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PUMA INACTIVATION PROTECTS AGAINST OXIDATIVE STRESS THROUGH p21/Bcl-X_L INHIBITION OF BAX DEATH

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Abstract

The tumor suppressor protein p53 activates growth arrest and pro-apoptotic genes in response to DNA damage. It is known that negative feedback by $p21^{Cip1/Waf1/Sdi1}$ represses p53-dependent transactivation of PUMA. The current study investigates PUMA feedback on p53 during oxidative stress from hyperoxia and the subsequent effects on cell survival mediated through p21 and Bcl-X_L. Deletion of *PUMA* in HCT116 colon carcinoma cells increased levels of p53 and p21 resulting in a larger G_1 population during hyperoxia. P21-dependent increase in Bcl-X_L levels protected *PUMA*-deficient cells against hyperoxic cell death. Bax and Bak were both able to promote hyperoxic cell death. Bcl-X_L protection against hyperoxic death was lost in cells lacking Bax, not PUMA, suggesting that Bcl-X_L acts to inhibit Bax-dependent death. These results indicate PUMA exerts negative feedback on p53 and p21, leading to p21-dependent growth suppressive and survival changes. Enhanced survival was associated with increased Bcl-X_L to block Bax activated cell death during oxidative stress.

Keywords

Free radicals; cell death

INTRODUCTION

Reactive oxygen species (ROS) play an important role as secondary messengers in signaling pathways and are generated through aerobic respiration or by the radiolysis of water. Since aerobic organisms must tolerate aberrant ROS formation due to electron leakage along the respiratory chain, anti-oxidant systems have developed to detoxify ROS thus preventing oxidative injury to the cell. Oxidation of intracellular macromolecules occurs when anti-oxidant defenses are overwhelmed by ROS production. Oxidative stress resulting from ROS contributes in the pathology of neurodegenerative diseases, cardiovascular dysfunctions,

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inflammation, cancer and promotes to the aging process [1]. DNA damage caused by oxidative stress may result in activation of growth arrest, DNA repair and/or cell death pathways. The tumor suppressor protein, p53, plays a critical role in regulating cell cycle checkpoints and apoptosis during genotoxic responses. The primary function of p53 is to transactivate gene targets involved in the damage response, since mice and cells expressing a transcriptionally inactive mutant of p53 lose the ability to undergo growth arrest and apoptosis during stress [2,3].

$P21^{Cip1/Waf1/Sdi1}$ (hereafter p21), is a transcriptional target of p53 which is responsible for initiating G_1 arrest by binding and inhibiting cyclin-dependent kinases (cdk) and proliferating cell nuclear antigen (PCNA). Expression of p21 enhances survival during exposure to apoptotic stimuli such as ionizing radiation, doxorubicin, cisplatin, nitrogen mustard, Fas activation, PGA_2 exposure, p53-overexpression and TNF activation [4–9]. While it is apparent that the extent of stress-induced damage is dependent on cell cycle phase of the cell, p21 possesses anti-apoptotic functions which are cell cycle-independent [10]. P21 has direct anti-apoptotic functions by blocking activation of procaspase-3, caspase-8 and ASK-1 [11,12].

Apoptosis is tightly orchestrated through the interactions of the Bcl-2 protein family which includes several p53 gene targets, such as p53-upregulated modulator of apoptosis (PUMA), Bax and NOXA. Bcl-2 family proteins are classified by homology between Bcl-2 domains (BH1-4) and functional outcome during apoptotic activation. The anti-apoptotic proteins (Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, A1) interact with pro-apoptotic multidomain members (Bax, Bak) and the BH3-only proteins (PUMA, NOXA, tBid, Bad, Bim, Bmf, Bik, Hrk) to regulate the release of pro-apoptotic stimuli from the mitochondria. It is clear that BH3-only molecules activate the pro-apoptotic multidomain proteins and that anti-apoptotic members prevent this activation. It still remains unclear which Bcl-2 proteins are engaged during resting state and/or stress conditions [13].

Unlike most instantaneous genotoxic agents, exposure to hyperoxia results in the persistent production of reactive species which cause cellular damage resulting in DNA damage [14]. Even though mixed apoptotic and necrotic cell death occurs, abundant findings implicate the Bcl-2 family as mediators of the hyperoxic cell death response. Fibroblasts isolated from $Bax^{-/-}$ $Bak^{-/-}$ mice have increased resistance against hyperoxic death [15] and ROS activation of Bax causes cytochrome c release in MLE-12 cells which can be blocked by Bcl-X_L overexpression [15,16]. Caspase-8-dependent cleavage of Bid to tBid in hyperoxia can be blocked by FLIP [17,18]. Hyperoxia stimulated expression of Bcl-X_L, Bcl-2 and A1 in mouse lungs and deletion of *A1* increased hyperoxic injury [19]. Overexpression of Bcl-X_L in Rat1a cells protected against LDH release and cell death while siRNA knockdown of Bcl-X_L in HCT116 wt cells increased cell death [15,20]. Also, Bcl-2 overexpression in L929 cells was able to prevent mitochondrial release of apoptosis inducing factor (AIF) and cell death [21]. Together, these findings implicate members of the Bcl-2 family in hyperoxia-induced cell death.

Hyperoxia has been shown to activate p53 and p21 and loss of p21 sensitizes mice and cells to hyperoxic death [22–24]. Recent studies show that the p21 pro-survival function in hyperoxia is uncoupled from its growth suppressive activity and involves the regulation of Bcl-X_L [20, 25]. Also, it is clear that p21 disruption can promote p53-dependent cell death which is thought to be mediated through increased PUMA transcription [26–28]. The current studies manipulate PUMA, Bcl-X_L, Bax, Bak and p21 in HCT116 colon cancer cells to investigate if PUMA regulates p53 activation of p21 utilizing hyperoxia as a model of persistent oxidative stress. Since p21 regulates Bcl-X_L, the pathway of Bcl-X_L inhibition of cell death was also investigated.

MATERIALS AND METHODS

Cell lines and exposures

The human colon carcinoma HCT116 wild-type (wt) and isogenic cell lines lacking *PUMA* (PUMA^{-}), *P21/PUMA* (p21^{-}/PUMA^{-}) or *BAX* (Bax^{-}) were obtained from Dr. Bert Vogelstein (Johns Hopkins Oncology Center and the Program in Human Genetics and Molecular Biology) and genetic deletions have previously been verified by our laboratory [20]. Cells were cultured in McCoy's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Cells were counted with a hemocytometer and plated at 5×10^5 cells per 100-mm dish and allowed to adhere overnight. The following day plates were exposed to normoxia (room air with 5% CO_2) or hyperoxia (95% O_2/5% CO_2) by being placed into a Plexiglas box (Belco Glass) that was sealed and flooded with gas at a flow rate of 5 L/min for 10 min and then maintained at a flow rate of 0.2 L/min [20]. Oxygen concentrations were monitored with a miniOXI for the duration of exposure (Catalyst Research Corporation).

Cell death and cell cycle measurements

Following treatment, cells were trypsinized, resuspended in medium and centrifuged before overnight fixation in 75% ethanol. Cells were treated with 1 mg/mL RNase for 30 min and resuspended in phosphate buffered saline containing 10 μg/mL propidium iodide (Sigma-Aldrich). Samples were analyzed using a BD FACSCalibur flow cytometer set to collect 10,000 events. The percentage of cells with subG_1 DNA content was determined by using CellQuest v3.3 software (BD Biosciences). The percentage of cells in G_1, S and G_2/M was determined using ModFit LT software (Verity Software House).

Western blot analysis

Cells were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 25 mM sodium fluoride, 25 mM sodium β-glycerophosphate, 0.1 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 0.3% IGEPAL CA-630, 0.1 μg/ml pepstatin A, 1.9 μg/ml aprotinin and 2 μg/ml leupeptin. Protein concentrations were determined by the Lowry method (BioRad). Cell lysates were diluted in 3X Laemmli Buffer and boiled for 5 minutes. Laemmli at 1X contains 50 mM Tris (pH 6.8), 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The extracted protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Pall Life Sciences). The membranes were then incubated with anti-p53 clone DO-1 (1:1000, Oncogene Research Products), anti-p53 ser-15 (1:1000, Cell Signaling), anti-p21 clone SX118 (1:500, BD Pharmingen), anti-Bax clone N-20 (1:500, Santa Cruz Biotechnology), anti-Bcl-X_L clone 2H12 (1:500, Sigma-Aldrich) and anti-Bak clone Ab-1 (1:500, Calbiochem) with actin (1:1000, Sigma-Aldrich) as a loading control. Membranes were then incubated in horseradish peroxidase conjugated secondary anti-mouse (1:4000, Southern Biotechnology) or anti-rabbit (1:5000, Jackson Immunoresearch) antibodies and visualized by chemiluminescence (Amersham Biosciences). Densitometric analysis was performed by quantifying band intensities and normalizing to actin using a Fluorochem8900 (Alpha Innotech).

RNAi treatment

Cells were plated overnight in 12-well plates at 7×10^4 cells per well in medium lacking antibiotics. Cells were transfected with annealed luciferase (Dharmacon), Bcl-X (5'-AAGAGAATCACTAACCAGAGA-3', Invitrogen) or Bak (SMARTpool, Dharmacon) oligonucleotides using Lipofectamine 2000 [20,29]. After 12 hrs cells were washed and exposed to room air or hyperoxia for 4 days.

Statistical analysis

Group means were compared by ANOVA with Fisher's PLSD using Statview software (Abacus Concepts). Values represent means ± standard deviation for three to four separate exposures to hyperoxia with $p \leq 0.05$ considered to be significant.

RESULTS

Loss of PUMA Activates p21-Dependent Growth Arrest in Hyperoxia

Since p21 is able to affect p53 activity during the DNA damage response, we studied the ability of PUMA to regulate p53 and p21 during oxidative stress caused by treatment in hyperoxia. HCT116 cells exposed to hyperoxia accumulated total p53, increased phosphorylation of p53 at serine-15 and increased levels of p21 (Fig. 1A). Disruption of the *PUMA* gene altered the response to hyperoxia characterized by increased p53 total and phosphorylated levels along with increased p21 levels. Cell cycle analysis was performed on PUMA$^-$ cells due to altered p21 expression in response to hyperoxia after 4 days. HCT116 wt and PUMA$^-$ cells had similar cell cycle profiles in room air (Fig 1C), but a greater number of PUMA$^-$ cells were found in G_1 in hyperoxia (Fig. 1B, C). The number of HCT116 wt cells in G_1 following hyperoxia was 24.3±1.6% compared to 38±2.83% of the PUMA$^-$ cells which had increased levels of p21 ($p = 0.03$). This finding is consistent with the ability of p21 to elicit growth checkpoints in G_1 during environmental stress. Since PUMA$^-$ cells expressed more p21 and accumulated in G_1, p21$^-$/PUMA$^-$ were used to test for p21-dependency. Fewer p21$^-$/PUMA$^-$ cells were in G_1 following hyperoxic exposure (12.7±3.13%), suggesting that these cell cycle changes are p21-dependent. These data suggest lack of *PUMA* leads to increased p53 and p21 levels which enhanced G_1 growth arrest during oxidative stress.

Cells Lacking PUMA are Protected Against Hyperoxic Death via p21/Bcl-X_L

P21 is able to protect cells against hyperoxia by regulating Bcl-X_L levels [20,25]. Since PUMA$^-$ cells have higher levels of p21, changes in Bcl-X_L and survival were examined. PUMA$^-$ cells had increased Bcl-X_L protein levels compared to HCT116 wt in room air and during the hyperoxic timecourse (Fig. 2A). To determine if changes in Bcl-X_L were p21-dependent, expression of Bcl-X_L was investigated in HCT116 wt, PUMA$^-$ and p21$^-$/PUMA$^-$ cells exposed to room air or hyperoxia. While hyperoxic treatment caused loss of Bcl-X_L, it remained higher in PUMA–cells. While Bcl-X_L remained elevated in p21$^-$/PUMA$^-$ cells, it was not as high as PUMA$^-$ cells, suggesting Bcl-X_L expression in PUMA$^-$ cells was regulated by both p21-dependent and independent mechanisms (Fig 2B,C). Since Bcl-X_L inhibits cell death under numerous stress stimuli including hyperoxia, cell survival studies were performed to test if PUMA$^-$ were protected against hyperoxia and Bcl-X_L knockdown was perfomed to see how PUMA$^-$ cells responsd to loss of Bcl-X_L. Successful RNAi knockdown of Bcl-X_L in HCT116 cells has been performed previously [20] and was performed in both HCT116 wt and PUMA$^-$ cells prior to 4 days of hyperoxia exposure. Efficient knockdown of Bcl-X_L levels was achieved with no effect of the transfection reagent alone (mock) or siRNAs targeting luciferase (Fig. 2D). PUMA$^-$ cells had reduced death after 4 days hyperoxia by measuring subG_1 DNA content (HCT116 mock siRNA, 32.54±6.38% death; PUMA$^-$ mock siRNA, 21.68±3.82% death; Fig. 2E). RNAi knockdown of Bcl-X_L increased death of both HCT116 wt and PUMA$^-$ cells, implicating that p21 regulation of Bcl-X_L affects survival of PUMA$^-$ cells exposed to hyperoxia. These data also indicate that PUMA is not required to cause cell during Bcl-X_L knockdown.

Bax and Bak promote hyperoxic cell death

Bax and Bak are Bcl-2 pro-apoptotic multidomain proteins which are required for cell death in response to a variety of stress stimuli [30,31]. Bax activation in response to hyperoxia has

been identified and hyperoxic cell death was reduced in murine embryonic fibroblasts from *Bax*$^{-/-}$*Bak*$^{-/-}$ mice, but it is not clear whether it is Bax, Bak or both proteins which are required to promote cell death [15,16]. Less cell death was seen in Bax^{-} cells after 2 and 4 days hyperoxia (Fig. 3A). Only 16.6±4.35% cell death occurred in Bax^{-} cells after 4 days hyperoxia compared to 28.92±5.47% cell death of HCT116 wt cells. After 4 days hyperoxic treatment, Bax^{-} cells had increased cell death compared to untreated controls indicating that cell death was not completely inhibited by genetic deletion of Bax. To test for Bak-dependent cell death in hyperoxia, RNAi was used to knockdown Bak in Bax^{-} cells. Bak protein levels increased in response to hyperoxia, but Bak siRNA treatment was sufficient to knockdown protein levels 87% in Bax^{-} cells (Fig. 3B). Cell death was reduced with Bak siRNA treatment indicating that Bak also promotes hyperoxic cell death (Fig. 3C). SiRNA knockdown of Bak also increased death of HCT116 wt cells exposed to hyperoxia (data not shown). Taken together these data show that Bax and Bak both activate cell death pathways in response to hyperoxia.

Bcl-X_L blocks Bax-dependent hyperoxic cell death

Bcl-X_L can block death by binding and inhibiting Bax and Bak or by inactivating specific BH3-only proteins [13]. To test if Bcl-X_L protected against Bax-induced hyperoxic cell death, RNAi targeting Bcl-X_L was performed in Bax^{-} cells. Bcl-X_L knockdown significantly increased hyperoxic death of HCT116 wt cells (luciferase siRNA, 23.39±6.34% death; Bcl-X_L siRNA, 48.11±8.57% death), while Bcl-X_L siRNA treatment had no change in the survival of Bax^{-} cells exposed to hyperoxia (luciferase siRNA, 16.93±1.55% death; Bcl-X_L siRNA, 17.72 ±1.27% death; Fig. 4A, B). An interesting observation was that siRNA knockdown of Bcl-X_L significantly increased the number of HCT116 wt cells in the G_1 phase (luciferase siRNA, 18.76±1.6% G_1; Bcl-X_L siRNA, 31.41±3.36% G_1) of the cell cycle and decreased the number of cells in the G_2/M phase (luciferase siRNA, 32.81±2.17% G_2/M; Bcl-X_L siRNA 13.79 ±0.02% G_2/M) after 4 days of hyperoxic treatment (Fig. 4C, D). Bax^{-} cells responded similarly to the Bcl-X_L siRNA treatment, justifying that Bcl-X_L knockdown was sufficient to elicit a cell cycle response even though there were no changes in survival (Fig. 4B, D). Cycling effects were not secondary to changes in viability, since Bax^{-} cells had similar changes in cell cycle with Bcl-X_L knockdown even though survival was unaffected. P21 expression was investigated due to its role in the G_1 checkpoint, but there were no changes in p21 expression during Bcl-X_L knockdown (data not shown). To test if Bcl-X_L was involved in G_1 progression or a G_2/M block, HCT116 wt cells were treated with colcemid to prevent progression through G_2/M. Cells treated with Bcl-X_L siRNA still had more cell in G_1 after colcemid treatment in hyperoxia suggesting that Bcl-X_L plays a role in promoting cell cycle progression through G_1 phase (data not shown). These results suggest that Bcl-X_L plays a role in cell cycle regulation independent of p21 and is able to inhibit Bax-dependent cell death during hyperoxia.

DISCUSSION

P53 plays a pivotal role in regulating the damage response, inducing growth arrest which prevents the replication of damaged DNA or programmed cell death which is important for eliminating defective cells [32]. It was previously established that p21 exerted negative feedback on p53 transactivation of PUMA [26–28]. As illustrated in Figure 5, we report that PUMA regulates p53 and p21 levels during oxidative stress and that this affects p21-dependent growth arrest and survival pathways. Loss of PUMA led to elevated expression of p21 and growth arrest in G_1 and decreased cell death was associated with higher Bcl-X_L levels in hyperoxia. Bax and Bak activation both contributed to hyperoxic cell death, but only Bax-dependent cell death was blocked by Bcl-X_L. Also, increased numbers of cells were seen in G_1 after Bcl-X_L knockdown without any changes in p21 expression.

The novel finding of PUMA feedback inhibition of p53 and p21 is consistent with the idea that pro-apoptotic factors exist to antagonize the protective effects of p21. Polyak and colleagues [28] showed that one colorectal cell line, DLD1, undergoes apoptosis in response to transfected p53 while HCT116 wt cells preferentially growth arrest. Tetraploid fusions between each cell type resulted in an apoptotic response to p53 that was described to be dominant because of a cell specific pro-apoptotic factor that inhibited p21-dependent survival. Another key piece of evidence suggesting that survival factors are important regulators of the p53 response is from data that loss of *P53* in mice or HCT116 cells does not alter the rate of cell death during hyperoxia [33,34]. The chronic nature of hyperoxia leads to a balanced increase in both p53-dependent survival (p21) and death (PUMA) pathways. These observations question if p53 itself can contribute in choosing the response to oxidative stress [35]. P53 may activate default pathways following stress and there are accessory signals which help coordinate the downstream response such as activation of caspase-8 via Fas signaling [17,35]. Alternatively, specific post-translational modifications, such as phosphorylation, may direct p53 activation of preferential response pathways. This is evidenced by different substrate specificity of the phosphatidylinositol 3-kinase-like kinases ATM and ATR in response to hyperoxia, ionizing radiation and UV light. Cofactors, such as Slug, p300 and p21, have been shown to preferentially direct p53 transactivation of gene targets [26,36–38]. There is also evidence that negative feedback p53 loops are integrated in the p53 response. It is known that p53 gene targets, cyclin G and HDM2, function together to promote degradation of p53 [39]. Also, p63 and p73 have overlapping functions to p53 and can regulate p53 gene transcription [37]. P21 and PUMA, respectively, regulate pro-survival and pro-death pathways and these studies demonstrate that PUMA has the ability to moderate p21/Bcl-X_L pro-survival signaling. It is suggested that p21 regulates p53 activity through $p14^{ARF}$ and it is possible that PUMA also targets a protein which regulates p53 stability.

Our studies also clearly show that both Bax and Bak are able to activate cell death in response to hyperoxia. This is consistent with multiple studies implicating Bcl-2 family proteins in the regulation of hyperoxic cell death [15–21]. Bax or Bak oligomerization must take place in order to initiate mitochondrial release of cytochrome c and AIF. Studies suggest that Bcl-X_L can inhibit Bax and Bak to block cell death, yet our data support the idea that Bcl-X_L works only via Bax inhibition under oxidative stress, since deletion of *BAX* completely blocked the death response to Bcl-X_L knockdown. Support for Bcl-X_L inhibition of Bak comes from experiments showing that Bcl-X_L binds Bak in healthy cells but UV light treatment causes Bak-dependent cell death via NOXA. While a direct genetic link between NOXA and Bcl-X_L was established, Bcl-X_L binding to Bak could be an artifact of detergent conditions during protein co-immunoprecipitation [40]. However, our studies investigate cell death pathways using a genetic approach with a physiological outcome measure, thereby, reducing the likelihood of experimental artifacts. Cell death was not abolished in Bax^{-} cells treated with Bak siRNA indicating that additional pro-death pathways including Bid cleavage may remain intact. Another important finding was that hyperoxia upregulated Bak protein levels. Bak may be regulated transcriptionally by WT1 or Sp3, or it may be post-translationally modified by interacting proteins [41,42]. Current studies are aimed at investigating the pathway of Bak upregulation by hyperoxia.

Currently, there are two working models for activation of Bax and Bak [13,43]. In the direct model of activation a genotoxic signal activates BH3 only proteins which then block anti-apoptotic protein inhibition of Bax and Bak causing cell death [44]. While it is clear that anti-apoptotic proteins block the activation of Bax and Bak, it is thought that the biochemical inhibition is due to direct binding and sequestration of Bax and Bak in this model. Recent evidence argues for a hierarchical model of activation where a higher class of BH3 only proteins disrupts anti-apoptotic protein inhibition of more potent BH3 only proteins. The free potent BH3 only proteins then stimulate Bax and Bak activation [45]. Our findings argue that PUMA

is not directly downstream of Bcl-X_L during hyperoxic death, since PUMA was not necessary to promote death in cells lacking Bcl-X_L. Studies by Ming et al. show that adriamycin stimulates Bcl-X_L dissociation from Bax in a PUMA-dependent manner [46]. During hyperoxia, it is possible that either PUMA is targeting a different anti-apoptotic protein upstream of Bax or that it activates Bak directly in this model. P21-independent regulation of Bcl-X_L was seen in PUMA$^-$ cells and one explanation is that PUMA binding to Bcl-X_L promotes degradation of Bcl-X_L, since NOXA binding to Mcl-1 initiates proteasome-dependent degradation of Mcl-1 [40]. The ability to visualize changes in Bcl-X_L degradation may be unique to hyperoxia due to the chronic nature of the stress given the relatively long protein half-life of Bcl-X_L [47]. Cytoplasmic p53 may promote cell death by engaging Bcl-X_L, Bcl-2 and Bax [48,49], but there is currently no evidence supporting that this pathway is functional during hyperoxia as p53 was not required to promote cell death with knockdown of Bcl-X_L (data not shown).

Similar to the dual-functions of p21, specific Bcl-2 family proteins integrate regulation of apoptotic and cell cycle pathways. It has been reported that Bcl-2 and Bcl-X_L are able to delay G_1 and S phase progression by driving cells into G_0 [50]. Also, many of the anti-apoptotic proteins have cell cycle effects which can be mediated through binding of Bcl-2 and Bcl-X_L. The precise mechanism for these growth effects is not known and appears to be cell specific. Interestingly, our studies found no cell cycle effects due to Bcl-X_L knockdown in room air, but increased cells in G_1 with hyperoxia. Since the effect is damage-dependent, one explanation for the unexpected Bcl-X_L changes in cell cycle is compounded due to hyperoxia-induced growth changes [51].

This study utilizes hyperoxia as a model of persistent oxidative stress to identify pathways which may be associated with chronic oxidative diseases such as atherosclerosis, neurodegenerative diseases, ischemia-reperfusion injuries and Crohn's disease. In summary, this study demonstrates that PUMA regulates p53 and p21 levels during oxidative stress resulting in cell cycle and survival effects. Our data suggest that survival was enhanced in PUMA$^-$ cells due to increased levels of Bcl-X_L which were partly dependent on p21. Hyperoxic exposure resulted in activation of Bax and Bak but Bcl-X_L inhibition of Bax activation prevented cell death. Since many disease processes and aging are associated with ROS accumulation and DNA damage, knowledge of which specific cell survival and death pathways are activated in response to oxidative stress could provide new opportunities for effective disease treatment.

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LIST OF ABBREVIATIONS

ROS, reactive oxygen species; PCNA, proliferating cell nuclear antigen; wt, wild-type.

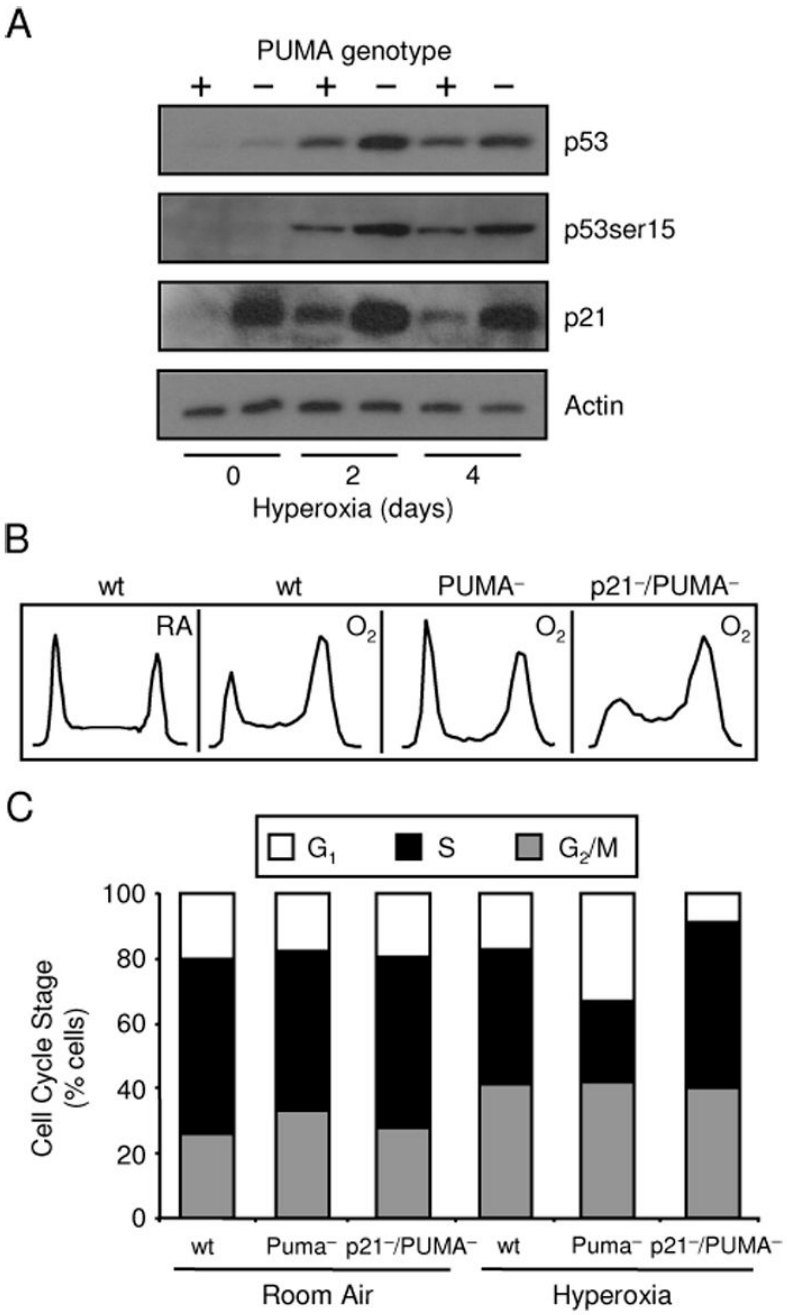

Figure 1. Loss of PUMA activates cell cycle checkpoints through p21

(A) Expression of p53, p53 ser-15 and p21 in HCT116 wt and PUMA$^-$ cells exposed to a hyperoxic time course with actin used as a loading control. Immunoblots were representative of three separate experiments. (B) Representative DNA histograms of HCT116 wt, PUMA$^-$ and p21$^-$/PUMA$^-$ cells exposed to 4 days of room air (RA) or hyperoxia (O_2). (C) Mean average of HCT116 wt, PUMA$^-$ and p21$^-$/PUMA$^-$ cells in G_1, S and G_2/M exposed to 4 days of room air or hyperoxia (n=3).

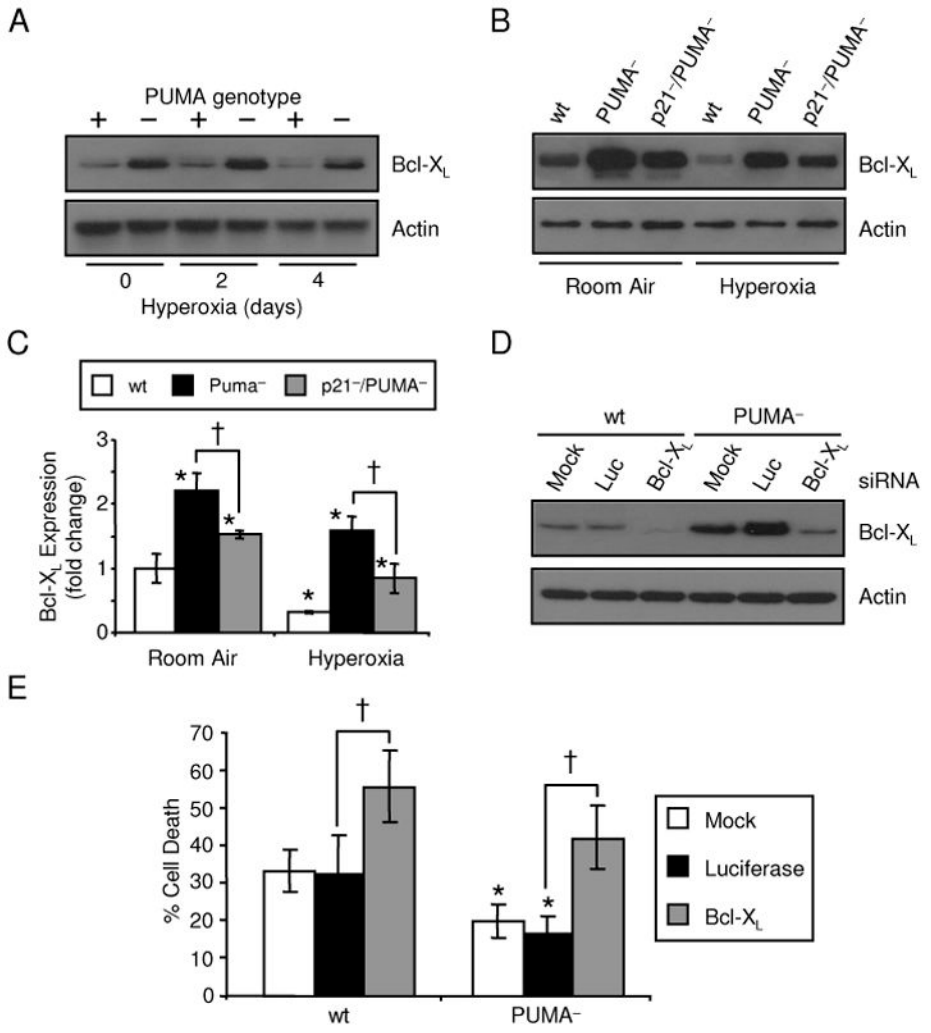

Figure 2. PUMA inactivation protects cells against hyperoxic cell death via p21/Bcl-X_L

(A) Bcl-X_L protein expression in HCT116 wt and PUMA$^-$ cells exposed to a hyperoxic time course with actin used as a loading control. (B) Representative immunoblots of Bcl-X_L and actin expression in HCT116 wt, PUMA$^-$ and p21$^-$/PUMA$^-$ cells exposed to 4 days of room air or hyperoxia. (C) Bcl-X_L levels represented in (B) were quantified by densitometry and normalized to actin. (D) Bcl-X_L and actin levels in HCT116 wt and PUMA$^-$ cells either mock transfected or treated with siRNAs targeting luciferase (Luc) or Bcl-X_L prior to hyperoxic exposure for 4 days. (E) Percentage of death in HCT116 wt and PUMA$^-$ cells transfected with Bcl-X_L siRNAs and treated with hyperoxia for 4 days (*p=0.04 compared between cell lines; †p=0.006 compared between siRNA treatment; n=3).

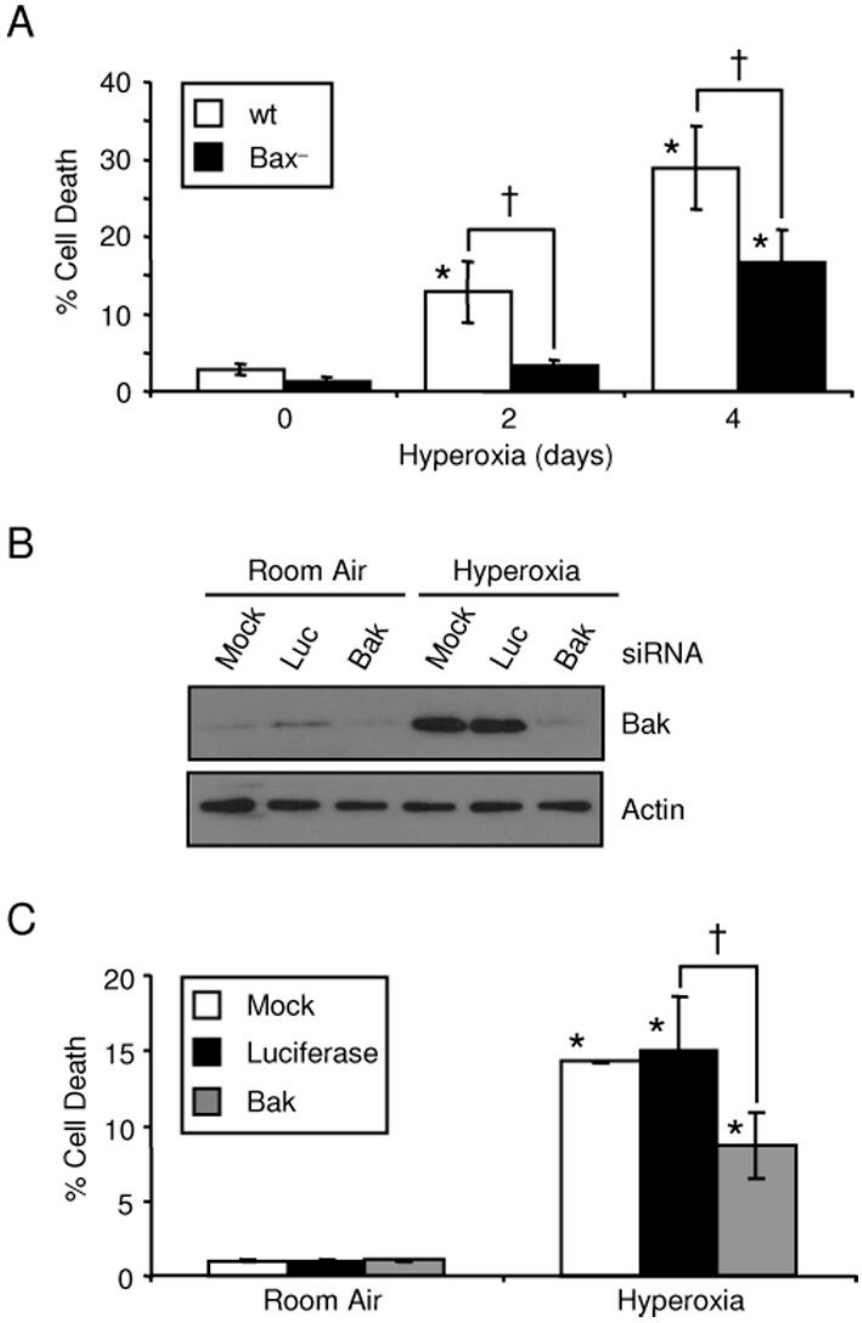

Figure 3. Bax and Bak promote hyperoxic cell death

(A) Percentage of cell death in HCT116 wt and Bax^{-} cells exposed to a hyperoxic time course. (B) Bak expression in HCT116 Bax^{-} cells either mock transfected or treated with siRNAs targeting luciferase (Luc) or Bak prior to hyperoxic exposure for 4 days. Actin expression was used as a loading control (C) Percentage of death in HCT116 Bax^{-} cells transfected with Bak siRNAs and cultured for 4 days in room air or hyperoxia (*p=0.0002 compared to untreated controls; †p=0.0013 compared between cell lines or siRNA treatment; n=3).

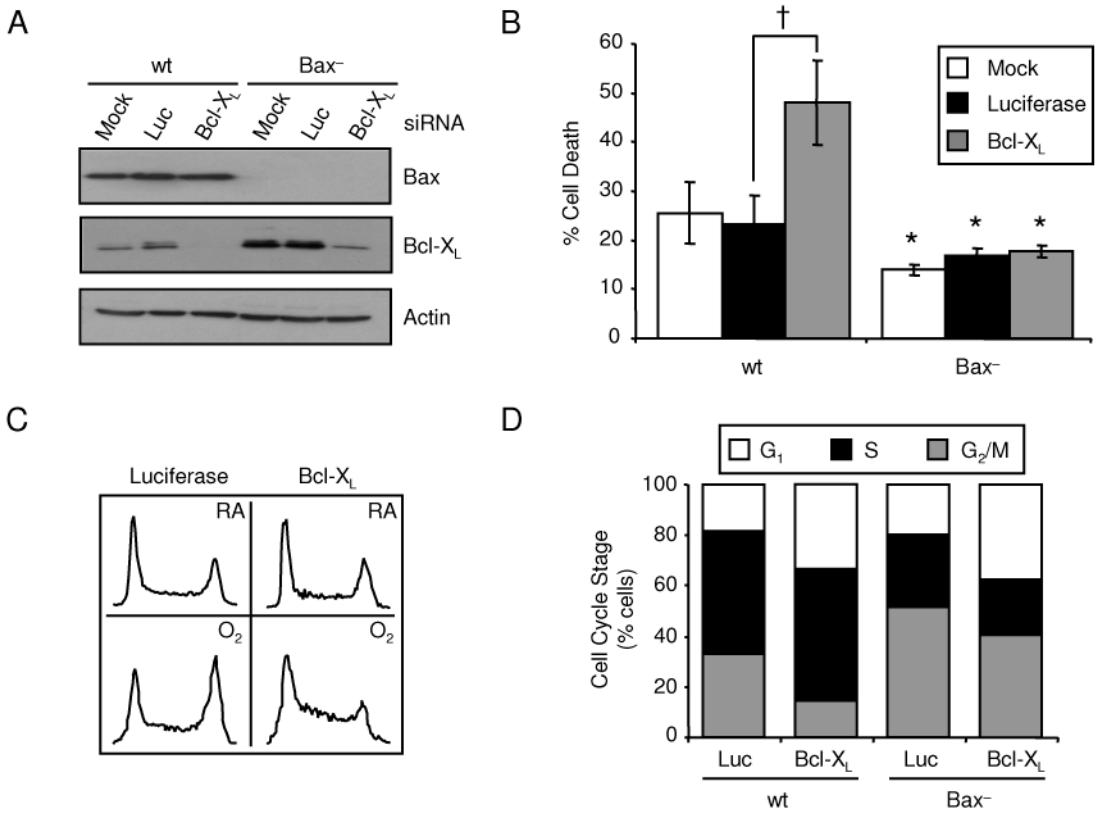

Figure 4. Bcl-X_L blocks Bax-dependent hyperoxic cell death

(A) Expression of Bax and Bcl-X_L in HCT116 wt and Bax$^-$ cells mock transfected or treated with siRNAs targeting luciferase (Luc) or Bcl-X_L prior to hyperoxic exposure for 4 days with actin used as a loading control. Immunoblots were representative of three separate experiments. (B) Percentage of cell death in HCT116 wt and Bax$^-$ cells transfected with Bcl-X_L siRNAs then exposed to hyperoxia for 4 days. (C) Representative DNA histograms HCT116 wt cells transfected with Bcl-X_L siRNAs then exposed to room air or hyperoxia for 4 days. (D) Mean average of HCT116 wt and Bax$^-$ cells in G_1, S and G_2/M after Bcl-X_L siRNA treatment cultured in room air or hyperoxia for 4 days (*p=0.02 compared between cell lines; †p=0.0004 compared between siRNA treatments; n=4)..

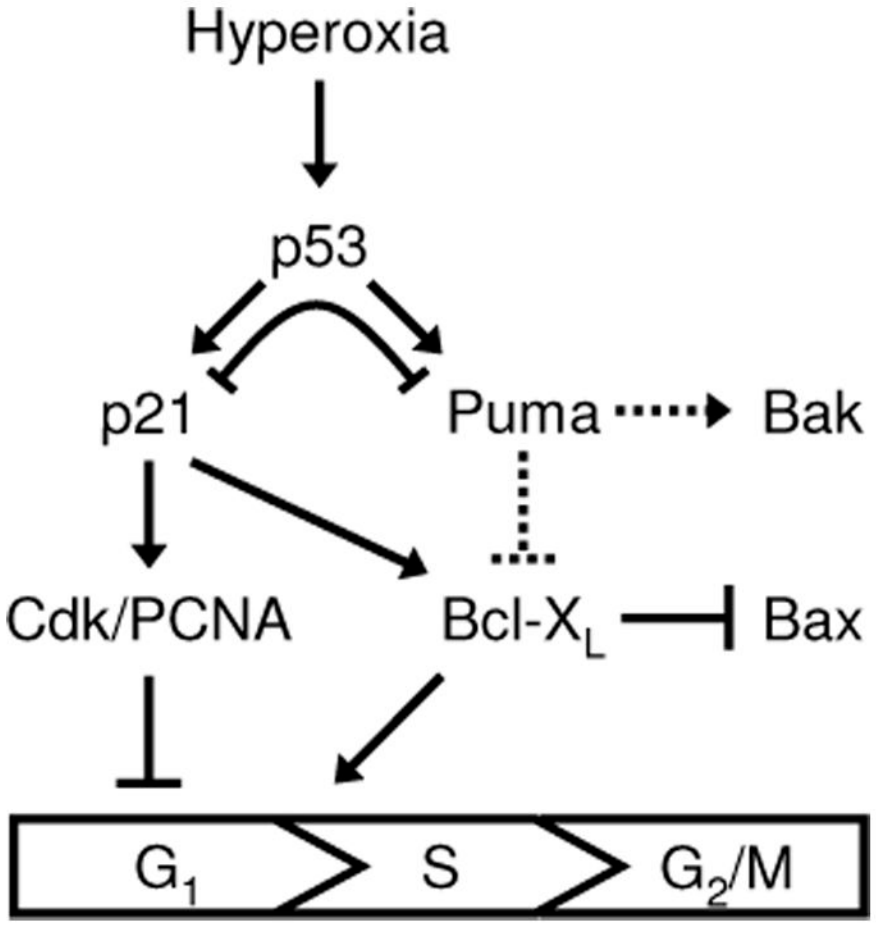

Figure 5. Model of cell survival and death pathways activated during hyperoxia

Hyperoxia-induced cell survival and death pathways downstream of p53. Solid lines represent interactions described in this study or previous studies and dotted arrows represent putative relationships. Arrows indicate pathway activation while perpendicular lines indicate pathway inhibition.