient, and determined their responses to HIF-1α expression. In agreement with our previous findings, transient hypoxia and HIF-1α expression inhibited DNA repair and induced DNA damage in all cell types examined; however, cumulative DNA damage only occurred in apoptosis-deficient, malignant cells transduced for sustained expression of HIF-1α or HIF-1α PAS-B itself. In keeping with the theory of apoptosis as a cancer barrier, only these apoptosis-deficient cells acquired anchorage-independent growth and epithelial-mesenchymal transition. Furthermore, these cells exhibited increased Akt activity and resistance to etoposide by inhibiting autophagy. Altogether, our results define an essential role for apoptosis to prevent HIF-1α-induced genetic alteration and thereby malignant progression.

Introduction

Genetic alteration plays an important role in the development and progression of cancer. Mutations in oncogenes and tumor suppressor genes alter gene function, leading to the neoplastic transformation. A stepwise accumulation of genomic changes occurring in a small subset of genes leads to the emergence of the complex cellular behavior that characterizes malignant tumors.1 DNA in all living organisms constantly undergoes numerous types of damage during cellular metabolism and by external DNA damaging agents. To respond to such attacks, cells devise ingenious mechanisms to repair and cope with the damage. Among a variety of DNA damage types, DNA double-strand breaks are of the most disruption, leading to broken chromosomes and cellular lethality if not repaired. Furthermore, erroneous repair of the DNA damage may result in chromosome loss or amplification, translocations and truncations.2

Numerous studies have indicated that defects in DNA repair genes are responsible for hereditary syndromes conferring genetic predisposition to cancers. However, the role of DNA repair in the majority of sporadic human cancers remains unclear, because mutations are rarely detected in DNA repair genes. We and others have shown that hypoxia inhibits DNA repair gene expression and promotes genetic changes, suggesting that microenvironmental factors play a critical role in tumor progression by suppressing DNA repair at the functional level.3,4 Furthermore, we reported that the hypoxia-inducible transcription factor, HIF-1α, inhibits DNA mismatch repair and double-strand break repair by downregulating MSH2 and NBS1, respectively.5,6 Notably, HIF-1α does so by an alternative mechanism that is independent of its DNA binding and transactivation domains,7 which is in stark contrast to the canonical mechanism of HIF-1α transcriptional activation.8,9 In fact, HIF-1α relies on its PAS-B subdomain to compete in the target gene promoter for Sp1 binding by displacing the transcription activator, c-Myc, that maintains DNA repair.5,6,8 Furthermore, HIF-1α PAS-B itself has been shown to be sufficient to inhibit DNA repair and induce DNA double-strand breaks. Moreover, we demonstrated recently that HIF-1α PAS-B is essential to mediate HIF-1α effects on genetic alteration and malignant progression in vivo.10,11

Although all these studies strongly indicate that this HIF-1α-c-Myc pathway is responsible for tumor hypoxia to promote
Results

Hypoxia or HIF-1α expression inhibits DNA repair gene expression and induces DNA damage in mouse cell lines. Previously, we demonstrated that hypoxia and HIF-1α expression result in NBS1 and MSH2 downregulation in human osteosarcoma U-2 OS cells and colon cancer HCT116 cells.13 To extend these findings to mouse cells, we used four mouse cell lines of different degrees of malignancy and apoptotic status. These cells include NIH/3T3; BMK epithelial cells BMK W2 (Bak+/− Bax+); and BMK D3 (Bak−/− Bax−);16 and Hepa 1-6. We first verified their apoptotic status by examining caspase 3 activation after the treatment with DNA damaging agents etoposide and actinomycin D. As expected, both NIH3T3 and BMK W2 displayed strong activation of caspase 3 after treatment, whereas BMK D3 and Hepa 1-6 showed no and little response, respectively (Sup. Fig. 1). The data confirmed that BMK D3 and Hepa 1-6 are apoptosis deficient.

Next, we subjected these cells to a 16-h hypoxic treatment and analyzed the expression of Nbs1 and Msh2 with real-time PCR. Results in Figure 1A show various degrees of hypoxic downregulation of Nbs1 and Msh2 genes in these cell types. Of note, BMK W2 and D3 exhibited a far greater inhibition of Nbs1 than the other two cell types and yet, a much smaller upregulation of Ca9, a classic hypoxia-inducible gene. These results indicate that hypoxic downregulation of DNA repair genes is not just limited to malignant cells, a finding consistent with our previous report that hypoxia also inhibits mismatch repair genes in normal primary human cells.5

To corroborate the role of HIF-1α in hypoxic suppression of DNA repair genes identified in human cells,5,6 we tested whether forced expression of a stable form of HIF-1α [HIF-1α(ΔODD)],17 in mouse cells would also inhibit DNA repair gene expression. Previously, we showed that HIF-1α PAS-B (abbreviated thereafter as PAS1B) is sufficient to inhibit DNA repair.6 Therefore, PAS1B was also tested along with PAS1B-VAT, a functional mutant resulting from substitutions of the three HIF-1α amino acid residues Val-317, Ala-321 and Thr-327 with the corresponding ones in HIF-2α6 (Fig. 1B). To that end, recombinant adenoviruses expressing HIF-1α(ΔODD), PAS1B and PAS1B-VAT were created. Owing to the very low efficiency of adenoviral infection in NIH/3T3 cells, we focused on the other three cell

Figure 1. Transient hypoxia and HIF-1α expression repress DNA repair gene expression in mouse cells. (A) NIH/3T3, BMK (W2, D3) and Hepa 1-6 cells were subjected to normoxic (N) or hypoxic (H) treatment (% O2, 16 h). Hypoxic effects on Nbs1, Msh2 and Ca9 transcript levels were examined in reference to Actb by real-time PCR. Representative results in triplicates are presented in mean ± SE. (B) A schematic representation of HIF-1α is shown. Sequence alignment of PAS1B residues 315–329 with those of PAS1B-VAT is depicted. PAS1B-VAT contains replacements of Val-317, Ala-321 and Thr-327 (underlined) with the corresponding HIF-2α residues. (C) Hepa 1-6 cells were subjected to adenoviral infections as indicated and analyzed for NBS1 protein levels. FLAG-tagged PAS1B and PAS1B-VAT levels were also examined. (D) Similar studies as above were performed with BMK W2 and D3 cells.

genetic alterations, hypoxia also serves as a biological cue in various developmental and physiological processes.14 Given that evasion of apoptosis is considered one of the hallmarks of cancer,15 we tested whether non-cancerous cells would experience genetic changes induced by hypoxia or the HIF-1α-c-Myc pathway and whether apoptosis would protect non-cancerous cells from acquiring genetic alterations. To that end, we selected mouse cell lines of various degrees of malignancy, including benign NIH/3T3 fibroblasts, baby mouse kidney (BMK) cells transformed with adenoviral protein E1A and dominant-negative p5316 and, finally, malignant mouse hepatoma Hepa 1-6 cells for this study.
types. Similar to the inhibitory effect by hypoxia, HIF-1α(ΔODD) expression reduced Nbs1 protein levels in Hepa 1-6 cells (Fig. 1C). Likewise, PAS1B but not PAS1B-VAT markedly decreased Nbs1 protein levels in all three cell types (Fig. 1C and D). Of note, equivalent expression of PAS1B and PAS1B-VAT was observed, confirming the specific role for an intact PAS1B in Nbs1 downregulation.

In keeping with this, hypoxic treatment as well as HIF-1α(ΔODD) and PAS1B expression all led to significant damage to DNA in both BMK W2 and D3 cells, as shown by the alkaline comet assay (Fig. 2A). This assay allows for visualization and quantification of DNA damage, because the damaged, unwound DNA fragments migrate out of the cell under the electric field, forming a distinct comet-like tail.18 There was a >3-fold increase in the percentage of comet tail DNA from hypoxia-treated cells and those expressing HIF-1α(ΔODD) and PAS1B (Fig. 2B and C). However, no such increase was observed in cells expressing PAS1B-VAT or green fluorescent protein (GFP). Collectively, these results indicate that HIF-1α PAS-B is necessary and sufficient to inhibit DNA repair and induce DNA damage in mouse cells.

Cumulative DNA damage induced by the HIF-1α-c-Myc pathway occurred only in apoptosis-defective cells. To further understand the role of HIF-1α in DNA damage, we created recombinant retroviruses carrying either PAS1B or PAS1B-VAT fused to the enhanced yellow fluorescent protein (EYFP) for sustained expression. After retroviral infection and selection, the transduced cells were pooled and analyzed for transgene expression by fluorescent microscopy (data not shown). Surprisingly, reduction of Nbs1 protein levels by PAS1B, as assayed by protein gel blotting, was observed only in the apoptosis-deficient cells, BMK D3 and Hepa 1-6; no such diminution was detected in BMK W2 and NIH/3T3, the apoptosis-proficient cells (Fig. 3A). As expected, both PAS1B-VAT and EYFP alone showed no effect. In accordance with these findings, the alkaline comet assays showed a 2.5-fold increase of DNA damage in the PAS1B-expressing BMK D3 but not BMK W2 cells (Fig. 3B).

To substantiate these findings in the context of HIF-1α, we engineered a recombinant retrovirus expressing a stable form of full-length HIF-1α through the destruction of Pro-402 and Pro-564, which are required for proteolysis through hydroxylation,19 and a negative-control virus expressing the HIF-1α VAT mutant.12 Again, transduced BMK D3 but not BMK W2 cells expressing HIF-1α showed Nbs1 downregulation (data not shown). More importantly, this was correlated with a ~2-fold increase in DNA damage as determined by the comet assay (Fig. 3C). As expected, HIF-1α VAT had no obvious effect, as shown previously in reference 12. Together with the earlier findings, these results suggest that apoptosis is critical in preventing cumulative DNA damage, even though acute DNA damage occurs irrespective of the status of apoptosis during transient hypoxia or HIF-1α expression.

Preferential inactivation of tumor suppressor genes by the HIF-1α-c-Myc pathway in apoptosis-deficient cells. We have shown recently that human cancer cells acquire common fragile site (CFS) instability specifically by the HIF-1α-c-Myc pathway.12 CFSs are specific regions of the genome that commonly form gaps and breaks on metaphase chromosomes following partial inhibition of DNA synthesis during replicative stress.20 Two of the most active CFSs in the human genome are *FRA3B* and *FRA16D*, which lie within the tumor suppressor genes *FHIT* and *WWOX*, respectively, and undergo frequent chromosome breakage and rearrangement in cancer cells, resulting in inactivation of the associated genes.21,22

To determine whether inactivation of tumor suppressor genes is also correlated with apoptosis deficiency, we first analyzed the expression of *Fhit* and *WWOX* genes of the murine orthologs *Fra14A2* and *Fra8E1*, respectively.23,24 Results in Figure 3D (top panel) show a complete loss of *Fhit* gene expression in the PAS1B-expressed BMK D3 and Hepa 1-6 cells. *WWOX* expression was significantly decreased in the transduced BMK D3 but modestly decreased in the transduced Hepa 1-6 cells. The
apoptosis-proficient cells, however, maintained Wwox expression, even though a modest reduction of Fhit expression was detected in the PAS1B-expressed BMK W2 cells.

To identify possible genetic aberrations responsible for the inactivation of Fhit and Wwox genes, we examined the two CFS loci, Fra14A2 and Fra8E1, with three sets of PCR primers per gene locus that span the regions harboring the exons used for examining gene expression (Fig. 3D, bottom part). Consistent with the results of gene expression, loss or weak amplification of the genomic loci by PCR was observed in the PAS1B-expressed BMK D3 and Hepa 1-6 cells. Furthermore, the decreased Fhit gene expression in the transduced BMK W2 cells was correlated with reduction of part of the exon 7. By contrast, no such changes were detected in the PAS1B-expressed NIH/3T3 cells. Thus, these results suggest that HIF-1α induces CFS instability preferentially in apoptosis-deficient cells.

HIF-1α confers malignant traits only in apoptosis-deficient cells. We recently demonstrated that HIF-1α promotes malignant progression of human tumor cells through the induction of genetic alterations via the HIF-1α-c-Myc pathway. To demonstrate the importance of apoptotic evasion in HIF-1α-induced malignant progression, we examined the effect of HIF-1α on anchorage-independent growth, a definitive in vitro characteristic that distinguishes tumorigenic cells from non-tumorigenic cells. Results in Figure 4A show a marked increase of colony size and number on soft agar, particularly with PAS1B-expressed BMK D3 cells (Figs. 4A and B), whereas those expressing EYFP and PAS1B-VAT had no such change, a finding in good correlation with their inability to induce DNA damage and genetic alteration. Furthermore, BMK D3 cells expressing HIF-1α but not HIF-1αVAT grew ~4 times more colonies than the parental cells (Fig. 4C and Sup. Fig. 2), whereas enhanced colony formation was also observed in apoptosis-deficient Hepa 1-6 cells (Fig. 4D), but not in apoptosis-proficient NIH/3T3 or BMK W2 cells. These results support the protective role of apoptosis against HIF-1α-induced malignant progression.

Epithelial-mesenchymal transition (EMT) is an important indicator of malignant progression, by which tumor cells of epithelial origin acquire motility for migration and intravasation through the functional loss of epithelial marker E-cadherin, a suppressor of invasion during tumor progression. In keeping with the antagonistic role of apoptosis in malignant progression,
the epithelial markers E-cadherin and β-catenin were unaffected at protein levels in BMK W2 cells by the HIF-1α-c-Myc pathway (Fig. 5A). By contrast, there was a marked reduction of E-cadherin and β-catenin levels in the transduced BMK D3 cells, concomitant with a gain of vimentin, a mesenchymal marker. Furthermore, immunofluorescent staining confirmed the loss of E-cadherin and the gain of vimentin in the transduced BMK D3 cells but not in the transduced BMK W2 (Fig. 5B). In aggregate, these results are consistent with an essential role of apoptosis in preventing cumulative DNA damage and genetic alterations induced by HIF-1α, thereby blocking malignant progression.

Gain of further survival advantage in apoptosis-deficient cells via the activation of the HIF-1α-c-Myc pathway. Tumor cells undergo malignant progression by acquiring additional growth advantages. Although evasion of apoptosis is advantageous for tumor cell survival, we asked whether PAS1B expression could confer additional growth advantage on BMK D3 cells. To that end, we examined cell viability under the treatment of chemotherapeutic agents. Figure 6A shows that BMK W2 cells had a ~60% survival rate after etoposide treatment, whereas BMK D3 cells exhibited a greater survival rate. However, PAS1B enhanced BMK D3, but not BMK W2, cell survival to ~90%. Hepa 1-6 cells also gained such survival (Sup. Fig. 3a). Together, these results indicate gain of additional growth advantage, specifically by apoptosis-deficient cells, as a result of activation of the HIF-1α-c-Myc pathway.

In support of this theory, we identified a striking increase of Akt activity, as shown by Akt abundance and phosphorylation, in PAS1B-expressed BMK D3 and Hepa 1-6 cells (Fig. 6B and Sup. Fig. 3b), which was not detected in the counterpart of BMK W2. To ascertain whether Akt activity contributes to etoposide resistance, we employed tricirbin, a cell-permeable tricyclic nucleoside that specifically inhibits the phosphorylation, activation and signaling of Akt-1, -2 and -3.39 Results in Figure 6C show that tricirbin treatment of BMK D3 markedly reduced cell survival and abrogated PAS1B-mediated resistance to etoposide. However, tricirbin hardly had any effect on BMK W2. In keeping with the essential role of Akt activity in cell survival and cancer development, this result underscores the importance of the Akt signaling pathway for the viability of BMK D3 cells.

To understand the role of the Akt signaling pathway in preventing cell death, we examined autophagy, another type of programmed cell death that is inhibited by the activation of the PI3K/Akt pathway. We asked whether the increased Akt activity in transduced cells contributes to the inhibition of autophagy. Figure 6D shows that etoposide treatment of BMK D3 and Hepa 1-6 cells produced punctate staining of LC3B, an indicator of autophagy that is concentrated in the vacuoles of cells undergoing autophagy.27 However, no such staining was detected in those cells with increased Akt activity, suggesting that the HIF-1α-c-Myc pathway inhibits autophagy. Furthermore, protein gel blot analysis confirmed the induction of LC3-II protein levels in BMK D3 but not the transduced cells (data not shown). Hence, the HIF-1α-c-Myc pathway promotes survival of apoptosis-defective cells by blocking autophagy.

**Discussion**

The importance of tumor microenvironment in malignant progression and metastasis has long been recognized.28 Specifically, hypoxia has been proposed to be a physiological selective pressure for tumor cells to evade apoptosis and to acquire p53 mutations.29 Furthermore, hypoxia and defective apoptosis have been shown to drive genomic instability and tumorigenesis.30 However, the mechanisms by which hypoxia induces genetic alteration still remain to be elucidated. We recently demonstrated that HIF-1α mediates hypoxic induction of genetic alterations by inhibiting DNA repair gene expression.31 Remarkably, HIF-1α does so even...
in normal human cells, which has raised an important question as to whether normal cells respond differently to hypoxic stress.

To address this question, we used mouse cell lines ranging from benign to malignant and confirmed that transient hypoxia or HIF-1α expression inhibits DNA repair and induces DNA damage. However, benign and malignant cells differed sharply upon sustained HIF-1α expression, a condition resembling the chronic hypoxic conditions in solid tumors. As a result, these malignant but not benign cells exhibited cumulative DNA damage and inactivation of tumor suppressor genes resulting from CFS instability, which correlates well with the gain ofanchorage-independent growth and EMT. Furthermore, these malignant cells also acquired additional growth advantage through the blockade of autophagy. By contrast, none of these happened in apoptosis-proficient cells, with the exception of CFS instability identified in the p53-defective, E1A-transformed BMK W2 cells. These observations argue that normal cells are generally safe under hypoxic stresses. This interpretation is in good agreement with the theory that cancer cells are derived from normal cells, and yet the transformation process could take decades in humans. However, what remains unanswered is how apoptosis-proficient cells are able to maintain DNA repair gene expression and thereby avoid cumulative DNA damage under sustained HIF-1α expression.

It is noteworthy that the gain of malignant traits observed in BMK D3 and Hepa 1-6 cells can be achieved by the overexpression of PAS1B. It is obvious that this small fragment of HIF-1α does not exist naturally, yet the strict requirement of the three residues (VAT) for its impact underscores the importance of the HIF-1α-c-Myc pathway in malignant progression, the “dark side” of...
HIF-1α functionality. Furthermore, in accordance with the effects of PAS1B overexpression, treatment of human osteosarcoma U-2 OS cells with long-term hypoxia gave rise to essentially identical results with respect to malignant progression. Although HIF-1α and HIF-2α are known to have a myriad of ways of promoting tumor growth and progression, the results from this study concur with the underlying cause of tumor progression, i.e., genetic alterations, which cannot be simply accounted for by the physiological role of HIF-1α in transcriptional regulation.

Several studies have shown that HIF-1α induces EMT during tumor progression and have even identified the transcriptional repressors of the CDH1 gene (encoding E-cadherin) that promote EMT. These repressors include TCF3, ZEB1/2 and Twist, which are regulated directly or likely by HIF-1α. In our recent study, we showed that the HIF-1α-c-Myc pathway specifically upregulates ZEB2. Although how PAS1B induces EMT in mouse cells warrants further investigation, suffice it to say that no direct transcription target of HIF-1α is likely involved. What remains unanswered is how HIF-1α selectively induces these transcriptional repressors for EMT by different means. Furthermore, how do apoptosis-proficient cells block the induction of EMT by the HIF-1α-c-Myc pathway? Despite the genetic difference in the pro-apoptotic genes between BMK D3 and BMK W2, we cannot rule out the possibility that other factors contribute to the differential effect of the HIF-1α-c-Myc pathway on the induction of EMT.

It was shown recently that hypoxia activates the autophagic pathway for metabolic adaptation. In this study, however, autophagy is inhibited by the HIF-1α-c-Myc pathway, presumably resulting from activation of the Akt pathway, thereby rendering cells more resistant to chemotherapeutic drugs. Autophagy is believed to be an important physiological process that eliminates excesses of damaged organelles by degradation. Under nutrient deprivation or hypoxic conditions, cells undergo autophagy to promote cell adaptation and

**Figure 6.** HIF-1α PAS-8 promotes cell survival by inhibiting drug-induced autophagy. (A) BMK cells and those transduced with PAS1B were treated with etoposide (VP16, 20 μM) for 24 h. Cell viability was assessed in quadruplicate and presented as mean ± SE. (B) AKT and ERK phosphorylation was determined in BMK parental and transduced cells by protein gel blot analysis with antibodies against phospho-AKT (pAKT), AKT, phospho-ERK (pERK), ERK and β-actin. (C) BMK parental and transduced cells were pretreated with 1 μM triciribine (TCN) before exposure to etoposide. Cell viability was assessed in triplicate and presented as mean ± SE. (D) BMK D3 and Hepa 1-6 cells were treated with etoposide for 48 h and examined for the induction of autophagy with LC3B antibody. Immunofluorescent microscopy shows lack of punctate staining of LC3B in PAS1B-transduced cells after etoposide treatment. Cell nuclei were stained with DAPI.
In contrast to its role of survival, increasing lines of evidence support the idea that defective autophagy facilitates cancer development. This paradox that the loss of a survival pathway promotes tumor growth has been eloquently explained by linking metabolic stress with the DNA damage response. Essentially, autophagy limits metabolic stress to protect the genome by maintaining metabolism, whereas defective autophagy increases DNA damage and genomic instability that ultimately facilitate tumor progression. Our finding that HIF-1α promotes malignant progression by inhibiting autophagy seems consistent with this theory.

In conclusion, we have shown that HIF-1α requires its PAS-B subdomain to inhibit DNA repair and, in turn, induce DNA damage in mouse cells. However, the consequence depends on the cell integrity itself; apoptosis protects against cumulative DNA damage, whereas apoptotic defects allow genetic alterations and malignant progression. Hence, these findings indicate that HIF-1α-induced genetic alteration is cell-context dependent and that evasion of apoptosis is a major determinant of malignant progression driven by the HIF-1α-c-Myc pathway.

**Materials and Methods**

**Cell culture and chemicals.** Baby mouse kidney (BMK) epithelial cells were kindly provided by Eileen White of Rutgers University. Hepa 1-6 cells and NIH/3T3 cells were obtained from the American Type Culture Collection (Virginia). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (or bovine calf serum for NIH/3T3), 100 unit/ml penicillin and 100 μg/ml streptomycin. Oxygen tensions in the incubator were either 20% O2 or 1% O2. Infection of recombinant adenoviruses was as previously described in reference 7. Etoposide (VP16) was purchased from Sigma-Aldrich (Missouri). Hygromycin was purchased from InvivoGen (California), neomycin and triciribine from VWR (Pennsylvania).

**Generation of transduced cell lines.** Recombinant adenoviruses that express FLAG-tagged HIF-1α PAS-B and its VAT (V317L-A321G-T327P) mutant were produced in the Gene Expression Laboratory of SAIC/NCI-Frederick (Maryland). Recombinant adenovirus expressing HIF-1αΔODD was as described previously in reference 7. Adenoviral infection was carried out with either a vehicle control or VP16 for 24–48 h. At the end of each treatment, cell viability in each well was measured by using Linominescent Cell Viability Assay kit (Promega, WI) and normalized to the vehicle-treated control. Triplicates were used for each experiment, and the experiments were repeated at least three times.

**Soft agar colony formation.** Anchorage-independent growth capability was determined by assessing the colony-formation efficiency of cells suspended in soft agar. Cells (1 x 10^5) were seeded in 0.35% overlay agar in 6-well plates coated with 0.5% underlay agar. Colonies were stained with crystal violet and counted 2–4 weeks after plating with the Kodak IS4000 system.

**Immunofluorescence microscopy.** Cells were washed with ice-cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 10 min, blocked and incubated with primary antibodies overnight at 4°C. After wash with PBS, the cells were incubated with Texas Red-conjugated secondary antibody for 1 h, followed by 10 min of incubation with 4',6-diamidino-2-phenylindole (DAPI). Slides were washed with PBS and mounted with Vectashield Hard Set Mounting Medium (Fisher Scientific, GA). Images were acquired using an Axiovert 200 fluorescent microscope.

**Polymerase-chain reaction (PCR).** Total RNA extraction and reverse transcription were performed essentially as described previously in reference 7. The PCR primer sets used for conventional reverse transcription-PCR were as follows: *Fhit* forward: 5'-CGG ACA GTG AGG ATG AGC TG-3', reverse: 5'-CCT CCT GGG AAG AAC GTG GA-3'; *Wwox* forward: 5'-CCG ACA GTG AGG ATG AGC TG-3', reverse: 5'-CCC ATC CAT ACG GCA AAT CTC C-3'.

**Antibodies and protein gel blotting.** Antibodies against Nbs1, β-catenin, p44/42 MAP kinase, phospho p44/42 MAPK, Akt, phospho Akt and LC3B were obtained from Cell Signaling (Massachusetts). Antibodies against Bax, Bak, Glut1, GFP, E-cadherin, vimentin were from Santa Cruz Biotechnology (California). Anti-FLAG and anti-β-actin antibodies were obtained from Sigma-Aldrich. Protein gel blot analysis was performed essentially as described previously in reference 19.

**The comet assay.** DNA damage was analyzed by using the comet assay kit (Trevigen, MD) following the manufacturer’s instructions. Density of DNA was measured in each cell with ImageGauge (Fujifilm, NY). The percentage of tail DNA was calculated by dividing the density of whole cell with the difference of densities between whole cell and nucleus. A total of 90 cells per slide were analyzed and presented as mean ± SE.

**Cell viability analysis.** To monitor the cytotoxicity, cells were plated in a 96-well plate and were treated with either a vehicle control or VP16 for 24–48 h. At the end of each treatment, cell viability in each well was measured by using Luminescent Cell Viability Assay kit (Promega, WI) and normalized to the vehicle-treated control. Triplicates were used for each experiment, and the experiments were repeated at least three times.
forward: 5'-GCA GGA GGC AGA ATG TGG AT-3'; reverse: 5'-ACA CTT GCT TCT CGT CAG T-3';
WexO (3)
forward: 5'-GAG CTT TGT GCT TCC CAG AG-3'; reverse: 5'-GGA TGT TCC CAG TGC TTC-3'.

For real-time PCR, cDNA was amplified by using the Taqman Universal Polymerase Chain Reaction Master Mix (Applied Biosystems, CA). Primers and probes for mouse
Car9 (Mm00198700_m1), Msh2 (Mm00500567_m1), Nbs1 (Mm00449861_m1) and Actb were purchased from Applied Biosystems.

References
17. Huang LE, Gu J, Schur M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O(2)-dependent degradation domain via the ubiquitoproteasome pathway. Proc Natl Acad Sci USA 1998; 95:7987-92.
30. Nelson DA, Tan TT, Rabson AB, Anderson D, Degenhardt K, White E. Hypoxia and defective apopto-

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Note
Supplemental materials can be found at: www.landesbioscience.com/journals/cc/article/16313